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Miniperspective

ATPases as Drug Targets: Insights from Heat Shock Proteins 70 and 90

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ATPases have long been suggested as attractive drug targets. Designing small molecule, ATP competitive inhibitors, akin to that used to target protein kinases, is an appealing strategy for ATPase drug discovery. This approach has been highly successful for the molecular chaperone heat shock protein (Hsp[a]) 90, resulting in multiple inhibitors entering clinical trials for the treatment of cancer. Despite its strong validation as a potential anticancer target, discovery and development of ATP competitive, small molecule inhibitors of another molecular chaperone, Hsp70, have been less successful. Our experience of drug discovery efforts targeting both Hsp90 and Hsp70 will be used to suggest how structural features of the ATP binding site, fragment hit rate, and affinity for ATP could be used to assess the "druggability" of other ATPases and ATP binding sites.

ATPases are a group of enzymes that utilize ATP hydrolysis, and the subsequent release of energy, to achieve a cellular function. The cellular functions involving ATPases are plentiful and diverse including initiation of DNA replication, DNA repair and remodeling, protein folding and chaperoning, protein degradation, intracellular transport, and ion transport. A large number of these enzymes represent attractive drug targets, and drugs targeting ATPases, such as proton pump inhibitors (e.g., omeprazole), the broad spectrum DNA gyrase inhibiting antibiotic ciprofloxacin or toposiomerase targeting chemotherapeutics (e.g., irinotecan and doxorubicin), are in widespread clinical use. Identification, and subsequent approval for clinical use, of ATP competitive, small molecule kinase inhibitors (such as imatinib, lapatinib, and sunitinib) suggests that the design of ATP competitive inhibitors will be an important and compelling way to drug ATPases.[1] Two families of molecular chaperones, heat shock protein 90 and heat shock protein 70, possess N-terminal nucleotide binding domains (NBD) and require ATPase activity for their functions. These two families of ATPases represent significant therapeutic targets for the treatment of cancer and are being extensively pursued.

The Hsp90 family of molecular chaperones comprise cytosolic Hsp90α and Hsp90β, endoplasmic reticulum Grp94 and mitochondrial Trap-1. In the cell, Hsp90α and Hsp90β exist as large multiprotein complexes with a variety of cochaperones including Aha1, Hsp70, Hip, Hop, Cdc37, and p23. The ATPase activity of Hsp90, in addition to its various cochaperones, is essential for maintaining the conformational maturation and stability of key signaling molecules involved in cell proliferation, survival, and transformation. The mechanism by which Hsp90 functions is complex, requiring the sequential binding and dissociation of various cochaperones as well as the hydrolysis of ATP to drive the chaperone cycle.[2]

Inhibiting the N-terminal ATPase activity results in the proteasomal degradation of client proteins followed by tumor growth arrest or death.[3,4] The ansamycin class of Hsp90 inhibitors, including geldanamycin and its semisynthetic derivative **1** (17-AAG, Figure 1), was the first set of inhibitors to demonstrate the power of targeting the Hsp90 NBD as an anticancer therapeutic strategy. **1** and the hydroquinone prodrug **2** (IPI-504) have demonstrated evidence of target modulation in melanoma, prostate, renal, multiple myeloma, and trastuzumab-refractory breast cancer.[5] Despite the clinical progression of **1**, this compound and those based on the ansamycin scaffold have potential limitations including poor solubility (necessitating complex formulations or the prodrug approach), limited bioavailability, and hepatotoxicity.[6] In addition to these limitations, the potential to develop clinical resistance through P-glycoprotein (P-gp) up-regulation or by mutation or loss of the NQO1 gene (NAD(P)H quinone oxidoreductase 1, DT-diaphorase) exists,[7] further limiting the potential clinical usefulness of this class of Hsp90 inhibitors. To overcome the limitations of the ansamycin derived Hsp90 inhibitors, significant drug discovery efforts have focused on identifying novel agents that bind to and inhibit the N-terminal ATPase site. Multiple agents suitable for intravenous administration, including **3** (NVP-AUY922, Novartis/Vernalis)[8−10] and STA-9090 (Synta, structure not disclosed), or via the oral route, including **4** (BIIB021, Biogen Idec),[11] SNX5422 (Pfizer),[12] XL888 (Exelixis, structure not discolosed), or IPI-493 (Infinity, structure not disclosed), are undergoing clinical trials (Figure 1).[12−15]

At least eight genes have been identified to code for Hsp70 family chaperones. Heat shock cognate (Hsc) 70 and Hsp70 are predominantly cytoplasmically expressed, and mortalin is located predominantly in the mitochondria and Grp78 in the endoplasmic reticulum.[16] These proteins have diverse biological functions including an involvement in nascent protein folding, preventing denatured protein aggregation and modulating the assembly/disassembly of protein complexes.[17,18] In most tissues, Hsc70 is generally ubiquitously and abundantly expressed and believed to undertake the housekeeping functions associated with this family of chaperones. In unstressed cells, Hsp70 is found at lower levels than Hsc70 but its expression is rapidly induced by the transcription factor heat shock factor 1 (HSF-1) following cellular stress. Following cellular stress (including the

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[a] Abbreviations: Hsc, heat shock cognate; Hsp, heat shock protein; HSF-1, heat shock factor 1; FP, fluorescence polarization; NBD, N-terminal nucleotide binding domain; P-gp, P-glycoprotein; SPR, surface plasmon resonance.

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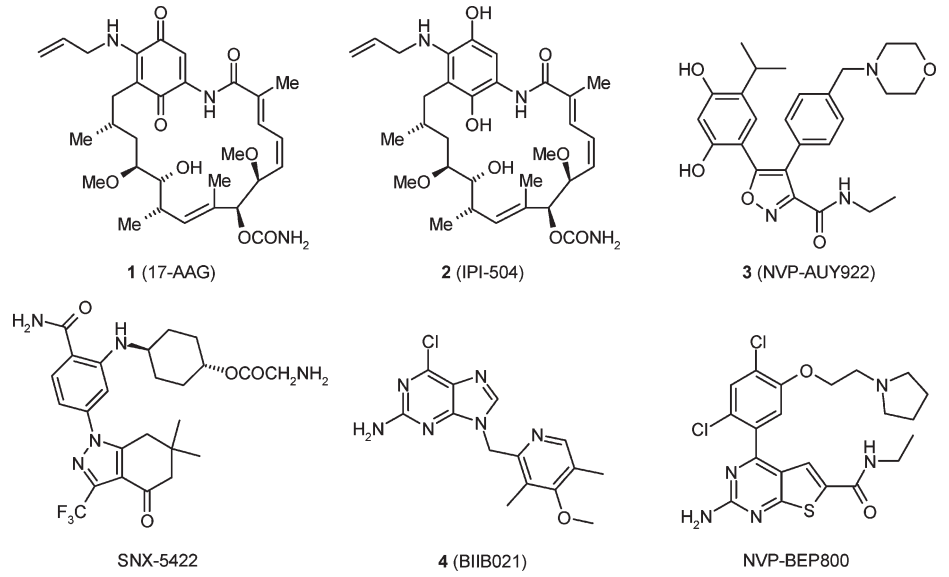

Figure 1. Chemical structures of key Hsp90 inhibitors.

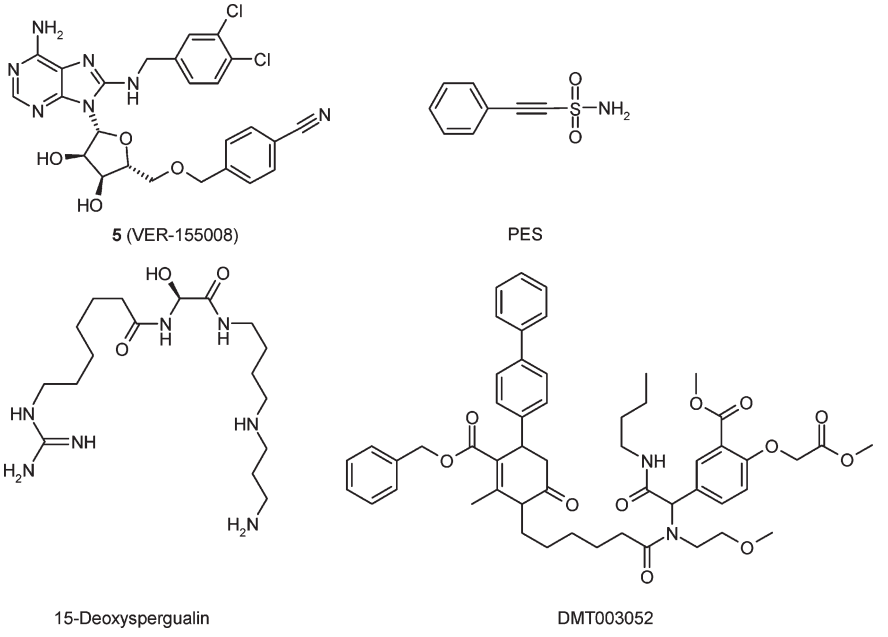

Figure 2. Chemical structures of Hsp70 inhibitors.

stress of oncogenesis), the expression levels of Hsp70 are increased. Hsp70 chaperones, like Hsp90, exist in large multiprotein complexes with various cochaperones including Bag-1, Hip, Hop, Hsp40, HspBP1, and Chip.

One of the major cellular roles of Hsp70 is to protect cells during periods of stress, and the chaperone functions of Hsp70 are often associated with prosurvival and antiapoptotic signaling. Hsp70 has been demonstrated to contribute to cancer cell survival via multiple antiapoptotic functions, and increased expression of Hsp70 has been implicated in resistance to cytotoxic chemotherapeutics. Selective knockdown of Hsp70 by RNAi or antisense results in cell arrest and death in a variety of cancer cell lines.[19,20] Furthermore, localized tumor cell application of Hsp70 antisense expressing adenovirus reduced the growth of orthotopic glioblastoma and breast carcinoma, as well as subcutaneous colon cancer xenografts in mice.[21] Hsp70 plays a key role in the Hsp90 chaperone machinery. Inhibiting the N-terminal ATPase domain of Hsp90 with small molecules results in Hsp70 induction, thereby potentially protecting the cells from Hsp90 inhibitor induced apoptosis.[22−24] Targeting both Hsc70 and Hsp70 with siRNA resulted in proteasome dependent degradation of Hsp90 client proteins, G1 cell-cycle arrest, and extensive tumor-specific apoptosis. Dual depletion of Hsc70 and Hsp70 increased the amount of tumor cell apoptosis following pharmacological Hsp90 inhibition with **1**.[25]

While many have suggested Hsp70 as an excellent target for therapeutic intervention in the treatment of cancer, no drug-like inhibitors have yet emerged.[26,27] Of the inhibitors so far described (Figure 2), only the ATP mimetic **5** (VER-155008) has been demonstrated to clearly bind in the ATPase pocket of Hsp70.[28,29] Of the other molecules, phenylethynesulfonamide (PES) binds to the C-terminal substrate binding domain.[30] The sites of action of 15-deoxyspergulin, dihydropyrimidines, and 3′-sulfogalactolipids have yet to be clearly defined.[31−36] Natural product inhibitors of Hsp90, namely, geldanamycin and its derivatives **1** and **2** along with radicicol, have been

Table 1. Fragment Hit Rate for Hsp90, Hsp70, Three Kinases, and Pin1[a]

enzyme		fragment hit rate (%)
Hsp90	ATPase	4.4
Hsp70	ATPase	0.4
PDPK1	kinase	4.5
CDK2	kinase	3.1
JNK3	kinase	4.0
Pin1	PPIase	0.4

[a] Data from ref 39.

known for over a decade. These have proved invaluable tools in understanding how potent inhibitors bind to the NBD of Hsp90 and have helped to map regions within the binding site critical for inhibitor potency ("hotspots"). Additionally, they have proved to be useful tool compounds for understanding the biology of Hsp90 inhibition in vitro and in vivo. In comparison, no natural product inhibitors targeting the NBD of the Hsp70 family of chaperones have yet been indentified.

Why Has It Been Difficult To "Drug" the ATPase Site of Hsp70 Compared to Hsp90?

Fragment Hit Rate as a Metric for Druggability. Various software tools, for example, SiteMap,[37] use complex algorithms to locate binding sites and predict the druggability of these binding sites. The software uses a complex algorithm that takes into account the size, enclosure, and hydrophobic/hydrophilic nature of the binding site to generate a druggability score (Dscore) for that site. In a large scale analysis of protein structures from the PDB, proteins with a Dscore less than 0.83 were classified as "undruggable", 0.83−0.98 as "difficult", and greater than 0.98 as "druggable".[38] Applying this technology to the ATPase sites of Hsp70 and Hsp90 results in Dscores of 0.95 and 1.04, respectively, placing these targets in the "difficult" and "druggable" categories.[39] Targets falling into the "difficult" category were characterized as containing above average hyrophilicity. To place this in perspective, the average Dscore for four serine/threonine kinases, a class of enzymes known to be highly druggable, was 1.02. On the face of it, this suggests that the two ATPases, Hsp70 and Hsp90, should be amenable to small molecule drug discovery though Hsp70 may prove more difficult because of the more hydrophilic nature of the binding site.

An alternative strategy first presented by scientists at Abbott[40] evaluates protein druggability by using 2D heteronuclear NMR to screen chemical libraries. The observed NMR hit rate was found to correlate closely with a number of surface properties calculated from the binding site. We too have interrogated our NMR fragment hit rate to evaluate the druggability of Hsp70 in comparison to Hsp90 and other known druggable targets such as kinases.[39] Vernalis' fragment library and approach have been described previously.[39,41] The fragments contained within this library have been selected for diversity of chemical functionality, solubility, synthetic tractability, and the absence of chemical functionalities incompatible with druglike properties. The fragment hit rate for Hsp90 was found to be 4.4% and for the three kinases analyzed 3.1− 4.5% (Table 1). In comparison, the fragment hit rate for Hsp70 was extremely poor at only 0.4%. This correlated closely with another difficult to drug target, the prolyl isomerase Pin1.[42] Despite a good predicted druggability score, our fragment hit rate suggests that identifying high affinity, druglike ligands for the NBD of Hsp70 from novel start points is likely to be challenging. The NBD of Hsp70 is lined with side chains from flexible residues, resulting in a highly dynamic binding site that is subject to remodeling dependent on the ligand bound. Adenine is enclosed by two solvent exposed arginines, which adopt different conformations upon ligand binding[28] and exhibit high B-factors in the apo structure.[43] Such features are currently not captured in the calculations by software such as SiteMap and may result in an overestimation of a binding sites druggability. The lack of fragment hits for Hsp70 cannot be attributed to poor fragment library design. A significant amount of effort has been invested in library design to ensure pharmacophore diversity representative of druglike chemical space and physicochemical properties that are complementary to the properties of a diverse set of target binding sites.[39] This analysis clearly emphasizes that it should be possible to identify small fragment hits that are specific to a wide variety of binding sites.

Table 2. Affinity of Hsp70, Hsp90, and Various Kinases for ATP

kinase	ATP K_M (μM)[a]
EGFR	17
FGFR	70
Akt2	254
Aurora B	34
CDK2/cyclin E	3.6
Chk1	1.4
ATPase	**ADP K_D (μM)**
Hsp90	150
Hsp70	0.11−0.5

[a] ATP K_M values are from ref 59.

ATP Affinity, a Hurdle to Cell Activity?

The observed efficacy of an ATP-competitive inhibitor will depend critically on the affinity of ADP and ATP for the site being targeted. In cells, the ATP concentration is typically about 1−5 mM. It therefore stands to reason that targets with a low affinity for ADP and ATP should be easier to inhibit than those with a higher affinity. This is exemplified in the collection of kinases for which inhibitors are in clinical development (Table 2). These kinases are a good test set because the ATP binding sites are largely very similar (thus of equal druggability) but which a wide range of affinities for ATP have been reported.

The affinity of Hsp70 for ADP and ATP is therefore a significant additional hurdle to the generation of cell active, ATP-competitive small molecule inhibitors targeting the nucleotide binding domain. Hsp90 exhibits a relatively weak affinity for ADP with the K_D of yeast Hsp90 measured at 150 μM.[44] Hsp70, in comparison, binds much more tightly to the nucleotide than Hsp90. The K_D of ADP for bovine Hsc70 using a filter binding assay was calculated at 110 nM,[45] human Hsc70 by isothermal calorimetery at 260 nM,[46] and human Hsp70 by surface plasmon resonance (SPR) to be 500 nM.[28] All were measured using purified proteins but with differing methodologies. The K_D is a dissociation constant derived from a thermodynamic equilibrium and the K_M (or Michaelis constant) a constant derived from kinetic measurements. For practical purposes, it is generally accepted that the K_D is approximately equal to the K_M.

For Hsp90, it has been possible to identify small molecule inhibitors with single digit nanomolar K_D values and correspondingly potent cellular activity (cellular IC_{50} less than 100 nM). Despite it proving possible to generate inhibitors of Hsp70 with K_D of about 50 nM, the corresponding cellular activity of these compounds has been poor with IC_{50} generally about 5−15 μM (Table 3).[28,29] For an Hsp70 inhibitor to achieve similar cellular responses to an Hsp90 inhibitor, the

increase in affinity required will be about 300 times more potent. This is simply derived from the ratio of K_D values of Hsp70 and Hsp90 for ATP. Given the highly polar nature of the Hsc70/Hsp70 ATP binding site, this will prove extremely challenging.

An important caveat to this analysis is that the determination of both the K_D for ATP and the inhibitor relies on conditions not truly representative of the cellular nature of the molecular chaperones. Hsp90 and Hsp70 exist in cells as large, dynamic multiprotein complexes in which the associated cochaperone partners switch during the chaperone cycle. As both isolated chaperones exhibit low intrinsic ATPase activity, inhibitor affinity has generally been determined by either indirect (e.g., fluorescence polarization, FP) or direct (e.g., SPR) binding methods.[8,28] For a given series of Hsp90 inhibitors, the measured affinity by FP has generally been closely correlated with cellular growth inhibition and Hsp90 mode of action. For Hsp70, this correlation is less clear and there still exists the distinct possibility that, by focusing on the isolated NBD, we are attempting to drug a cellularly irrelevant form of the enzyme. Further investigation is required to correlate Hsp70 NBD structure, in vitro enzyme inhibition, and cellular activity.

Hsp70 and Hsp90 Have Very Different Nucleotide Binding Domains

Three major structural families of ATPases have been identified and characterized.[1] Most of the ATPases for which structures have been described contain the classical mononucleotide binding motif known as the Walker motif. In comparison, Hsp90 belongs to a smaller subset of GHKL ATPases whose binding site is characterized by a left handed $\beta-\alpha-\beta$ (Bergerat) fold. The GHKL ATPases are named after key family members: gyrase B, Hsp, histidine kinase, and MutL. Hsp70 belongs to a third subset of ATPases that contain an actin fold.[1] In this group of ATPases, the nucleotide binds in a cleft formed at the interface of two domains with a loop containing conserved residues and two β-hairpins forming interactions with adenine and the phosphate groups, respectively.

The very nature of the ATP binding pocket is a clear reason why targeting the NBD of Hsp70 has proved particularly challenging so far. Hsp70 and Hsc70 exhibit a high degree of structural identity (>99%) in the NBD, and therefore, small molecule inhibitors targeted against the NBD of Hsp70 are likely to inhibit Hsc70 with equipotency. The following discussion will focus on the binding site of Hsp70, but the points and ideas raised here are equally applicable and relevant to Hsc70. Since Hsp90 and Hsp70 belong to two very distinct classes of ATPases, the binding sites for these two enzymes are structurally very different (Figure 3). In Hsp70, the β and γ phosphates of ATP (which correspond to a large fraction of the ATP binding affinity) are stabilized in a buried binding mode by very polar interactions with the protein, several of which are water mediated and chelate the cations K^+ and Mg^{2+}.[47,48] Targeting this polar pocket while retaining sensible druglike characteristics presents a significant challenge. Gaining potency via polar interactions is difficult in general because these interactions are already formed with water in the unbound state. Therefore, it is frequently argued that little is gained per interaction in the bound state. Generating "good" multiple polar interactions (via hydrogen bonds) is equally difficult, as they need to be directional and precise.[38,49] Binding to Hsp70 via such interactions, while still trying to meet Lipinski and Veber's criteria of "orally bioavailable druglikeness", is extremely challenging. Trying to mimic these interactions has resulted in compounds with high polar surface area, the number of O + N atoms being >10, and a high molecular weight. For example, **5** has a PSA of 164.4, O + N atoms of 11, and a molecular weight of 555. In contrast to Hsp70, ATP is bound in Hsp90 with the adenine portion buried toward the bottom of the pocket and the α-, β-, γ-phosphates pointing out into the solvent (Figure 3).[50,51] The γ-phosphate of N-terminal bound Hsp90 can interact with residues located within the middle domain of Hsp90 termed the catalytic loop which are critical for ATP hydrolysis.[52,53] This has resulted in a nucleotide binding domain with well balanced hydrophilic/hydrophobic character and has allowed the discovery and development of potent, druglike molecules (e.g., NVP-BEP800, Figure 1, PSA = 93, O + N = 7, and MW = 480).[54,55] Therefore, the highly polar, charged nature of the Hsp70 NBD has made the discovery of potent, cell permeable, druglike molecules exceptionally challenging.

Table 3. K_D and Cellular Potency of Hsp90 and Hsp70 Inhibitors Targeting the NBD[a]

compd	target	K_D (nM)	IC_{50} BT474 (μM)
3	Hsp90	1.7	0.031
4	Hsp90	1.7	0.14
5	Hsp70	300	10.4

[a] Data are from refs 9, 11, and 28.

Lessons for Targeting the ATP Binding Domain of the ATPome

From our observations discussed above, can any lessons and insights be drawn from our experience of successfully developing druglike molecules of the NBD of Hsp90 but being unsuccessful in developing equivalent inhibitors of Hsp70? A naive conclusion may be to suggest targeting only the GHKL family of ATPases and avoiding those that fall into the actin class of ATPases. However, these two groups make up only a

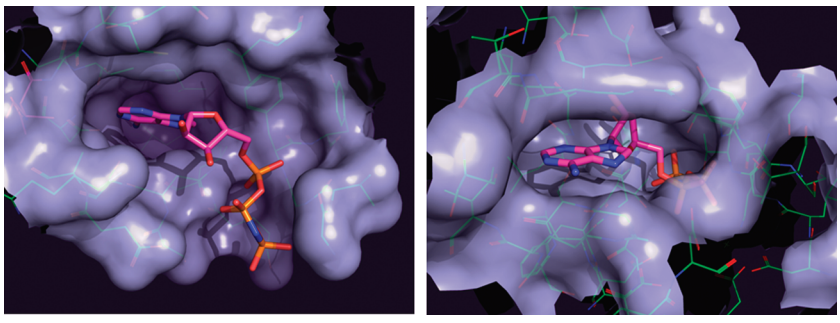

Figure 3. Comparison of ATP binding sites of Hsp90α (left) and Hsp70 (right) with ADPnP bound. X-ray crystallographic structures were visualized using PyMOL and are orientated such that they are viewed looking from solvent down into the binding site.

small proportion of the total ATPome. It should, however, be possible to evaluate the druggability of the ATPome in a more rational way. On the basis of our experience of Hsp90 and Hsp70 small molecule drug discovery, the following ideas could form an initial basis for assessing target druggability.

Evaluation of the Structure of the NBD. A thorough evaluation of the binding mode of ATP along with those factors that contribute to ATP binding should suggest whether the discovery and design of potent, druglike small molecules against any given ATPase could be challenging. For example, nucleotide binding domains where the phosphates of ATP or ADP are buried in the pocket and are critical to ATP affinity, as is the case with Hsp70, are likely to be challenging targets.

Affinity for ATP. ATPases that demonstrate a high affinity for ATP are likely to pose a higher hurdle for obtaining cellular activity than those that bind ATP with lower affinity. For example, purified Hsp70 demonstrates an affinity for ADP around 300-fold greater than purified Hsp90, suggesting that inhibiting Hsp70 in cells is likely to be much more challenging. Assessing the ADP or ATP affinity of a cellularly relevant form of the target (preferably in the presence of any cofactors) should be a high priority. The possibility that the target ATPase exists in forms that have different affinities for ADP or ATP cannot be discounted, and identification of a low affinity ADP or ATP form could enhance the target's overall druggability.

Form of Enzyme. Following from above, many ATPases exist in large and/or dynamic multiprotein complexes. Understanding the biological mechanism and role of any cofactors may be critical in successfully drugging a given ATPase.

Fragment Hit Rate. Fragment hit rate is starting to gain credence as a potential measure of target druggability. Early investment in a target is required before the fragment hit rate is known and could serve as a useful filter to set priorities for target progression.

Future Perspectives for Targeting Hsp70

Drugs targeting the Hsp70 family of chaperones have the potential to be critical therapeutic targets for a wide range of diseases, not just cancer. Given the difficulty of designing and discovering potent, selective, cell active small molecule inhibitors targeted against the NBD of these chaperones, what other avenues remain open for exploitation? Targeting the substrate binding domain is one obvious approach, and this is where the small molecule PES is suggested to bind. However, like the NBD, this site is charged and highly polar in nature with no crystal structure yet published, and it may therefore prove as challenging to drug as the NBD. Disrupting the protein−protein interactions of Hsp70 with specific cochaperones, such as Hop or BAG-1, is another potential area that may be worth exploring. Supporting this approach, short peptides derived from the C-terminus of BAG-1 can disrupt the interaction between Hsp70 and BAG-1 and decrease the growth of breast cancer cells.[56] Disrupting protein−protein interactions with small molecules is notoriously challenging and an area of drug discovery that is still in its infancy, and thus, this approach will not be without its own challenges. An alternative may be to target the cochaperones directly, as disrupting one part of the chaperone cycle may be sufficient to inhibit Hsp70 function and elicit an antitumor response. No independent enzymatic activity for any of Hsp70 cochaperones has, however, so far been detected. A final approach could be to target the transcription factor HSF-1 responsible for Hsp70 up-regulation in response to stress, oncogenesis, and Hsp90 inhibition. Several tool compounds that inhibit HSF-1 have been identified. These have been shown to inhibit HSF-1 activation and trimerization, the binding of activated HSF-1 to the DNA heat shock element, or to inhibit HSF-1 mediated transcription or translation.[31,57,58]

Genetic and biochemical data support Hsp70 as an exciting therapeutic target for a wide range of therapeutic indications and not just cancer. Hsp70 family members are involved in a wide range of cellular processes in normal cells as well as disease states. Therefore, a potential issue for targeting the Hsp70 family of molecular chaperones is one of diseased versus normal cell selectivity. Inhibiting the global function of Hsc70 and Hsp70 (as would be the case with a small molecule inhibitor targeted against the NBD) could prove too toxic to normal cells to be of any therapeutic benefit. Similar arguments were presented in the early days for Hsp90 inhibitors, but tumor cells have been shown to have a higher dependence on the Hsp90 chaperoning pathway for survival, and these fears have so far proved unfounded. Potent small molecule inhibitors with appropriate pharmaceutical and pharmacokinetic properties targeting Hsp70 and Hsc70 as well as distinctive processes such as the antiapoptotic function of Hsp70 are needed to address these questions.

In summary, discovering small molecule, ATP competitive inhibitors of the nucleotide binding domain of Hsp70 has proved extremely challenging. However, the lessons learned from attempting to drug this class of ATPases leave us better equipped to evaluate the druggability of novel ATPases and ATP binding proteins.

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Biography

Andrew J. Massey graduated with an M.A. in Natural Sciences from Cambridge University, U.K., in 1997. He then received a Ph.D. in 2001 at the ICRF (now Cancer Research UK) Clare Hall Laboratories with Dr. Peter Karran, studying DNA repair and the cytotoxic effects of cisplatin and DNA thiobases. Following a short stint as a postdoctoral research fellow at Clare Hall, he joined Vernalis (then RiboTargets) in 2002 where he is currently head of the oncology team. He has coled drug discovery programs against the molecular chaperones Hsp90 and Hsp70 and more recently the checkpoint kinase Chk1.

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