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# Targeting Allosteric Control Mechanisms in Heat Shock Protein 70 (Hsp70)

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## Abstract

Heat shock protein 70 (Hsp70) is a molecular chaperone that plays critical roles in protein homeostasis. Hsp70's chaperone activity is coordinated by intra-molecular interactions between its two domains, as well as inter-molecular interactions between Hsp70 and its co-chaperones. Each of these contacts represents a potential opportunity for the development of chemical inhibitors. To illustrate this concept, we review three classes of recently identified molecules that bind distinct pockets on Hsp70. Although all three compounds share the ability to interrupt core biochemical functions of Hsp70, they stabilize different conformers. Accordingly, each compound appears to interrupt a specific subset of inter- and intra-molecular interactions. Thus, an accurate definition of an Hsp70 inhibitor may require a particularly detailed understanding of the molecule's binding site and its effects on protein-protein interactions.

### Keywords

Chaperone; protein folding; proteostasis; irreversible inhibitors; dynamics; protein-protein interactions

## INTRODUCTION

Heat shock protein 70 (Hsp70) is a highly conserved chaperone that is broadly involved in protein homeostasis and quality control. This chaperone has been linked to molecular pathways of protein folding, trafficking, disaggregation, turnover and many other aspects of a protein's life cycle [1–4]. Indeed, Hsp70 is a central "hub" of a larger protein homeostasis (*i.e.* proteostasis) network, which includes hundreds of chaperones, co-chaperones and related systems that, together, maintain the health of the proteome [5]. There is a growing appreciation that the proteostasis network includes many untapped drug targets, a perspective enforced by recent clinical successes with proteasome inhibitors [6–8] and autophagy inducers [9]. However, other possible targets in the network, including Hsp70, heat shock protein 90 (Hsp90) and proteins involved in the unfolded protein response (UPR),

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**CONFLICT OF INTEREST**

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have proven more difficult to safely inhibit [10–12]. Another issue is that these "challenging" targets are inexorably linked to each other as part of the broader proteostasis network, often making it difficult to anticipate the impact of perturbing a specific node [13]. In this review, we focus on Hsp70 as a model for this type of target complexity and we highlight how recent examples of allosteric Hsp70 inhibition are illuminating both the challenges and opportunities.

## MULTI-DOMAIN ALLOSTERY AND DYNAMICS IN HSP70

Hsp70 is composed of an N-terminal, 45 kDa, nucleotide-binding domain (NBD) and a C-terminal, 35 kDa, substrate-binding domain (SBD). The NBD is divided into two lobes (I and II) that are further split into four subdomains (IA, IIA, IB, IIB). In turn, the SBD is comprised of a beta-sandwich domain (βSBD) that contains the substrate-binding cleft and an alpha-helical "lid" domain (αSBD) that regulates affinity for misfolded proteins (*i.e.* substrates or "clients"). The SBD and NBD are linked to each other through a short, hydrophobic linker. Hsp70 is highly conserved across all kingdoms of life. In prokaryotes, the ortholog is termed DnaK. In eukaryotes, the major Hsp70 paralogs in the cytosol are Hsc70 and Hsp72, while BiP is found in the ER and mortalin in the mitochondria. For simplicity, we will often use the inclusive term "Hsp70" when referring to the conserved structural and biochemical properties of these chaperones.

The Hsp70/DnaK system has served as a model for studying the allosteric mechanisms of multi-domain proteins because the NBD and SBD engage in well-defined, two-way cooperativity [14–16]. The two domains are tightly docked to each other in the ATP-bound state, which causes the αSBD to adopt an "open" configuration (Fig. 1) [14–15]. Hydrolysis uncouples the NBD from the SBD so that they now move independently in solution, tethered by the linker [17]. In this state, the αSBD domain closes onto the βSBD, increasing its affinity for substrates (Fig. 1). Thus, the net effect of ATP cycling is that the NBD and SBD are iteratively coupled and then decoupled, sampling the tight- and weak-binding states [18–19]. The linker is critical to this allosteric communication, as are a series of weak, intra-molecular contacts throughout the chaperone [14, 20–22].

Another key to understanding Hsp70's biology is that it functions as part of a multi-protein complex in the cell. Members of the J protein class of co-chaperones (sometimes termed Hsp40s) bind between the NBD and SBD using a highly conserved J domain. This inter-molecular protein-protein interaction stimulates uncoupling of the NBD from the SBD and accelerates hydrolysis of ATP (Fig. 1) [23]. Likewise, members of the nucleotide exchange factor (NEF) family, such as Hsp105, Bag1, Bag2 and Bag3, bind to the IB and IIB subdomains, stimulating release of ADP from the NBD and substrate from the SBD [24–28]. Thus, a series of interactions between Hsp70, J proteins and NEFs, combine to regulate ATP and substrate cycling (Fig. 1). In addition to these contacts, some Hsp70s interact with tetratricopeptide repeat (TPR) co-chaperones, a family that includes CHIP, FKBP51, HOP and PP5 [29]. Binding of Hsp70 to TPR co-chaperones appears to have less of an impact on ATP/substrate cycling; rather, these proteins recruit specific enzymatic functions to the complex [30]. For example, CHIP is an E3 ubiquitin ligase involved in substrate turnover by the proteasome [31–32], while PP5 is a phosphatase that acts on Hsp70 substrates [33]. Each

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of these TPR-domain co-chaperones binds to the C-terminal “EEVD” motif present in the cytoplasmic Hsp70s, such that only one can be bound at a given time. Finally, the substrates of Hsp70 are predicted to include nearly all proteins with exposed hydrophobic regions [34], including short peptide segments and even partially folded proteins [3, 35]. This promiscuity allows Hsp70 to engage in many types of interactions, a necessity to maintain the global proteostasis network. Adding to this complexity, there are >45 different J proteins and dozens of NEFs and TPR domain co-chaperones expressed in human cells – creating a complicated web of possible multi-protein complexes.

Hsp70 is linked to many diseases, largely by genetic studies or expression analyses. Recent reviews have detailed the evidence linking Hsp70 to cancer, neurodegenerative disease, auto-immunity, infection and other disorders [11, 36–41]. The breadth of potential indications for Hsp70 inhibitors isn’t too surprising, given the central role of this chaperone in the proteostasis network. However, this breadth of possible indications also brings up a key challenge – if Hsp70 is involved in so many pathways, then how can one safely tune its function to correct a specific disease while avoiding collapse of other pathways? The key to rationally achieving this goal may be to eventually understand how individual co-chaperones, such as the J proteins, Bag proteins and TPR-containing proteins, recruit Hsp70 into distinct roles. The classic example of this selectivity comes from pioneering work on the role of Hsp70 in clathrin-mediated endocytosis. A specific J protein, auxilin, is tightly associated with clathrin and it recruits an Hsp70 into the clathrin triskelion, where the chaperone is required to remodel that structure during vesicle budding [42]. If one desired to interrupt Hsp70’s role in endocytosis, it is clear that targeting auxilin or the auxilin-Hsp70 interface would be the desired approach because it would presumably leave other aspects of Hsp70 structure-function unscathed. However, this type of detailed molecular understanding is rare and, typically, the specific way to achieve an outcome through targeting Hsp70 complexes is uncertain.

In the absence of granular details about the connectivity of the proteostasis network, recent work has focused, instead, on developing broad classes of Hsp70 “inhibitors”. These molecules are defined by their ability to bind Hsp70 and interrupt its readily measurable biochemical activities, such as ATP turnover, which can be readily measured *in vitro*. However, it isn’t clear if chemical inhibition of ATP turnover directly correlates with all chaperone functions [43]. Rather the power of these molecules may lie in their ability to empirically link specific, *in vitro* biochemical mechanisms (*e.g.* nucleotide hydrolysis) to cellular outcomes. In other words, the molecules discovered thus far might be considered “tool compounds” for learning about the structure-function relationships in this system. This approach aims to produce definitions of Hsp70 function that are based on pharmacology, a strategy patterned after success in the study of GPCRs and ion channels [44].

## MULTIPLE ALLOSTERIC BINDING SITES ON HSP70

In the cell, Hsp70 might be best considered the “core” of a dynamic, multi-protein sub-network composed of co-chaperones, nucleotides and substrates. Dramatic allosteric transitions and changes in both intra- and inter-molecular interactions accompany the motions of Hsp70 and these activities are powered by nucleotide turnover. In the face of this

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orchestrated series of motions, it is not surprising that numerous inhibitors of Hsp70 have been discovered [45–46] or that many of these inhibitors have non-degenerate binding sites [47–52]. In theory, Hsp70 is rich in possible allosteric regulatory sites and, as it transitions through an ATP hydrolysis cycle, these sites are expected to appear/disappear [16, 30]. In addition, binding to co-chaperones would be expected to "hide" some possible sites (*e.g.* at the newly buried contact surface), while possibly revealing other sites (*e.g.* when induced allostery reveals a previously buried site). Viewed this way, each Hsp70 conformer and each Hsp70 complex with its co-chaperones might be considered an independent drug target.

In this review, we will focus on three of the classes of Hsp70 inhibitors, exemplified by MKT-077, VER-155008 and YK5 (Fig. 2). We will discuss how emerging evidence suggests that they each disrupt Hsp70 function through a complex, "domino effect" on allostery, dynamics and protein-protein interactions. We discuss how each of these inhibitors, because of their distinct binding sites, might have both *similar* and *different* effects on Hsp70 structure-function. A major theme (or speculation) is that each of these Hsp70 inhibitors, because of its unique properties, might be expected to have some similar, but also some dissimilar, effects in cells and animals. Thus, by better understanding the molecular mechanisms of an inhibitor, we might be able to better select the "right tool for the job". It is worth noting that there have been many other inhibitors of Hsp70 reported [53–57]. To focus the discussion, we have selected three that have good evidence of selectivity and well-defined binding sites, while recent reviews cover the broader field [58–60].

## ALLOSTERIC INHIBITORS BASED ON MKT-077

MKT-077, 1-ethyl-2-((*Z*)-((*E*)-3-ethyl-5-(3-methylbenzo [*d*]thiazol-2(3*H*)-ylidene)-4-oxothiazolidin-2-ylidene)methyl)pyridin-1-ium chloride, is a cationic rhodacyanine that was first discovered by Fuji Film [61]. It was subsequently found to have promising anti-cancer [62–63] and antimalarial activities [64]. The major cellular targets of MKT-077 were identified as mortalin, the mitochondrial Hsp70, and Hsc70, a cytoplasmic paralog [62]. It is likely that the delocalized cation of MKT-077 is responsible for the targeting of the compound to mitochondria, where it binds to mortalin. However, this localization appears to be incomplete [65], which is likely why Hsc70 is also a known target. Although the compound has anti-proliferative activity against cancer cells from many tissues, MKT-077 has little cytotoxicity against either normal human fibroblasts or immortalized epithelial cells [66]. This selectivity is thought to originate from increased dependence of cancer cells on Hsp70s, because of their elevated requirements for protein synthesis and high levels of oxidative stress [67]. Based on its promising pre-clinical selectivity, MKT-077 was advanced into Phase I clinical trials in patients with advanced solid tumors in the 1990s; however, the study was terminated due to renal toxicity in a subset of patients [68]. Despite this setback, MKT-077 remains an interesting scaffold for studying Hsp70 structure-function.

MKT-077 has been studied in a limited number of medicinal chemistry campaigns. A single replacement of the ethyl group on the pyridinium nitrogen with a methyl group yielded YM-01 (Fig. 3), which is more potent than MKT-077 in a number of cellular and animal systems, including cancer xenograft models [65, 69], tau turnover assays [70], and polyglutamine-induced proteotoxicity models [71–72]. This molecule had more exposure to

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the cytoplasmic isoforms of Hsp70, which might give rise to some of this increased efficacy [65]. In addition, a neutral MKT-077 derivative, YM-08, was developed to improve blood-brain barrier permeability [73]. More recent modifications have retained the cation and, instead, focused on improving microsomal stability and reducing renal toxicity [74–76]. JG-98, a next-generation MKT-077 derivative, binds 60-fold tighter to Hsp70 than YM-01 ($K_D$ ~ 86 nM) and its anti-proliferative activity is improved 3- to 20-fold in various cancer cell lines ($EC_{50}$ ~ 0.3 – 4 μM) [77].

NMR titrations and docking calculations revealed that MKT-077 and its analogs bind within a highly conserved, hydrophobic pocket adjacent to the nucleotide-binding cleft [47, 78]. The enhanced affinity of JG-98 (Fig. 3) for Hsc70 appears to originate from favorable contacts with a deep pocket composed of residues T13, T14, K71, V146, Y149, E175 and T226, as well as a secondary interaction with V82, P147, Y149 and F150, (Fig. 4A; Hsc70 numbering). Throughout the binding site, favorable hydrophobic interactions are made with the benzothiazole and phenyl moieties, while electrostatic interactions with the delocalized cation are also observed. The pocket that accepts the benzothiazole is relatively narrow and this observation has played out in the structure-activity relationships, with large groups on the rings decreasing activity. However, small electron-withdrawing groups on the benzothiazole provide resistance to metabolism by liver microsomes and are still accepted in the pocket [76]. The rhodacyanine appears to play a role in orienting the other rings, with few direct contacts.

Interestingly, the binding site of MKT-077 is not overlapping with the nucleotide-binding cleft and docking studies suggest that both MKT-077 analogs and ADP can be bound simultaneously [77]. Despite this, analogs of MKT-077 block the ATPase activity of Hsp70s *in vitro* and they also interrupt its ability to refold model substrates [71]. How do they inhibit Hsp70 functions if they don’t compete with nucleotide? One clue comes from NMR titration experiments, which reveal that MKT-077 and its analogs bind Hsc70 in the ADP-state, but not to the ATP-bound state or the apo conformation [47, 76]. The structures of Hsp70 isoforms in the ADP- (PDB: 3C7N) [79] and ATP-bound states (PDB: 4B9Q) [15] provide a compelling reason for this selectivity. The entrance to the MKT-077 binding pocket is formed by Y149 and T226 when Hsp70’s NBD is in its “open”, ADP-bound state (Fig. 4A). Upon binding of ATP; however, rotations in lobes I and II of the NBD dramatically re-organize the binding pocket, such that it “collapses”. Indeed, the former binding pocket becomes largely occupied by hydrophobic side chains (Fig. 4B). In addition, the gate residues (*i.e.* Y149 and T226) swivel to block the entrance to the binding site [47]. Thus, the binding site of MKT-077 analogs is only accessible in the ADP-bound state and these compounds appear to favor that specific conformer of Hsp70.

## MKT-077 analogs allosterically inhibit interactions between Hsp70 and Bag domain proteins

What are the effects of stabilizing the ADP-bound state on Hsp70 structure-function? As mentioned above, nucleotides bind at the bottom of a deep cleft between lobes I and II and are coordinated by residues from all four subdomains (Fig. 4C) [80]. The ADP-bound state keeps the αSBD in the “closed” conformation, enhancing affinity for substrates (see Fig. 1).

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Thus, one outcome of MKT-077 treatment is to promote the substrate-binding affinity of Hsp70 [71]. Chemically disrupting the normal "catch-and-release" dynamics of Hsp70 then limits re-folding of damaged clients *in vitro* [81] [71], consistent with biochemical studies that suggest multiple turnovers are needed to refold substrates [43, 82]. Thus, binding of MKT-077 analogs has an impact on the *in vitro* chaperone functions of Hsp70 by altering its internal dynamics and nucleotide cycling.

What about effects on co-chaperone binding? To explore this question, we used molecular dynamic simulations (MDS) [83–84] to monitor how the NBD might change in response to MKT-077 and its analogs. MD trajectories suggested that the major consequence of compound binding is rotation of subdomain IIB, which is even more dramatic than the rotation that occurs in the ADP-bound state (PDB: 3C7N) (Fig. 4C). A major consequence of this rotation is that the position of the residues at the "top" of the IB and IIB subdomains are dramatically shifted. Specifically, the positions of residues E268, K271, S275 in subdomain IIB and T13, T14, and Y15 in subdomain IB are moved out of their normal orientation. This movement is important because these residues are required for binding the Bag domain [85]. Indeed, comparing the crystal structure of apo-Hsc70 bound to the Bag1M domain (PDB: 1HX1) with the compound-bound $Hsc70_{NBD}$ model (by aligning the Cα atoms of the IIA subdomain) shows that many of the key interactions are significantly disrupted (Fig. 4D). Electrostatic interactions between the Bag domain and Hsp70's NBD are normally mediated by E212, D222, R237, and Q245 in the Bag domain, which form hydrogen bonds with residues R262, R261, E283/Y294, and S286 in Hsc70 (Fig. 4D) [85]. Indeed, mutations in R262A and E283A (in Hsc70) or E212A, D222A, R237A, and Q245A (in Bag1) are known to disrupt binding [25], [85]. Likewise, MKT-077 is predicted to cause major re-organization of residues R261, S286, and Y294 and dramatically perturb the hydrogen-bonding network (Fig. 4D, right). Because the other Bag domains are thought to bind similarly to Bag1 [86], MKT-077 might be expected to broadly disrupt the interactions between Hsp70 and other members of the Bag family of NEFs, a prediction that has recently been experimentally confirmed [69]. Thus, although MKT-077 analogs bind >20Å away from the Hsp70-NEF interaction surface, they trap a conformer that disfavors this protein-protein contact.

## ALLOSTERIC INHIBITORS BASED ON VER-155008

Work by medicinal chemists at Vernalis has produced a chemical probe for Hsp70 with a distinct binding site. Their tactic was to identify an ATP-competitive inhibitor, but they realized that the challenges in this approach are that Hsp70 has a tight affinity for ATP ($K_D$ ~ nM) and the ATP-binding site is quite hydrophilic, especially compared to that of Hsp90 [87]. Despite these obstacles, the Vernalis group successfully screened an adenosine analogue library using a fluorescence polarization (FP) assay [52] and structure-guided expansion of an active hit yielded compound VER-155008 (Fig. 5A). It was confirmed that VER-155008 competes with ATP for binding to Hsp70 with an $IC_{50}$ of 0.5 μM, and it inhibits Hsp70- and Hsc70-mediated luciferase refolding *in vitro* [50, 88]. It also inhibits the growth of several human breast and colon cancer cell lines with $GI_{50}$ values ranging from ~5 to 14 μM, and it induces degradation of suspected Hsp70 substrates, such as Her2 and Raf-1, in both HCT116 and BT474 cells. These activities might be expected from Hsp70

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knockdowns [89], supporting Hsp70s as major cellular targets. Although VER-155008 still requires further optimization to improve its potency and pharmacokinetic properties, the molecule provides another compelling case study.

### VER-155008 binding approximates the ADP-bound state of the NBD

The structure of VER-155008-bound Hsc70 has been solved using both $Hsc70_{NBD}$/Bag1 (by soaking) and $Hsp70_{NBD}$ alone (by co-crystallization) [50, 52]. However, the structure was different in these cases, likely because of the strong influence of Bag1. Analysis of the VER-155008 bound Hsc70 structure suggests that the adenine moiety overlays with ATP and the $N^1$ of the adenine ring forms a hydrogen bond with Ser275 (Fig. 5B). The $O^{2'}$ of the ribose part makes a direct hydrogen bond with Lys271, whereas $O^{3'}$ forms a hydrogen bond with Asp234 via a water molecule. It is only the π-stacking interactions between the side chain of Arg272 and the dichlorobenzene and the interactions between Tyr15 and the 4-cyano-benzyloxymethyl moieties that appear to differentiate the binding modes (Fig. 5C). In this state, the IB and IIB subdomains are in the ADP-like configuration (Fig. 5D). However, treatment with VER-155008 did not significantly impact substrate binding, except at high concentrations [50], suggesting that the compound-bound chaperone does not act entirely like the ADP-bound state.

If VER-1550088 is competitive with nucleotide, why do we refer to it as an allosteric inhibitor? The answer comes from observations that ATP hydrolysis is only indirectly linked to many important chaperone functions, such as substrate refolding [90]. Thus, even though the compound is orthosteric for one co-factor (*e.g.* nucleotide), it is expected to have primarily allosteric impacts on distal functions. In other words, it isn't the interruption of ATP cycling *per se* that is important, but the effects of compound binding on downstream activities.

## ALLOSTERIC INHIBITORS BASED ON YK5

Recently, Rodina *et al.* reported their discovery of a new druggable site in the NBD located between lobes IB and IIB [91]. They identified this site after searching through approximately 25 available structures of Hsp70 paralogs in various nucleotide states. A superimposition of these structures most resembled the ADP-bound state, where the NBD was in the "open" conformation. Searching for druggable pockets in this consensus state revealed a potential binding site that is distinct from the site bound by either MKT-077 or VER-155008. However, like MKT-077, this site is only predicted to be available in the ADP-bound state because of rotations in lobes I and II. Exploration of this site revealed a potentially reactive cysteine at position 267 (the $TAC^{267}ERAK$ sequence is shown in Fig. 6) and this cysteine was then exploited in the rational design of a 2,5′-thiodipyrimidine series featuring a terminal acrylamide, leading to the identification of compound YK5 (Fig. 6A). YK5 was shown to selectively and irreversibly interact with Hsp70 in cancer cells, in particular with the cytosolic isoforms and not the mitochondrial or ER-resident isoforms (Grp75 and Grp78, respectively), where Cys267 is not conserved.

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### Effects of YK5 on binding to co-chaperones

*In vitro*, YK5 only modestly inhibits ATP hydrolysis by the combination of Hsc70, a J protein (DnaJA1) and a NEF (Hsp110), while having no significant effect on ATP turnover by the combination of Hsc70 and DnaJA1 alone [91]. However, YK5 was shown to inhibit Hsc70-mediated refolding of denatured luciferase *in vitro*, suggesting that it does interfere with the productive interaction with substrates. These findings suggest that YK5 might have relatively minimal effects on the protein-protein interactions with J proteins or NEFs, while primarily impacting substrate interactions or intra-molecular contacts within Hsp70. Indeed, YK5 has been useful in understanding which proteins specifically assemble on Hsp70 in cancer cells [92].

After the discovery of YK5, more potent, irreversible inhibitors were developed that also exploit Cys267 [51]. Derivatives of the 2,5′-thiodipyrimidine and 5-(phenylthio)pyrimidine class were assembled and armed with an electrophilic acrylamide moiety. Like YK5, these molecules are designed to interact with a pocket formed by V59, F68, H89, R261, R264, L237 and V238 in the vicinity of Cys267. This site is less hydrophilic than the nucleotide-binding cleft and may be more suitable for development of potent analogs. In the initial reports, compounds with anti-proliferative activities of 0.9 ± 0.2 μM in Kasumi-1 myeloid leukemia cells were reported [51]. In addition, one of the analogs (compound 17a) was tested for activity against >400 kinases and only c-Met was identified as a possible target. This selectivity is likely due to the unique nature of the allosteric site on Hsp70, which does not resemble the kinases.

Importantly, these compounds bound irreversibly to Hsp70 in lysates, but they did not bind Hsp90 [51]. Treatment with YK5 significantly reduces the levels of oncogenic Hsp70/Hsp90 substrates, such as HER2 and Raf-1, presumably by destabilizing Hsp70 interactions. It was also shown that in lysates of breast cancer SKBr3 cells, one derivative dose-dependently depleted the amount of Hsp70 bound to the TPR domain co-chaperone, HOP, with an $IC_{50}$ value very similar to that needed to inhibit growth. This result suggests that YK5 analogs employ a mechanism that involves disruption of the Hsp70-HOP-Hsp90 complex. It isn't yet clear why binding of these molecules to the region around Cys267 in the NBD might impact interactions between Hsp70 and HOP, which occur at the end of the disordered, C-terminal tail region [91]. In a companion paper, a new series of reversible inhibitors, lacking the acrylamide moiety, was developed (Fig. 6B) [93]. These non-covalent inhibitors were also shown to bind Hsp70 and they appeared to operate via similar mechanisms to their irreversible counterparts. Thus, covalent binding is not required for the activity of this series.

## COMPARISONS BETWEEN THREE ALLOSTERIC INHIBITORS

Hsp70 is a dynamic, two-domain protein that functions in concert with its co-chaperones [16]. The chaperone itself is highly mobile and there are many important contacts made between its NBD and SBD. In this ensemble of possible structures, a compound might be expected to "trap" a state as a consequence of its binding. In turn, this trapped state might be expected to communicate with the local network of protein-protein interactions [94]. For example, the ATP-bound state is known to interact with J proteins and NEFs, while the ADP-bound state has tight affinity for substrates [24, 95]. Thus, binding of small molecules

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at two different sites might produce distinct effects on Hsp70 structure/function by favoring specific conformers and shifting the ensemble of bound co-chaperones.

To explore this concept, we reviewed the literature around three distinct chemical inhibitors of Hsp70: MKT-077, VER-155008 and YK5. These molecules were chosen because they have been shown to be relatively specific for Hsp70 in cells and they were each known to bind different sites on the protein. MKT-077 and VER-155008 appear to trap an ADP-like state. However, the MKT-077 bound state was not exactly the same as the natural nucleotide-bound state, at least by modeling simulations, and this configuration has a dramatic effect on binding to Bag co-chaperones. YK5 was designed based on an ADP-bound structure, but experimental evidence suggests that YK5 might favor a conformer of Hsp70 that is not well-defined as either ATP- or ADP-bound. Thus, MKT-077, VER-155008 and YK5 provide a suite of molecules with distinct binding sites and mechanisms.

What are the impacts of enforcing a specific Hsp70 conformer on chaperone functions? *In vitro*, MKT-077 and VER-155008 interrupt ATP cycling and refolding, while YK5 has less dramatic effects. MKT-077 blocks Hsp70-NEF interactions, while VER-155008 and YK5 are expected to have less dramatic effects on this protein-protein interaction. Thus, *in vitro* measurements of Hsp70 biochemistry (*e.g.* ATP turnover, binding to co-chaperones) may begin to "bin" the small molecules into distinct categories. However, side-by-side studies are severely lacking (other than important work by the Bukau group [50]) and more work is needed to understand how many categories of Hsp70 inhibitors might be possible. These are early days.

Taking this speculative thought-process a step further, one might also assume that different Hsp70 inhibitors could have non-overlapping effects on chaperone function in the cell. This hypothesis is starting to be tested. For example, trapping the ADP-bound state with MKT-077 analogs appears to catalyze the degradation of some misfolded Hsp70 substrates, including polyglutamine-expanded androgen receptor [71] and tau [70], while having comparatively less effect on the undamaged versions [96]. Likewise, some oncoproteins, including kinases, seem to require constant cycling of Hsp70 to avoid proteasomal degradation [96]. Thus, any compound that interferes with cycling might have degenerate effects on the stability of these proteins. Indeed, treatment with VER-155008, YK5 and MKT-077 all destabilize some chaperone-dependent oncoproteins in cancer cells [76, 88, 91].

## THE FUTURE OF HSP70 INHIBITOR RESEARCH

The ultimate goals of Hsp70 inhibitor research are to: (a) learn how this chaperone is involved in disease and (b) produce clinical candidates for use in treating these diseases. In addition to the Hsp70 inhibitors discussed here, a number of others have been identified [97–101]. Unfortunately, none of these molecules has yet advanced to clinical trials (with the exception of MKT-077, which failed in Phase I). However, a number of series are in active pre-clinical development. The path for these molecules may be long and, in our opinion, the current compounds may ultimately be more useful as chemical probes than drugs. This conclusion is based, in part, on the complexity of the Hsp70 system and, as we have

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highlighted in this review, on the diversity of possible mechanisms. Indeed, it seems prudent and important to develop and fully characterize a number of different tool compounds. Out of that process, the field might uncover the safest and most effective ways of using Hsp70 as a therapeutic target.

Another frontier area in the study of Hsp70 inhibitors is paralog specificity. As mentioned above, there are Hsp70 paralogs found in each cellular compartment (*e.g.* mitochondria, ER, *etc*) and it is often not clear whether specific paralogs should be targeted or whether pan-Hsp70 inhibitors will be most effective. There needs to be significantly more work done to address this question, likely using a combination of paralog-specific inhibitors and genetics.

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## Biography

**Jason E. Gestwicki**

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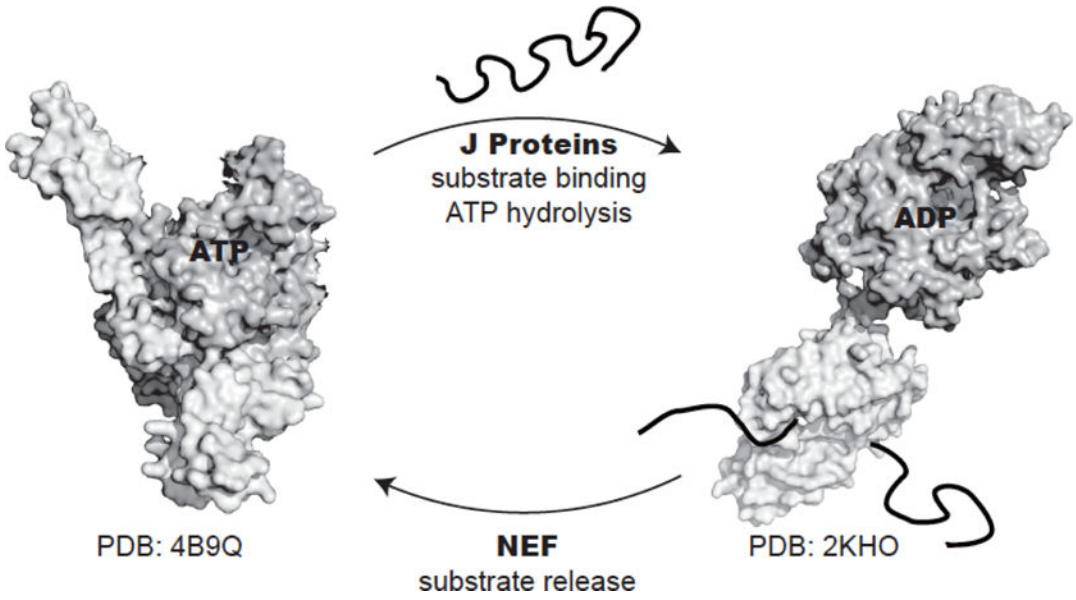


**Fig. 1.**

Illustration of the ATPase cycle of Hsp70. Structural insights have been made into how the NBD and SBD of Hsp70 are arranged during hydrolysis. Many of these insights have come from studies on the highly conserved prokaryotic Hsp70, DnaK. The NBD (dark gray) and SBD (light gray) are docked to each other in the ATP-bound state (left; 4B9Q) and they move independently in the ADP-bound state (right; 2KHO).

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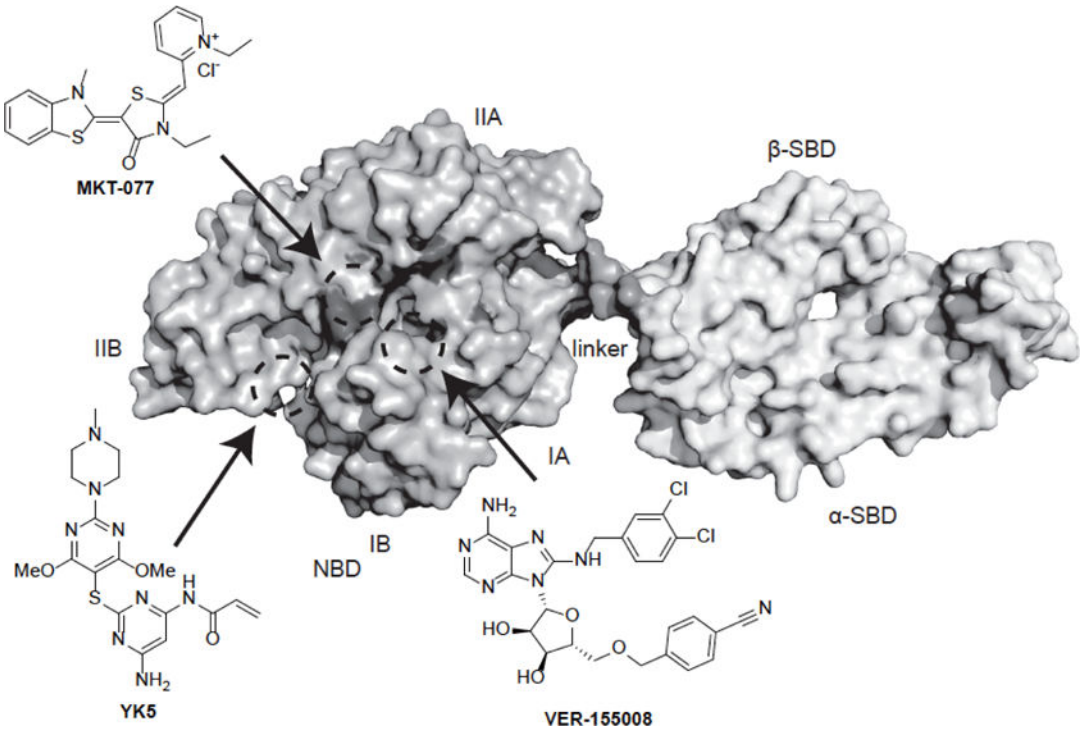


**Fig. 2.**

Three classes of Hsp70 inhibitors: MKT-077, VER-155008, and YK5. These molecules bind in three distinct pockets of Hsp70 (denoted by circles). For clarity, only the ADP-bound form is shown (PDB: 2KHO). The locations of the NBD, SBD and subdomains are shown.

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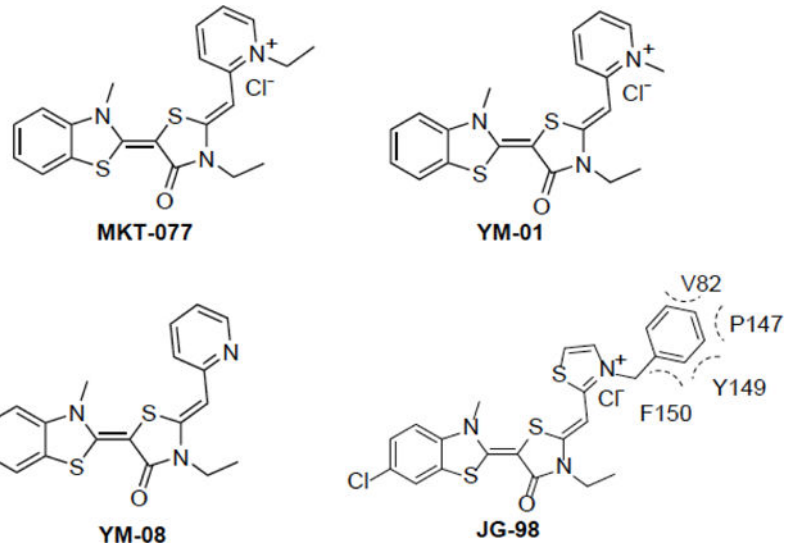


**Fig. 3.**

Chemical structures of MKT-077 and its analogs. The residues in Hsc70 that interact with the terminal phenyl group of JG-98 to add additional binding energy are shown.

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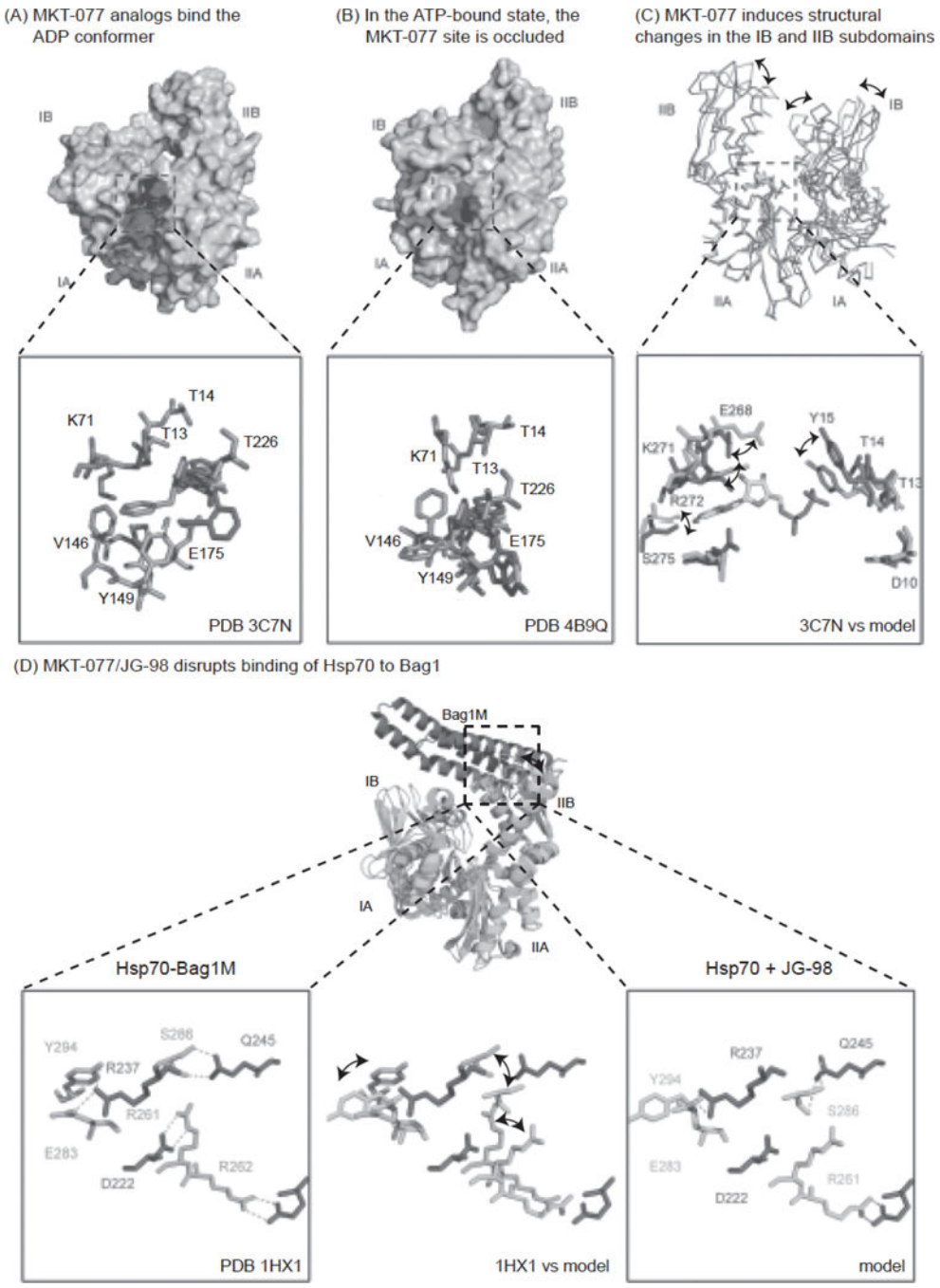


**Fig. 4.**

MKT-077 binds to the ADP-form of Hsp70 and disrupts binding to Bag co-chaperones. (A). MKT-077 and its analogs bind to a pocket of Hsc70's NBD in the "open" state. The gate residues are highlighted. (B) The binding site for MKT-077 and its analogs is occluded in the ATP-bound, "closed" state, with the gate residues collapsed. (C) Binding to MKT-077 induces rotations in subdomain IIB and closure of the nucleotide-binding cleft. The key motions of nearby residues in the binding site are shown compared to Hsc70's NBD in the ADP state (PDB 3C7N). (D). Binding to MKT-077 disrupts the hydrogen-bonding network

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that normally links Hsc70 to Bag1M. (Dark Gray: Bag1M, Light Gray: Hsc70) no compound (left; PDB 1HX1) or JG-98 bound Hsc70 (right). The MKT-077 binding images were generated from docking (DOCK 3.6) (see ref 77).

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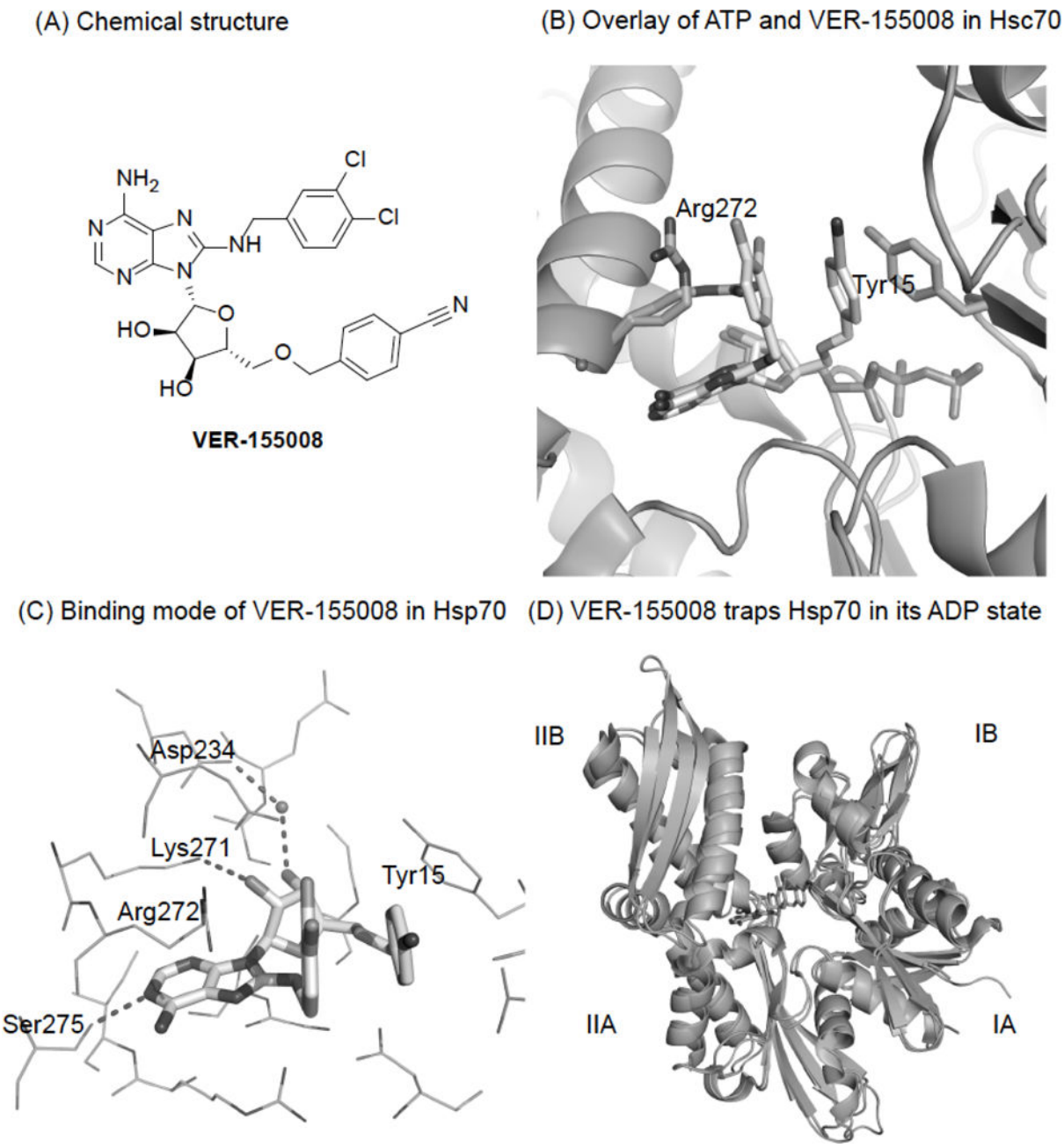


**Fig. 5.**

Ver-155008 is an ATP-competitive inhibitor of Hsp70 and it binds in the nucleotide-binding pocket. (A) Chemical structure of Ver-155008. (B) Overlay of the X-ray crystal structure of Hsc70 bound to ATP and Bag1 (PDB 3FZF) with the structure of Hsc70 and Bag1 bound to VER-155008 (PDB 3FZL). Other than a repositioning of Arg272, ATP and VER-155008 are in the same pose and Hsc70 is largely unperturbed. (C) Binding mode of VER-155008 (PDB 4IO8), with key contact residues highlighted. Hydrogen bonds are indicated by dotted lines. (D) Overlay of Hsp70's NBD bound to ADP (PDB 2KHO) or VER-155008 (PDB 4I08), showing that VER-155008 traps Hsp70 in its ADP state.

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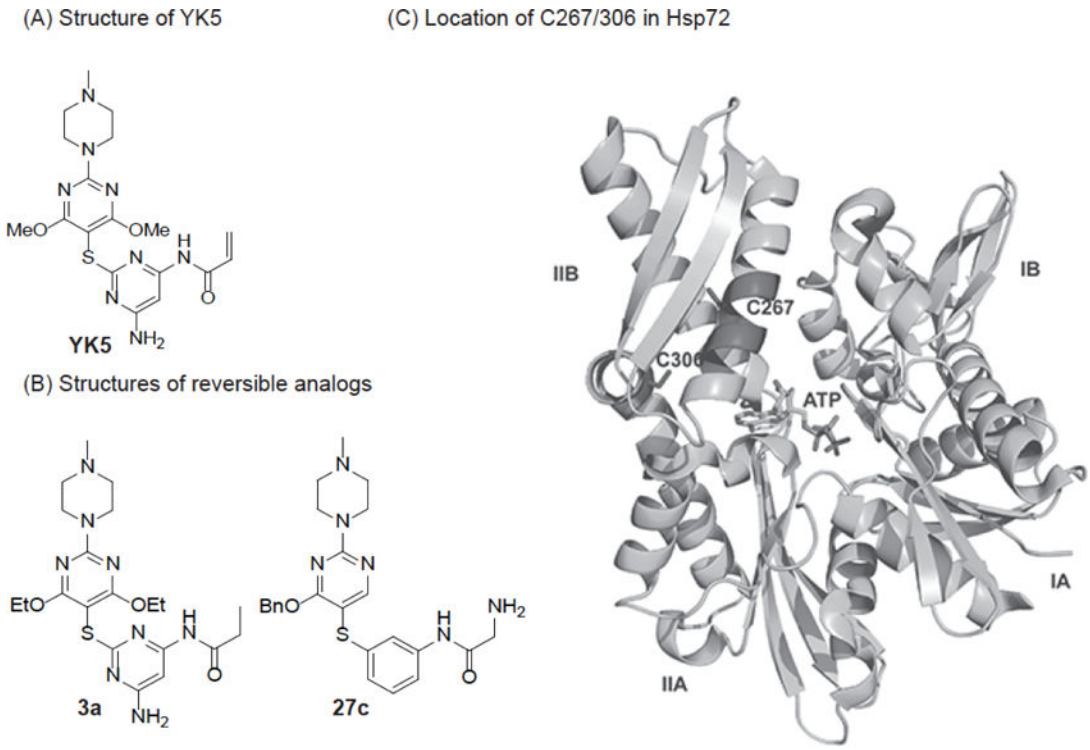


**Fig. 6.**

The Hsp70 inhibitor, YK5, binds to an allosteric site near Cys267. (A) Chemical structure of the irreversible inhibitor, YK5. (B) Chemical structures of reversible inhibitors, 3a and 27c, which lack the electrophile. (C) The structure of the NBD of Hsp70 (3JXU), highlighting the position of the nucleotide and C267 and C306. The YK5-binding site $TAC_{267}ERAK$ is also shown (dark gray).