Letters

Novel Adenosine-Derived Inhibitors of 70 kDa Heat Shock Protein, Discovered Through Structure-Based Design†

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Abstract: The design and synthesis of novel adenosine-derived inhibitors of HSP70, guided by modeling and X-ray crystallographic structures of these compounds in complex with HSC70/BAG-1, is described. Examples exhibited submicromolar affinity for HSP70, were highly selective over HSP90, and some displayed potency against HCT116 cells. Exposure of compound **12** to HCT116 cells caused significant reduction in cellular levels of Raf-1 and Her2 at concentrations similar to that which caused cell growth arrest.

Heat shock proteins (HSPs[a]) are molecular chaperones that assist protein folding processes within the cell.[1,2] They are made up of families containing both heat-inducible and constitutively expressed members, defined by their different molecular weights; the 70 kDa HSP isoforms (HSP70) are one such family.[3]

There is growing evidence to support HSP70 as a potential anticancer target.[4] An antisense construct targeting HSP70 proved effective at causing cell arrest and death in a variety of human tumor cell lines.[5] Locoregional application of adenovirus expressing antisense HSP70 cDNA eradicated xenografts of glioblastoma, breast, and colon carcinoma. Analysis of the expression levels of cytosolic HSP70 family members in human cancer and normal cell lines, and their subsequent knock-down using RNAi technology, also revealed the importance of HSP70 in human cancer.[6]

HSP70 isoforms are composed of an *N*-terminal ATPase domain, which binds ATP (**1**) and hydrolyses it to ADP (**2**), a substrate-binding domain, and a *C*-terminal domain.[7−10] The presence of a peptide in the substrate binding domain stimulates the ATPase activity of HSP70, increasing its normally slow rate of ATP hydrolysis. HSP70 is a weak ATPase, and maximal activity requires cochaperones, such as HSP40.[11] When ATP is hydrolyzed to ADP, the binding pocket of HSP70 closes, tightly binding the peptide chain, preventing it from aggregating.

A nucleotide exchange factor, such as BCL-2-associated athanogene-1 (BAG-1), stimulates the release of ADP, binding of fresh ATP, and opening of the binding pocket to allow substrate release.[12]

Molecules demonstrated to modulate HSP70 function to date do so by affecting the interaction of HSP70 with its cochaperones.[13,14] There are no inhibitors known to bind to the ATPase domain of HSP70, and it was thought that inhibition of this site would be a suitable point for disrupting the function of this protein for potential use in the treatment of cancer.

A fluorescence polarization (FP) assay was developed in which measurement of binding competition with a fluorescein-labeled ATP-based probe, N^6-(6-amino)hexyl-ATP-5-FAM (K_D 0.4 μM) was determined. The HSP70 IC_{50} of endogenous ligands ATP (**1**) and ADP (**2**) were found to be 0.5 μM (Table 1). Although these values were close to the limit of detection of the assay (HSP70 conc 0.4 μM), this format was considered to be appropriate for the discovery of ATP-competitive compounds.

Adenosine (**3**), a fragment of ATP (**1**), was chosen as a start-point for initial investigations, and commercially available adenosine analogues were screened in the FP assay. In comparison with compound **3** (HSP70 IC_{50} 560 μM), the 8-amino derivative **4**[15] (HSP70 IC_{50} 9.4 μM) was approximately 60-fold more potent.

Compound **4** was also shown to inhibit the growth of human colon tumor 116 (HCT116) cells in a sulforhodamine B (SRB) growth inhibition assay (GI_{50} 0.05 μM).[16,17] The cytotoxic activity of **4** has been previously reported,[18] but the mismatch between HSP70 FP IC_{50} and HCT116 GI_{50}, and later PD marker studies (see below), indicated that the cytotoxic mechanism of action of compound **4** must be predominantly through a route other than HSP70 inhibition.

The crystal form of HSP70 obtained gave low resolution diffraction data and was not amenable to the rapid generation of ligand-bound structures. Analogous HSP70 isoforms were therefore investigated for their suitability as surrogates. The 70 kDa heat shock cognate (HSC70), which is constitutively expressed, shares high sequence and structural homology with HSP70 in the ATPase domain. Encouragingly, crystals of HSC70 in complex with BAG-1[19] were suitable for soaking of ligands and collection of diffraction data.

X-ray crystal structures of both **1**/HSC70/BAG-1 and **4**/HSC70/BAG-1 were obtained, and compound **4** was shown to overlay with the adenosine moiety of ATP (**1**) (Figure 1).[20] An intramolecular hydrogen bond was observed between the 8-amino substituent on the purine ring and the 5-hydroxymethyl substituent of the tetrahydrofuran ring of **4**. It was postulated that this preorganizes the bound conformation of the ligand by influencing the syn/anti equilibrium of the two rings as well as stabilizing the bound conformation itself.

Elaboration of the 8-amino group of compound **4** was investigated initially (Table 1). *N*-Methylation (**5**) and *N*-benzylation (**6**) retained potency in the HSP70 FP assay. Further substitution on the benzene ring of compound **6** was explored, and it was shown that the 3,4-dichlorobenzyl analogue **7** had similar enzyme potency to compound **4**, with reduced activity in the SRB assay (GI_{50} 26 μM). An X-ray crystal structure of

† Coordinates of HSC70/BAG-1 complexes with compounds **1**, **4**, **7**, **12**, and **15** have been deposited in the Protein Data Bank under accession codes 3FZF, 3FZH, 3FZK, 3FZL, and 3FZM.

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[a] Abbreviations: BAG-1, BCL-2-associated athanogene-1; FP, fluorescence polarization; HCT116, human colon tumor 116; HSC70, 70 kDa heat shock cognate; HSP70, 70 kDa heat shock protein; SPR, surface plasmon resonance; SRB, sulforhodamine B.

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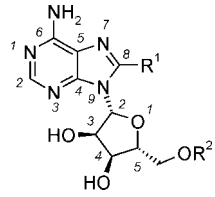
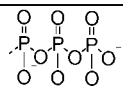
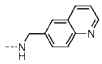
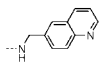
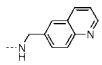
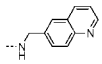
Table 1. Biological Data for HSP70 Inhibitors



	R^1	R^2	HSP70 FP IC_{50} (μM)[a]	HSP70 K_D (μM)[a]	HCT 116 GI_{50} (μM)[a]
1	H		0.5	ND[b]	-
2	H		0.5	0.5	-
3	H	H	560	-	-
4	NH_2	H	9.4	11	0.05[c]
5	NHMe	H	17	-	13
6		H	10	-	>80
7		H	9.1	-	26
8		H	4.9	-	>80
9		H	2.9	-	>80
10		Me	3.2	-	>80
11			2.4	-	>80
12			0.5	0.3	5
13			1.3	0.2	>80
14			1.7	4.2	13
15			0.9	0.05	>80
16			0.6	0.06	27
17			<0.4	0.12	18

[a] All IC_{50}, K_D and GI_{50} values are the mean of at least two determinations and are rounded to two significant figures where appropriate. [b] K_D could not be determined due to compound behavior on chip surface. [c] Low GI_{50} due to predominantly non-HSP70 mechanism.

compound **7** in complex with HSC70/BAG-1 (Figure 2) showed that a movement in the Arg272 residue allowed for an additional π-stacking interaction with the 3,4-dichlorobenzyl substituent while maintaining the intramolecular hydrogen bond. Tyr15 also occupied a different position in this crystal structure, with 75% occupancy. With a view to increasing enzyme potency by optimizing π-stacking with Arg272, bicyclic analogues **8** and **9** were synthesized. Encouragingly, the quinolyl analogue **9** was 3-fold more potent in the HSP70 FP assay in comparison with compound **4**.

Study of the X-ray structure of compound **7**/HSC70/BAG-1 indicated that modification of the 5-hydroxymethyl substituent

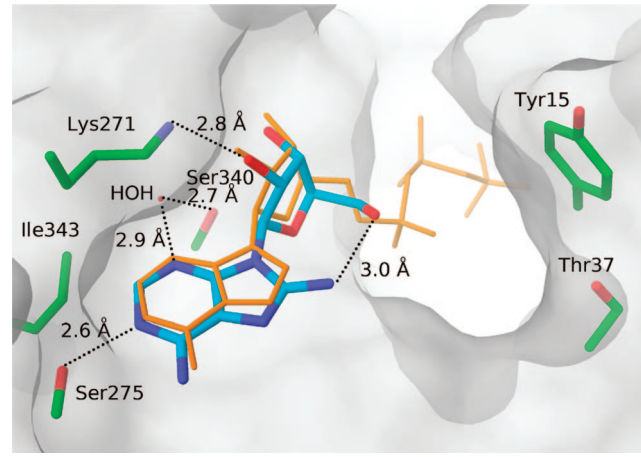


Figure 1. X-ray crystal structure of **4** (blue)/HSC70/BAG-1 (green) superimposed with ATP (**1**, orange), from an X-ray crystal structure of ATP (**1**)/HSC70/BAG-1.

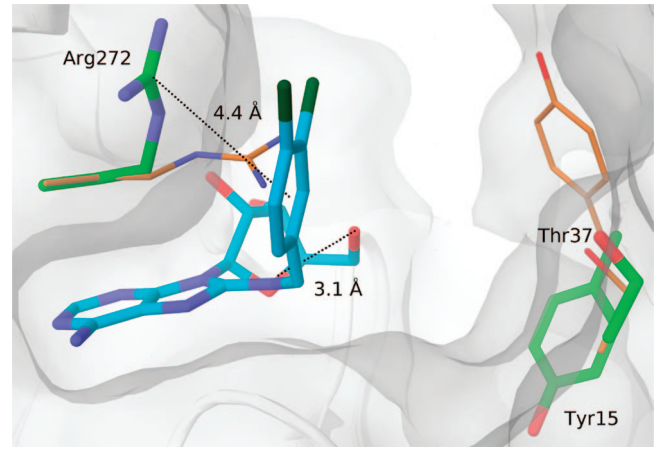


Figure 2. X-ray crystal structure of compound **7** (blue) in complex with HSC70/BAG-1 (green) showing π-stacking between the ligand and Arg272. Alternate conformers of Tyr15 and Arg272 from the X-ray structure of compound **4**/HSC70/BAG-1 are shown in orange.

of the tetrahydrofuran ring could enable interactions with proximal Tyr15 or Thr37 residues, and so synthetic elaboration at this position was investigated. The methyl analogue **10** (HSP70 IC_{50} 3 μM) was 3-fold more potent than compound **7**, and benzyl substitution (**11**) was similarly well-tolerated in the HSP70 FP assay.

In silico docking studies with rDock[21,22] and analysis of superimposed X-ray structures suggested that it was possible to optimize π-stacking between Arg272, Tyr15, and the 8- and 5-substituents of the adenosine-derived ligands. Virtual libraries of ligands were created with MOE;[23] only docked poses that maintained the experimental binding mode for 8-aminoadenosine (**4**) were considered. Substituents were prioritized for synthesis based on interactions with the protein. In particular, combinations of aromatic rings with electron withdrawing or donating groups were chosen to explore stacking interactions.

Incorporation of a 4-cyano group on the 5-benzyloxymethyl moiety of compound **11** was investigated, and gave rise to a further increase in HSP70 potency. Compound **12** had an HSP70 FP IC_{50} of 0.5 μM and an HCT116 GI_{50} of 5 μM and was the most potent example on cells in this series. Figure 3 illustrates the elaborate π-stacking network seen in the X-ray structure obtained of compound **12**/HSC70/BAG-1. The additional potency of this compound is likely due to water displacement and

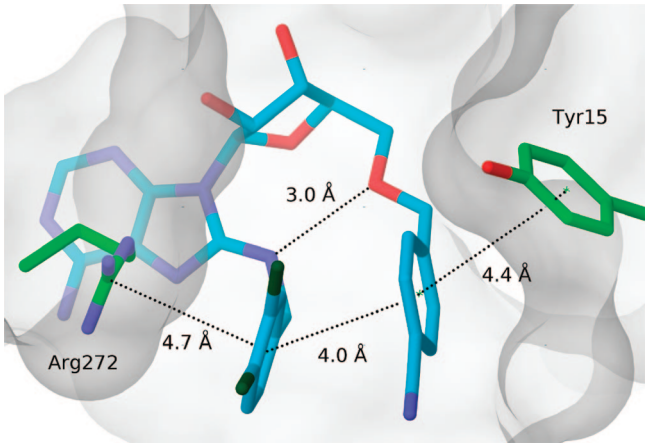


Figure 3. Inter- and intramolecular π-stacking interactions in the X-ray crystal structure of compound **12**/HSC70/BAG-1 in addition to the intramolecular H-bond between the 8-NH and 5-CH_2O of **12**.

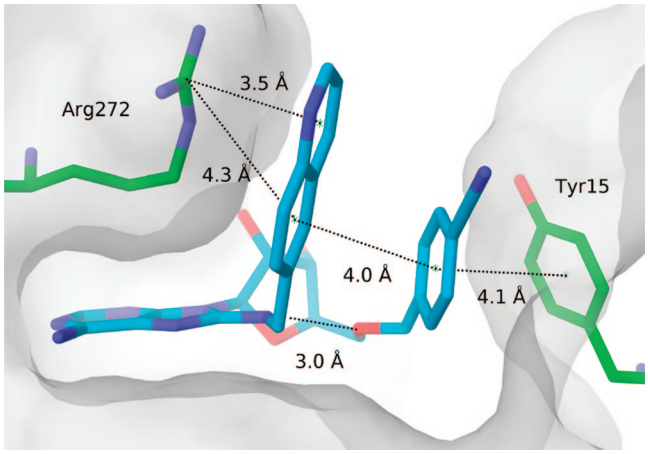


Figure 4. Improved π-stacking in the X-ray structure of compound **15**/HSC70/BAG-1.

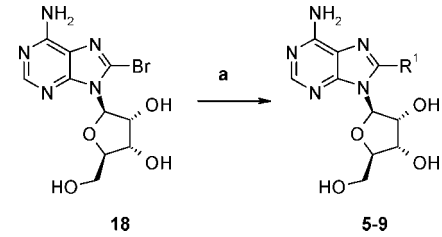
the stabilization of the bound ligand by two intramolecular interactions (H-bond and π-stacking).

As the limit of detection of the FP assay had been reached, an alternative method of measuring ligand binding was required. A direct binding assay was developed using surface plasmon resonance (SPR) in which a double His-tagged HSP70 construct was immobilized on a Biacore sensor chip. The HSP70 K_Ds of compounds **2**, **4**, and **12** were determined to be 0.5, 11, and 0.3 μM, respectively. This was in line with expectations for compounds with FP IC_{50}s equal or greater to the concentration of protein in the assay. For those compounds with an HSP70 FP IC_{50} <1 μM, the K_D was determined by SPR and used to generate subsequent SAR.

Further analogues of **12** were synthesized to optimize intramolecular π-stacking between the 8-benzylamino and 5-benzyloxymethyl substituents or interaction between the 5-substituent and Tyr15/Thr37 (compounds **13**−**17**). Compound **15** (HSP70 K_D 0.05 μM), which incorporates the preferred quinolyl substituent from compound **9** and the benzonitrile moiety of compound **12**, was the most potent ligand in this series. The X-ray structure of this compound with HSC70/BAG-1 (Figure 4) demonstrated the improved overlap between Arg272 and the quinoline ring.

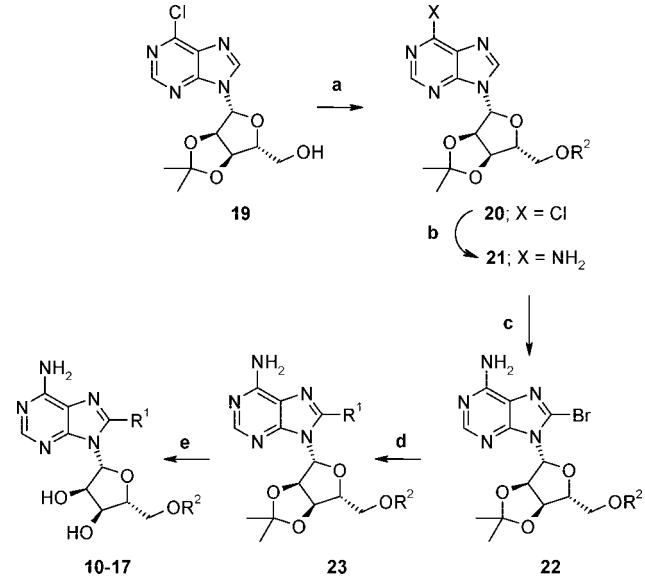
Compounds **5**,[24] **6**,[25] and **7**−**9** were synthesized by treatment of 8-bromoadenosine **18** with the corresponding amine, using a procedure based on the literature[25] synthesis of compound **6** (Scheme 1).

Scheme 1[a]



[a] Reagents: (a) R^1H, EtOH, 170 °C, μwave, 18−90%.

Scheme 2[a]



[a] Reagents: (a) R^2Br, DMF; NaH, 85%; (b) 8 N $NH_4OH_{(aq)}$, 100 °C, μwave; 57%; (c) Br_2, 10% $Na_2HPO_{4(aq)}$, 1,4-dioxane, 75%; (d) R^1NH_2, EtOH, 150 °C, μwave, 74%; (e) H_2O, CF_3CO_2H; MP-carbonate, 59%.

Compounds **10**−**17** were synthesized according to the procedure outlined in Scheme 2. Compound **19**[26] underwent alkylation with the corresponding halide in the presence of sodium hydride. The 6-Cl of the product **20** was converted to the primary amine by treatment with ammonium hydroxide to give intermediates of type **21**, which selectively underwent bromination at the 8-position upon treatment with bromine to give compounds of type **22**. Treatment of **22** with the corresponding amine, followed by deprotection of the acetonide moiety of intermediates **23** with trifluoroacetic acid, afforded **10**−**17**.

It has been demonstrated recently that dual targeting of HSC70 and HSP70 small interfering RNAs results in degradation of the HSP90 client proteins, Raf-1 and Her2.[27] To add support to the premise that the new compounds exert their antiproliferative effects via inhibition of HSP70, the effect of compound **12** on the PD markers Raf-1 and Her2 (known clients of HSP90 and down-regulated upon HSC70/HSP70 inhibition by siRNA) was explored. As shown in Figure 5, exposure of HCT116 cells to compound **12** caused significant reduction in cellular levels of Her2 and Raf-1 at concentrations similar to that which caused cell growth arrest. Compound **4** did not down-regulate Her2 at 2 × GI_{50}, indicating that the cytotoxic mechanism of action of this compound must be through a route other than HSP70 inhibition. Additionally, all examples in Table 1 had HSP90 FP IC_{50} > 400 μM.[28]

In conclusion, a first-in-class series of adenosine-derived HSP70 inhibitors that target the ATPase binding domain has

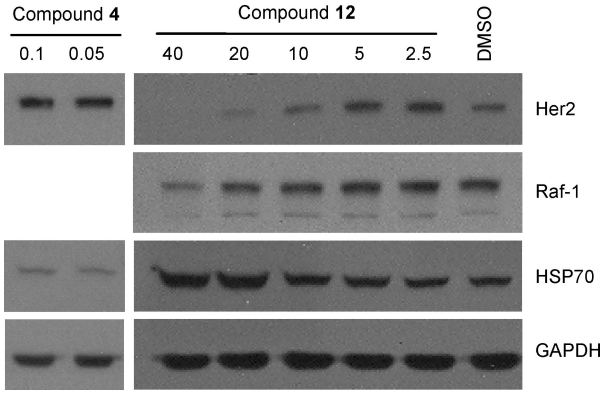


Figure 5. Effects of compounds **4** and **12** on expression of Raf-1, HSP70, and Her2 in HCT116 cells, as determined by Western blot. Compounds were dosed for 24 h at concentrations of 0.05 or 0.1 μM or 2.5−40 μM. GAPDH was used as a loading control.

been described. X-ray crystal structures of these compounds in complex with HSC70/BAG-1, a surrogate construct for the target enzyme, rationally explained the observed improvements in HSP70 potency. Exposure of compound **12** to HCT116 cells caused significant reduction in cellular levels of HSP90 client proteins at concentrations similar to that which caused cell growth arrest. Further reports will describe SAR in the wider series as single agents and in combination with HSP90 inhibitors.

Acknowledgment. We thank Rod Hubbard, James Davidson, and Terry Hart for valuable discussions, Heather Simmonite for expert assistance with compound structure determination by NMR spectroscopy, and Loic Le Strat for chromatographic support.

Supporting Information Available: Synthetic procedures with supporting spectroscopic and chromatographic data, assay protocols, X-ray crystallographic parameters, and protein production methods. This material is available free of charge via the Internet at http://pubs.acs.org.

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(20) Figures were made with Maestro v 8.5 (Schrödinger, LLC: New York).
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