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Identification of Hsp70 modulators through modeling of the substrate binding domain

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ABSTRACT

The design, synthesis and preliminary activity of small molecular weight modulators of the heat shock protein 70 (Hsp70) are described. The compounds provide a starting point for the synthesis of novel tools to decipher Hsp70 biology.

Heat shock proteins (Hsps) are a family of highly conserved molecular chaperones responsible for the folding of nascent protein chains, for the refolding of misfolded proteins, and for the degradation of polypeptide substrates that are unable to achieve their native conformations.[1] Hsp70s have also become important pharmaceutical targets due to their profound roles in multiple disease states.[2] For example, Hsp70s promote the growth of cancer cells[3] and inhibit cancer cell senescence.[4] Hsp70s are overexpressed in several cancers of different origin, and overexpression correlates with increased cell proliferation, poor differentiation, lymph node metastases, and poor therapeutic out-come in breast cancer.[5] Additionally, Hsp70 is overexpressed after cerebral ischaemia.[6] Hsp70 upregulation might also prove valuable as the chaperone helps select aberrant, toxic proteins for degradation.[7] Thus, novel small molecule Hsp70 agonists or antagonists could become valuable leads for drug discovery.

Relatively few small molecules that interact with Hsp70 have been identified. Nevertheless, several products from Biginelli and Ugi multicomponent reactions (MCR) are known that alter various steps in the Hsp70 kinetic cycle.[8] The active compounds modulate the ATP hydrolytic rate, an event that is catalyzed by the N-terminal, ATPase domain in Hsp70s. The binding and hydrolysis of ATP, and the release of ADP are linked to the binding and entrapment of polypeptide substrates in the C-terminal half of Hsp70. Some of these agents also inhibit the proliferation of transformed cell lines and the growth of the malarial parasite, which—like cancer cells—requires high levels of Hsp70s for survival.[9] Other Hsp70 modulators have been identified that are peptide mimics.[10] To our knowledge, the efficacy of these peptide mimics has not been further optimized. Thus, in order to expand the repertoire of Hsp70 modulators, we now describe our efforts toward the in silico design of small molecules that interact with the Hsp70 peptide recognition site. In this study, we have modeled compounds into known Hsp70 pdb structures instead of using 'real' iterative X-ray crystallography studies due to lack of access to the latter and due to its limited predictive capabilities. Hsp70 peptide substrates exhibit overall hydrophobic character, and the binding of these hydrophobic peptides stimulates ATP hydrolysis.[11] Some well-defined substrates contain a tri-Leu motif.[12] The high resolution crystal structure of the peptide binding domain of a bacterial Hsp70, DnaK, in complex with a tri-Leu-containing peptide served as a starting point to design substrates that might mimic the requirements for peptide binding site recognition.[13] We chose the bacterial Hsp70 homolog since the structure of the peptide binding domain from human Hsp70 in complex with a bound peptide substrate has not been reported. However, a comparison of the amino acid sequences from several Hsp70 proteins from different organisms show high homology in the substrate recognition site (Supplementary data). Additionally, canonical Hsp70 peptides are

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known to bind and activate the ATPase activities of *Escherichia coli*, yeast and human Hsp70s.[14] Thus, the peptide recognition sequence in a bacterial Hsp70 is a valid starting point for the design of modulators that might exhibit agonist or antagonist effects on peptide binding.

The interaction of the recognition motif with the bacterial Hsp70 is shown in Figure 1. A highly hydrophobic channel of 14 Å length accommodates a hydrophobic elongated 5-mer peptide, NR**LLLT**G (denoted in bold using the single letter amino acid code). The recognition motif is highly hydrophobic and is flanked with hydrophilic amino acids directed toward the occluded bound water. Clearly, all L and the T residues make extensive van-der-Waals interactions to Hsp70 (Fig. 1).

The peptide also forms an extensive hydrogen bond network involving the backbone amide protons (H-bond donors) and carbonyls (H-bond acceptors) (Fig 1). Overall, the interaction is dominated by strong shape complementarity of the hydrophobic, isovaleric side chains in addition to the hydrogen bond network. A substrate mimic and (ant)agonist design must therefore recapitulate the shape complementarity and the electrostatic interactions (van-der-Waals and hydrogen bonding) in order to bind. In addition to the hydrophobic core of the peptide (LLLT), many peptides that bind and modulate Hsp70 function also contain flanking basic amino acids.[15] Thus, synthetic approaches to mimic peptide recognition should allow for the introduction of basic amino acid side chains. For the design of recognition side binders we choose the Ugi isocyanide-based MCR since it allows for the fast and efficient assembly of molecules resembling the LLLT motif.[16] Ugi reaction products are also known to be peptide mimetic and therefore in a general sense resemble the model peptide. In a second step, such molecules can be morphed into heterocyclic motifs by a great variety of MCR modifications. However, Ugi products show fewer secondary amide bonds compared to peptides and therefore possess altered physico-chemical and biological properties, for example, better water solubility and enhanced protease resistance. We reasoned that a Ugi reaction product of isobutyric aldehyde, L derived isocyanide, an elongated primary amine, and a carboxylic acid should be well suited to mimic the hydrophobic nature, the shape of the RLLLT peptide, and the required hydrogen bond network. Additionally, the primary Ugi reaction product allows for further modification via the ester group, for example, the introduction of basic side moieties to mimic the flanking basic amino acid side chains (Scheme 1). Consequently, we generated a 3D model of the synthetic target and modeled it into the Hsp70 peptide binding site using MOLOC software (Fig. 2).[17]

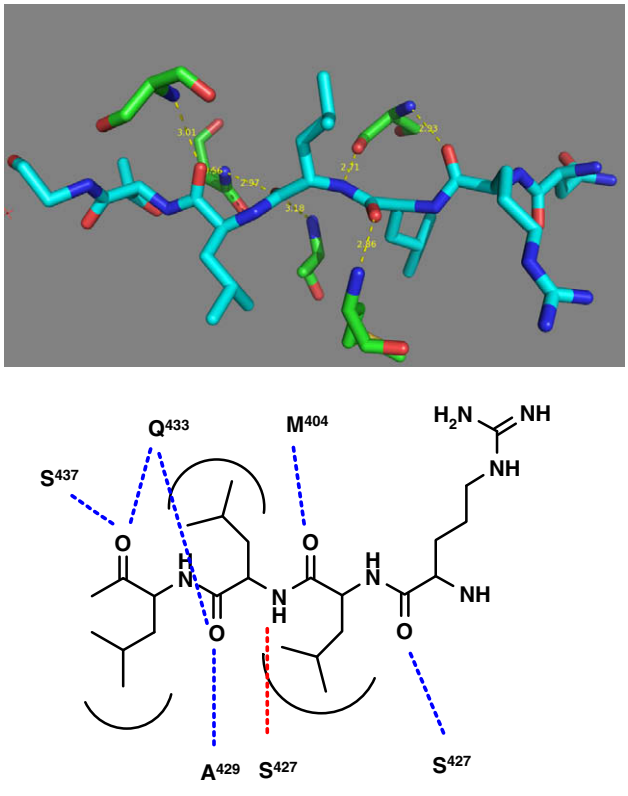
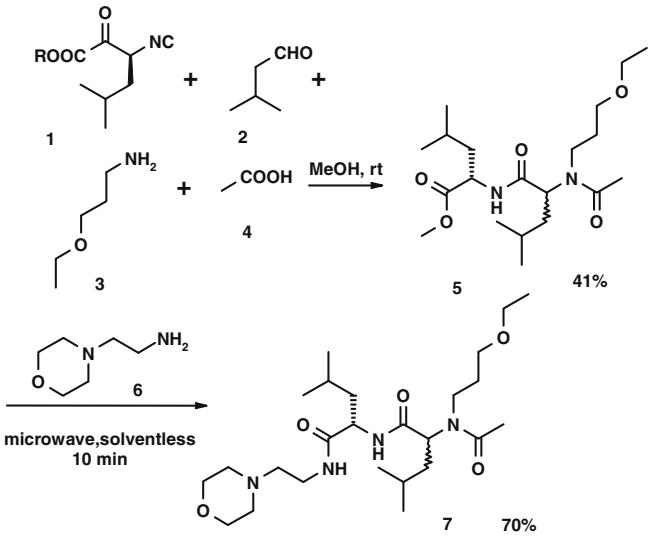


Figure 1. X-ray crystal structure and schematic representation of the binding site of the peptide NRLLLTG (blue sticks) to Hsp70 (green lines) showing the hydrogen bond network (pdb identifier:1DKZ). The peptide forms 7 hydrogen bonds with residues within the peptide binding pocket of Hsp70. Of note, neither the amide protons of N-terminal L nor of the opposite C-terminal L are involved in hydrogen bonding. Rendering software PyMol is used.

As anticipated, the model suggests that there is a good shape overlap between the recognition sequence of the peptide and ***S,S*-7**. The amides in both structures perfectly overlap, thus allowing ***S,S*-7** to undergo a similar hydrogen bonding network as observed in the structure depicted in Figure 1. The Ugi backbone also lacks the hydrogen bonds of the two NH amides in the peptide-Hsp70 structure by providing a tertiary amide at this position. The morpholino ethyl amide moiety of ***S,S*-7** forms hydrophobic interactions with Hsp70 and helps confer water solubility. The propylethylether moiety of ***S,S*-7** again provides additional van-der-Waals contacts with a hydrophobic patch at the Hsp70 surface, thus mimicking the hydrophobic $(CH_2)_3$ fragment of the R side chain of the peptide.

Next, we efficiently synthesized the target compound by a short two-step protocol involving a first Ugi reaction and a recently described direct amidation of the ester group. The Ugi reaction of the racemic L derived isocyanide **1**, isobutyric aldehyde **2**, amine **3** and acetic acid **4** gave the expected product **5** in 41% yield as a 65:35 mixture of two diastereomers. These were separated by silica gel chromatography. Next, we introduced the morpholino ethyl amide side chain using a direct amidation protocol, thus avoiding the laborious and classical saponification–activation–amidation sequence.[18] The reaction was performed under solventless conditions using microwave. This protocol has been shown to be valuable for the direct amidation to produce arrays of screening compounds in our laboratory.[19]

We then screened the designed compounds for their ability to interact with Hsp70 using a robust, single turnover assay that specifically measures the rate of ATP hydrolysis.[8a] In this assay, the



Scheme 1. Synthesis of a designed Hsp70-binding small molecule via an Ugi MCR.

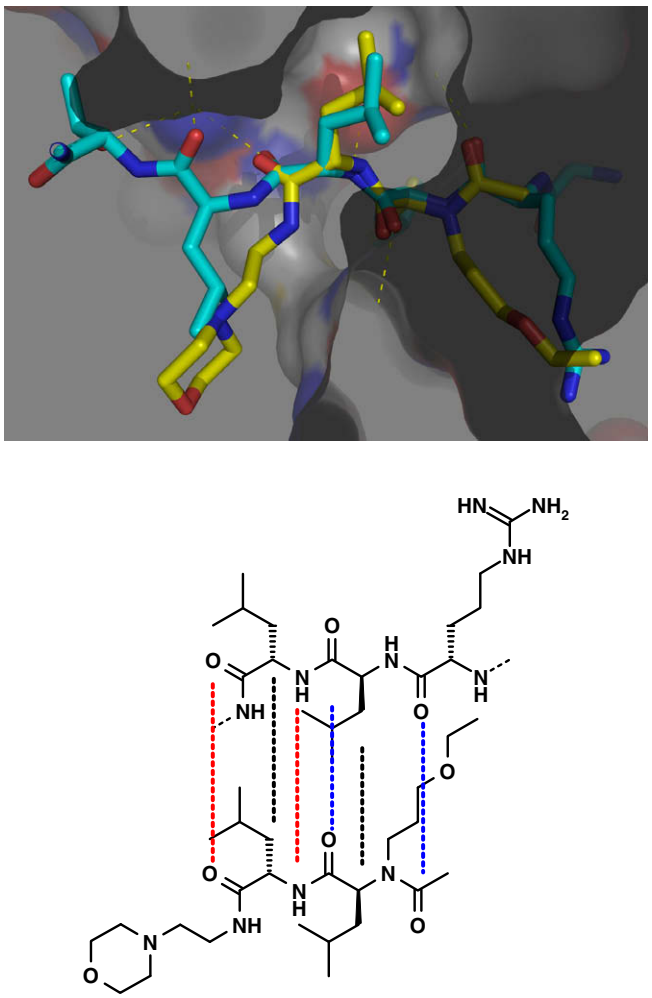


Figure 2. Above: Cut-away view of compound ***S,S*-7** (yellow sticks) modeled into the peptide binding channel of Hsp70 (surface representation) and overlapped with the recognition peptide (blue sticks) using pdb structure 1DKZ. The hydrogen bond network is shown as dotted yellow lines. Below: Comparison of the fragments of ***S,S*-7** and the LLR moiety of the peptide.

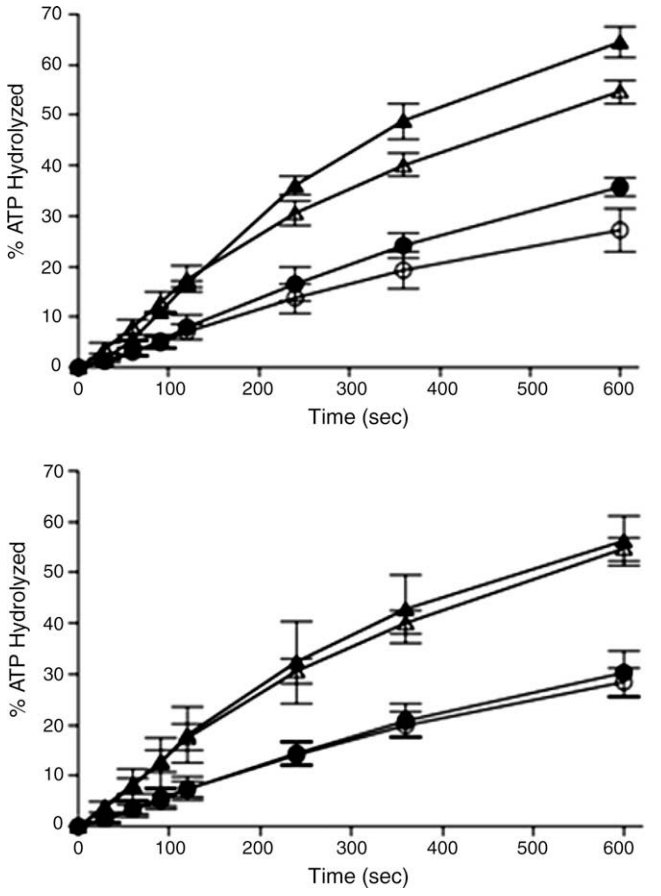


Figure 3. Diastereoslective Hsp70 activation. Reactions to measure the single-turnover hydrolysis of yeast Hsp70, Ssa1p, were assembled and performed as previously described[8a] in the absence or presence of 10 μM ala-p5, a peptide activator of Ssa1p ATP hydrolysis,[20] and in the absence or presence of 300 μM of the two diastereomers (above and below) of compound **7**. Open circle, no addition; Open triangle, ala-p5; Closed circle, compound; Closed triangle, Compound and ala-p5. Reactions lacking an added reagent contained DMSO to maintain the reaction volume. Data represent the means of 3–6 independent experiments, +/− SD. The data indicate that 10 μM of ala-p5 represents a sub-saturating concentration in this assay.

ala-p5 peptide (ALLLSAPRR) stimulates the rate of ATP hydrolysis by ~40% (Fig. 3) when used at a final concentration of 10 μM. The compounds were employed at a final concentration of 300 μM in order to most effectively assess their impact in the presence of the peptide. Consequently, we next added our compounds into this assay in the presence or absence of the peptide in order to discover whether the agents exhibited antagonist- (inhibition) or agonist (activation)-like activity. This analysis was possible because a concentration of peptide was chosen such that either activity could be measured (Fig. 4).

First, we observed that each compound was soluble even at the highest assay concentrations (~1 mM), since by design we included the morpholino ethyl side chain ***S,S*-7**. Second, we found that one diastereomer of compound **7** activated Hsp70 in either the presence of absence of peptide (Fig. 3). The other diastereomer, however, showed no effect despite very high structural resemblance. This result shows that compound **7** selectively activates Hsp70. We speculate that **7** binds within the peptide recognition channel and accelerates ATP hydrolysis. In the presence of limiting peptide, the agent acts as a peptide agonist and potentiates the effect of the peptide substrate.

In summary, we have described for the first time a small molecular weight peptide-binding mimic that enhances Hsp70 activity.

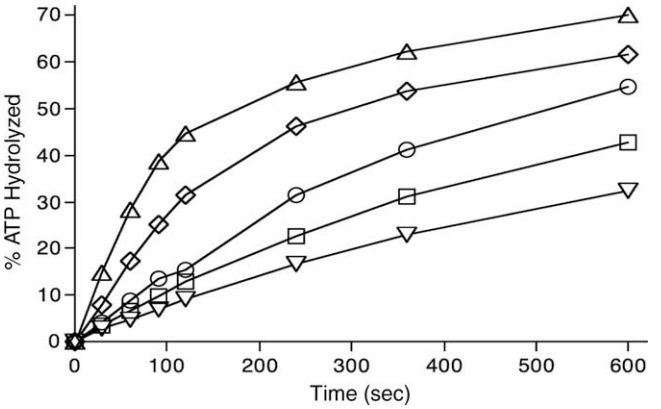


Figure 4. Single turnover ATPase reactions with the yeast Hsp70, Ssa1, were performed as described in the legend to Figure 3 in the presence of the following concentrations of peptide ala-p5: inverted triangle, DMSO control; square, 4 μM; circle, 10 μM; diamond, 50 μM; triangle, 200 μM.

Although the potency of the compound is poor, this result serves as a starting point for further enhancement of our rationale struc-

ture-based design, and it has to be considered that the M_w of compound **7** is only a fraction of the M_w of a bona fide peptide substrate for Hsp70. In addition, the compound was efficiently synthesized using a two-step sequence employing a key Ugi MCR. Clearly, compound **7**—although showing encouraging protein-based activity and an unusual agonistic action—requires further investigation to dissect its mode-of-action. Also, libraries based on higher numbers of compounds will be produced, which will allow us to isolate compounds with increased potencies beyond the current hit. Current efforts in our laboratories are directed towards these goals.

Supplementary data

Supplementary data (sequence alignment of Hsp70 from different species, synthetic protocol, analytical data of the new compounds) associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2009.04.062.

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