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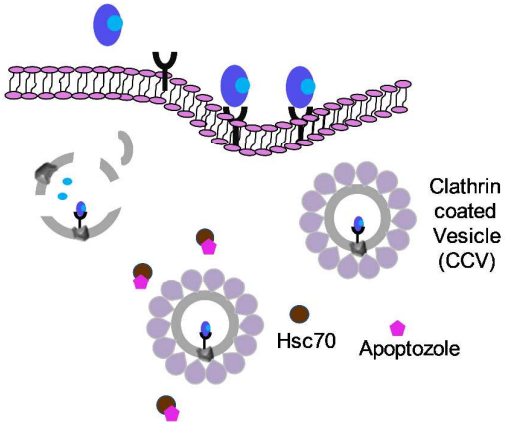
Graphical Abstract



Az blocks the association of Hsc70 with clathrin and consequently suppresses the disassembly of CCVs during clathrin-mediated endocytosis.

Probing the effect of an inhibitor of an ATPase domain of Hsc70 on clathrin-mediated endocytosis

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Keywords: chemical biology, combinatorial chemistry, Hsc70, small molecule inhibitors, structure-activity relationship

Abstract

Hsc70 is known to be involved in clathrin-mediated endocytosis (CME) by which cells take up various extracellular materials. More specifically, this protein promotes the disassembly of clathrin-coated vesicles (CCVs) by direct binding to clathrin during CME. As ATPase activity of Hsc70 is required for its association with clathrin, we have investigated the effect of an inhibitor (apoptozole, Az) of an ATPase domain of Hsc70 on CME. The results of biochemical studies show that Az binds to Hsc70 and Hsp70 without binding to other types of heat shock proteins. Structure-activity relationship studies provide information on the structural features responsible for inhibition of the ATPase activity of Hsc70. The results obtained from cell experiments reveal that Az disrupts the interaction of Hsc70 with clathrin in cells, thereby leading to accumulation of transferrin in CCVs and suppression of release of free Fe^{3+} from CCVs during transferrin receptor-mediated endocytosis.

Introduction

Endocytosis is a biological process by which cells take up diverse extracellular materials that cannot pass through the hydrophobic plasma membrane.[1,2] One major endocytic process in most cells is clathrin-mediated endocytosis (CME). In this process, extracellular materials are internalized into cells through which they are packed into clathrin-coated vesicles (CCVs).[3,4] CME is involved in a variety of cellular processes including signal transduction, neurotransmission and the modulation of various plasma membrane activities. Importantly, large biomolecules, therapeutic macromolecules and nanoparticles, which are conjugated by ligands binding to cell surface receptors, are delivered into cells through CME for therapeutic and diagnostic applications. In this event, the initial association of the ligand attached to cargo with the corresponding cell surface receptor leads to assembly of clathrin on the cell membrane in order to form clathrin-coated pits, which are subsequently converted to CCVs (Fig. 1). Clathrin coats in the cytosol are then disassembled to release cargos, a process which is promoted by binding of heat shock cognate 70 (Hsc70) to an unfolded exposed hydrophobic segment of clathrin.[5-9]

Hsc70, a constitutive member of the Hsp 70 family, has chaperone activities, such as protein folding, protein translocation across membranes, degradation of misfolded proteins, and regulation of the assembly and disassembly of protein complexes.[10-12] This protein is composed of an N-terminal ATPase domain and a C-terminal substrate binding domain (SBD) that interacts with peptide/protein substrates. The two domains are structurally coupled in such a way that hydrolysis of ATP to ADP by ATPase activity of Hsc70 induces conformational alterations in the adjacent SBD, which leads to an enhancement of binding to substrates.[13] In general, ATP-bound Hsc70 interacts with substrates weakly but the ADP-bound form has a strong affinity for substrates. It has been shown that ADP-bound Hsc70

exhibits a high affinity for clathrin during the CCV uncoating process and that the ATPase activity of Hsc70 is required for its binding to clathrin.[14]

To gain a better understanding of the complex clathrin-mediated trafficking events in cells, researchers have sought a method to disrupt this process by using chemical or genetic tools. Such inhibition studies have contributed to an understanding of the basic mechanisms of CME as well as modes of entry of therapeutic and diagnostic agents into cells. Even though some progress on an understanding of CME has been made by using a genetic approach, this methodology often has drawbacks such as the relatively long experimental times and occurrence of premature cell death. These limitations can be overcome when small molecules, which modulate the specific protein function in cells (chemical biology approach), are used because of relatively simple experimental procedures and their ability to reversibly regulate protein functions.[15,16] In this study we have investigated the effect of apoptozole (Az), a small molecule inhibitor of an ATPase domain of Hsc70 (Fig. 2A),[17,18] on CME. Because the ATPase activity of Hsc70 is essential for the disassembly of clathrin coats during CME, it is anticipated that Az may have an influence on this process. Although several chemical inhibitors for CME have been used to elucidate this process,[19,20] to our knowledge, Hsc70 inhibitors have not been employed for this purpose. In the effort described below, we have provided evidence to support a proposal that Az blocks the association of Hsc70 with clathrin and, consequently, suppresses the disassembly of CCVs during CME.

Results and discussion

The results of our previous study showed that primary cellular targets of Az were Hsc70 (a constitutive form) and Hsp70 (an inducible form), which are two major cytosolic members of the Hsp70 family and have high sequence and structural homology, and that Az bound to an

ATPase domain of these proteins to inhibit their ATPase activities.[17,18] Because Hsc70 is known as a more direct protein associated with CME than Hsp70,[5-9] the current study focusing on Hsc70 was carried out to investigate the effect of Az on this biological event.

We initially examined if Az bound to other types of heat shock proteins such as Hsp90, Hsp70 and Hsp40. To this end, an Az-conjugated resin was individually incubated with purified Hsp90, Hsc70, Hsp70 and Hsp40 (Fig. S1) in the absence and presence of Az (50 or 100 μM). As shown in Fig. 2B, whereas Hsc70 and Hsp70 bind to the Az resin, Hsp90 and Hsp40 do not. Also, we incubated the Az resin with HeLa cell lysates in the absence and presence of Az (50 or 100 μM) and then analyzed bound proteins by using the corresponding antibodies. The results of western blot analysis showed that Hsp60 and Hsp90 did not bind to the Az resin (Fig. 2C). These results indicate that Az binds to Hsc70 and Hsp70 but it does not bind to other types of heat shock proteins.

To investigate binding of Az to Hsc70 at atomic resolution, saturation transfer difference (STD) NMR studies were performed. STD method relies on selective saturation of protons of the protein and quick propagation of the saturation onto the bound ligand across the entire protein. In this process, the intensity of hydrogens of the ligand is reduced depending on their proximity to the protein. Therefore, STD NMR studies are particularly suitable for obtaining ligand specific binding information.[21,22] STD NMR spectra were taken with a sample containing 10 μM ATPase domain of Hsc70 and 1 mM Az-L (more soluble than Az in aqueous solutions, Fig. 2A) in 20 mM of deuterated Tris buffer (pD 7.0). Analysis of STD NMR spectra indicates that protons attached to benzene rings of Az have large STDs (Fig. 3), suggesting that they are positioned in close proximity to the binding site of Hsc70. On the contrary, other protons, such H1–H6 and H9, display only a small level of saturation, indicating that they only slightly contribute to binding to the protein. The results of STD

study suggest that Az binds to the ATPase domain of Hsc70 presumably through interactions of aromatic rings of Az with residues in the protein binding site.

In an effort to understand the structural features responsible for binding of Az to Hsc70, fourteen members (**1**-**14**) of a focused imidazole library were prepared on the solid support (Fig. 4A).[17,18,23-25] To investigate the effect of Az analogues on inhibition of the ATPase activity of Hsc70, an ATPase domain of Hsc70 was treated with each analogue. The results of a Malachite green assay,[17] which is frequently employed to measure monophosphate released from ATP, showed that **1** and **4**, which contain two electron withdrawing groups (CF_3 or CO_2Me) on a benzene ring at R_1, displayed higher inhibitory activities than analogues with electron donating (**5**-**7**) or a single electron withdrawing group (**2**, **3**) (Fig. 4B). Imidazole derivatives **8** and **9**, in which 4-methoxy groups present at R_2 and R_3 positions in Az are replaced by electron withdrawing groups (Cl or Br), exhibited lower inhibitory activities than Az and **10**, which bears electron donating 4-methyl groups. In addition, derivatives (**11**-**14**) containing larger functional groups, such as dimethylamino group and fused aromatic rings, than a methoxy group at these positions displayed reduced inhibitory activities in comparison to Az, a possible result of steric hindrance between these moieties and the binding site of the protein. This structure-activity relationship study suggests that even though the size and position of substituents attached to phenyl rings in Az may affect binding to Hsc70, incorporation of electron withdrawing groups at R_1 and electron donating groups at R_2 and R_3 is important to sustain good inhibitory activities toward Hsc70. It should be noted that because hydrolysis of ATP by Hsc70 and dissociation of hydrolyzed ADP from an ATPase domain of Hsc70 are quite slow in the absence of co-chaperones,[10-13] Az has the relatively low inhibitory activity toward the protein. However, an in vivo system contains co-chaperones of Hsc70 which accelerates hydrolysis of ATP and dissociation of ADP from

Hsc70,[10-13] and thus Az efficiently suppresses Hsc70 activity in cells as shown previously[17] and below.

We then examined the binding ability of Az to Hsc70 in cells. For this purpose, HeLa cells were incubated with 500 nM Cy3-conjugated Az (Cy3-Az, Fig. 2A) for 1 h and then treated with Hsc70 antibody. Analysis of confocal microscopy images indicates that fluorescence of Cy3-Az is overlapped with Hsc70 in the cytoplasm with the Pearson's correlation coefficient of 0.89 (Fig. 5A). It should be noted that Cy3-Az was not completely co-localized with Hsc70 because Az binds to both Hsc70 and Hsp70 which HeLa cells express. In contrast, pretreatment of cells with excess Az (5 μM) for 1 h induced a great decrease in the fluorescence intensity of Cy3-Az in cells (Fig. 5B), indicating that Az recognizes cellular Hsc70.[17]

The observation of inhibition of the ATPase activity of Hsc70 by Az led us to expect that Az might affect CME, presumably by abrogating the interaction of Hsc70 with clathrin, a process which is ATP-dependent. To test this proposal, HeLa cells were treated with various concentrations (0 - 1.0 μM) of Az for 12 h at 37 ^{o}C and then the cell lysates were incubated with clathrin antibody overnight at 4 ^{o}C. The resultant immunocomplexes were captured with protein G conjugated beads. Co-immunoprecipitated Hsc70 was detected with Hsc70 antibody. The results showed that the amount of Hsc70 associated with clathrin gradually decreased with increasing concentrations of Az (Fig. 6), indicating that Az disrupts the association of Hsc70 with clathrin in cells.

This exciting result prompted us to further probe the effect of Az on transferrin receptor (TfR)-mediated endocytosis. TfR is one of the well studied cell-surface transmembrane proteins which undergo clathrin-mediated endocytosis.[26] The principal role of this receptor includes the cellular acquisition of irons through the TfR-mediated endocytosis of holo-

transferrin (an iron bound form, Fe^{3+}-Tf). The binding of holo-Tf to TfR on the cell surface triggers endocytosis of Fe^{3+} (Fig. 1). A complex of holo-Tf with TfR is subsequently internalized inside cells via clathrin-coated pits and then is routed into the endosome. Upon uncoating of CCVs by binding of Hsc70 to clathrin, the endosome becomes acidic (pH ~ 5.5), which leads to weakening the binding of Fe^{3+} to Tf and, as a consequence, the release of Fe^{3+} from the protein. The complex of apo-Tf (iron-free Tf) and TfR then returns to the cell surface where they dissociate at extracellular physiological pH. Exported Tf binds to additional iron and participates in another round of iron delivery to cells. Thus, a Tf-TfR system is a suitable model for studies of the effect of Az on CME.

To examine whether Az affects Tf recycling during TfR-mediated endocytosis, HeLa cells were treated for 4 h with 500 nM Az or 50 nM Wortmannin (an inhibitor of phosphoinositide 3 kinase) as a positive control which is known to interfere with the Tf recycling process.[5] The treated cells were then incubated with biotin-conjugated Tf for 15 min. The cell image analysis after staining with Cy-3 labeled streptavidin showed that whereas untreated cells exhibited low fluorescence intensity, the increased fluorescence intensity was observed in Az treated cells, a phenomenon which was also seen in Wortmannin treated cells (Fig. 7A). We also measured the amount of the internalized Tf into cells in the absence or presence of Az. HeLa cells were treated with various concentrations of Az (0 - 500 nM) or 50 nM Wortmannin for 12 h at 37 ^{o}C and then incubated with biotin-Tf for 0.5 h. The internalized biotin-Tf protein was detected using horseradish peroxidase (HRP) labeled streptavidin. The amount of internalized Tf into cells was increased in an Az dose-dependent fashion (Fig. 7B). These results indicate that the internalized Tf is accumulated in CCVs since Az blocks the CCV uncoating process by inhibiting the ATPase activity of Hsc70 and, consequently, suppresses recycling of Tf. However, in the absence of Az, the internalized Tf

is discharged outside cells through the CCV recycling pathway (see Fig. 1).

Tf mainly acts as a transporter of Fe^{3+} into cells via TfR-mediated endocytosis and regulates a level of free Fe^{3+} in biological fluids. We thus examined the effect of Az on the release of Fe^{3+} from CCVs in the cytosol. To detect Fe^{3+} in cells, we used a Fe^{3+} fluorescent probe **FS1** (Fig. 8A) which does not respond to other metal ions including Fe^{2+}.[27] This probe was prepared according to the known procedure.[27] As expected, strong red fluorescence was observed upon addition of the probe to a solution of Fe^{3+} (Fig. 8B). However, when **FS1** was added to a solution of holo-Tf (Fe^{3+}•Tf), the fluorescence intensity was very weak, indicating that the probe does not detect Tf bound Fe^{3+} (association constant of Tf and $Fe^{3+} \sim 10^{22}$ M^{-1})[28] and thus is a suitable probe for detection of free Fe^{3+} dissociated from Tf during TfR-mediated endocytosis.

To examine whether Az affects the release of Fe^{3+} from CCVs, HeLa cells were exposed to 500 nM Az for 4 h and then incubated with 50 μg/mL holo-Tf for 30 min followed by incubation with 25 μM **FS1** for 2 h. As shown in Fig. 8C, untreated cells exhibited very weak fluorescence, indicating that cells rarely contain free Fe^{3+}. However, the cells treated with holo-Tf in the absence of Az showed strong red fluorescence, showing that cells take up holo-Tf via endocytosis and then free Fe^{3+} is released from the complex in the cytosol. In contrast, Az treated cells exhibited significantly attenuated fluorescence intensity in comparison to that in untreated cells. This suggests that free Fe^{3+} is only slightly liberated from the complex because the uncoating process of CCVs is suppressed by Az and thus a majority of holo-Tf is trapped in CCVs. The results of time-dependent experiments also support the reduced release of free Fe^{3+} in the cytosol in the presence of Az (Fig. 8D).

Conclusions

In this work, we have demonstrated that Az binds to isoforms (Hsc70 and Hsp70) of the Hsp70 family without binding to other types of heat shock proteins including Hsp40, Hsp60 and Hsp90. A systematic structure-activity relationship studies shed light on the structural requirements necessary for inhibition of the ATPase activity of Hsc70. Our investigations have also shown that Az disrupts association of Hsc70 with clathrin in cells. Furthermore, it has been found that Az promotes the accumulation of Tf in CCVs and suppresses release of free Fe^{3+} from CCVs during TfR-mediated endocytosis. These results suggest that Az interferes with CME by blocking the disassembly of CCVs which is an ATP-dependent process. To our knowledge, it is the first time that a Hsc70 inhibitor is employed to probe the effect on CME. Because CME is an important biological process for cellular uptake of various extracellular materials, it is anticipated that Az can serve as a new chemical tool to gain a better understanding of clathrin-mediated trafficking events in cells and will be of considerable value to researchers in this field.

Experimental

Cell imaging using Cy3-conjugated Az. HeLa cells were maintained in culture media (RPMI-1640 media supplemented with 10% fetal bovine serum, 100 units/mL penicillin and 100 μg/mL streptomycin). The cells were incubated with 500 nM Cy3-Az for 1 h at 37 ^{o}C. After washing with phosphate buffered saline (PBS) to remove remaining Cy3-Az, the cells were fixed with 4% paraformaldehyde in PBS for 15 min and permeabilized with PBST (PBS containing 0.1% Triton X-100) for 10 min. The cells were incubated in a blocking solution (PBS containing 0.1% Triton X-100 and 0.1% FBS) for 1 h. The cells were treated with mouse Hsc70 monoclonal antibody (1:100, Santa Cruz Biotechnology, Santa Cruz, CA) in PBST for 1 h at room temperature and then washed with PBST. The cells were treated with

FITC labeled anti-mouse IgG (1:400, Abcam, Cambridge, MA) in PBST for 0.5-1.0 h at room temperature. For competition experiments, HeLa cells were pre-incubated with 500 nM Az for 1 h at 37 ^{o}C. After washing with PBS, the cells were incubated with 500 nM Cy3-Az for 1 h at 37 ^{o}C. The cells were imaged by using confocal microscopy (LSM510 META, Carl Zeiss, Berlin, Germany).

Inhibition of ATPase activity of Hsc70 by compounds. Stock solutions of Malachite green (0.081% w/v), polyvinyl alcohol (2.3% w/v), and ammonium heptamolybdate tetrahydrate (5.7% w/v in 6 M HCl) were prepared and stored at 4 ^{o}C. Three solutions were mixed with water in the ratio of 2:1:1:2 to prepare the Malachite green reagent. For determination of the ATPase activity of Hsc70, a master mixture of an ATPase domain of Hsc70 was prepared in assay buffer (100 mM Tris-HCl, 20 mM KCl, and 6 mM $MgCl_2$, pH 7.4) as final concentration of 1 μM. An aliquot (10 μL) of this mixture was added into each well of a 96-well plate. To this solution was added each compound in assay buffer, and the plate was incubated for 30 min at room temperature. To start the reaction, 1 μL of 4 mM ATP was added to the solution. The final concentrations were 1 μM protein and 200 μM ATP in 20 μL of assay buffer. After 3 h incubation at 37 ^{o}C, 80 μL of Malachite green reagent was added into each well. The samples were mixed thoroughly and incubated at 37 ^{o}C for 15 min, and 10 μL of 34% sodium citrate was added to stop the nonenzymatic hydrolysis of ATP. The absorbance was determined at 620 nm on a SpectraMax 340 PC 384 (Molecular Devices, Sunnyvale, CA).

Inhibitory effect of Az on Hsc70-clathrin interaction (co-immunoprecipitation). HeLa cells were lysed with NP-40 buffer (50 mM HEPES, pH 7.4, 150 mM NaCl, 1 mM EDTA,

1% NP-40, 10% glycerol and 1 tablet of complete protease inhibitor cocktail (Roche Applied Science, Mannheim, Germany). HeLa cells in culture media were treated with various concentrations (0 - 1.0 μM) of Az for 12 h at 37 ^{o}C, and then lysed with NP-40 buffer. The cell lysates were incubated with mouse anti-clathrin monoclonal antibody (1:400, Millipore, Germany) overnight at 4 ^{o}C. overnight at 4 ^{o}C. Immunocomplexes were precipitated with 40 μL of a Dynabead protein G bead (Invitrogen) for 0.5 h at room temperature. After washing with NP-40 buffer, samples were denatured for 10 min at 80 ^{o}C with SDS-PAGE sample loading buffer and separated by 10% SDS-PAGE. Co-immunoprecipitated Hsc70 with clathrin was determined by western blot by using rat anti-Hsc70 monoclonal antibody (1:4000, Abcam, Cambridge, MA). Quantitative data were analyzed by using Origin version 8.0 (Microcal Inc, Northampton, MA).

Cell imaging of Tf. HeLa cells in culture media were treated with or without 500 nM Az or 50 nM wortmannin (Sigma) for 4 h at 37 ^{o}C for 4 h. After washing with culture media, the cells were incubated with 5 μg/mL of biotin-Tf for 15 min at 37 ^{o}C. The cells were fixed and permeabilized as described above. The cells were incubated with Cy3-streptavidin (1:200, Sigma) in PBST buffer. After washing with PBS, biotin-Tf in the cells was detected by using confocal microscopy or a fluorescence microplate reader (Molecular Probes, Eugene, OR). Quantitative data were analyzed by using Origin version 8.0.

Detection of internalized Tf in cells. HeLa cells in culture media were treated with various concentrations (0 - 500 nM) of Az or 50 nM Wortmannin for 12 h at 37 ^{o}C. After washing with culture media, the cells were incubated with 10 μg/mL biotin-Tf for 0.5 h at 37 ^{o}C. Internalization was stopped by incubation of the cells on ice. After washing with PBS, the

cells were lysed with lysis buffer (50 mM Tris-HCl, pH 7.4, 1% (v/v) NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA and 1 tablet of complete protease inhibitor cocktail). The cells were then briefly sonicated and centrifuged (4 ^{o}C, 13,000 rpm, 30 min). Proteins were separated by 10% SDS-PAGE and transferred onto nitrocellulose membrane (Hybond^{TM}, Amersham Biosciences, UK). Membranes were blocked in TBS buffer containing 0.1% Tween-20 and 5% non-fat skim milk for 1 h at room temperature and then incubated with HRP labeled streptavidin (Pierce, Rockford, IL) overnight at 4 ^{o}C. After washing with PBS, the treated membranes were visualized by using the ECL kit (WestZol, iNtRON Biotechnology, Korea).

Fluorescence detection of free Fe^{3+} in cells. HeLa cells in culture media were incubated with 500 nM Az for 4 h at 37 ^{o}C. After washing with culture media, the cells were treated with 50 μg/mL holo-Tf (Fe^{3+}-Tf, Sigma) for 30 min. After washing with culture media, the cells in culture media were stained with 25 μM **FS1** for 2 h at 37 ^{o}C. Excess **FS1** was removed by washing with PBS. The cells were imaged by using confocal microscopy. **FS1** fluorescence intensity (λ_{ex}: 519 nm, λ_{em}: 560 nm) was measured by using a fluorescence microplate reader.

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References

1. S. D. Conner and S. L. Schmid, *Nature*, **2003**, 422, 37-44.

2. S. Mayor and R. E. Pagano, *Nat. Rev. Mol. Cell Biol*., 2007, **8**, 603-612.

3. M. Lakadamyali, M. J. Rust and X. Zhuang, *Cell*, 2006, **124**, 997-1009.

4. H. T. McMahon and E. Boucrot, *Nat. Rev. Mol. Cell Biol*., 2011, **12**, 517-533.

5. I. Rapoport, W. Boll, A. Yu, T. Bocking and T. Kirchhausen, *Mol. Biol. Cell*, 2008, **19**, 405-413.

6. T. G. Chappell, W. J. Welch, D. M. Schlossman, K. B. Palter, M. J. Schlesinger and J. E. Rothman, *Cell*, 1986, **45**, 3-13.

7. E. Eisenberg and L. E. Greene, *Traffic*, 2007, **8**, 640-646.

8. S. L. Newmyer and S. L. Schmid, *J. Cell Biol*., 2001, **152**, 607-620.

9. J. Heuser and C. J. Steer, *J. Cell Biol*., 1989, **109**, 1457-1466.

10. J. C. Young, V. R. Agashe, K. Siegers and F. U. Hartl, *Nat. Rev. Mol. Cell Biol*., 2004, **5**, 781-791.

11. M. P. Mayer and B. Bukau, *Cell. Mol. Life Sci*., 2005, *62*, 670-684.

12. F. U. Hartl and M. Hayer-Hartl, *Science*, 2002, **295**, 1852-1858.

13. J. F. Swain, G. Dinler, R. Sivendran, D. L. Montgomery, M. Stotz and L. M. Gierasch, *Mol. Cell*, 2007, **26**, 27-39.

14. K. Prasad, J. Heuser, E. Eisenberg and L. Greene, *J. Biol. Chem*., 1994, **269**, 6931-6939.

15. L. I. Zon and R. T. Peterson, *Nat. Rev. Drug Discov*., 2005, **4**, 35-44.

16. C. A. MacRae and R. T. Peterson, *Chem. Biol*., 2003, **10**, 901-908.

17. H. J. Cho, H. Y. Gee, K.-H. Baek, S.-K. Ko, J.-M. Park, H. Lee, N.-D. Kim, M. G. Lee and I. Shin, *J. Am. Chem. Soc.*, 2011, **133**, 20267-20276.

18. D. R. Williams, S.-K. Ko, S. Park, M.-R. Lee and I. Shin, *Angew. Chem. Int. Ed.*,

2008, **47**, 7466-7469.

19. D. Dutta and J. G. Donaldson, *Cell. Logist.*, 2012, **2**, 203-208.

20. L. von Kleist, W. Stahlschmidt, H. Bulut, K. Gromova, D. Puchkov, M. J. Robertson, K. A. MacGregor, N. Tomilin, A. Pechstein, N. Chau, M. Chircop, J. Sakoff, J. P. von Kries, W. Saenger, H.-G. Kräusslich, O. Shupliakov, P. J. Robinson, A. McCluskey and V. Haucke, *Cell*, 2011, **146**, 471-484.

21. B. Meyer and T. Peters, *Angew. Chem. Int. Ed.*, 2003, **42**, 864-890.

22. A. Viegas, J. Manso, F. L. Nobrega and E. J. Cabrita, *J. Chem. Edu.*, 2011, **88**, 990-994.

23. D. R. Williams, G.-H. Kim, M.-R. Lee and I. Shin, *Nat. Protoc*., 2008, **3**, 835-839.

24. D. R. Williams, M.-R. Lee, Y.-A. Song, S.-K. Ko, G.-H. Kim and I. Shin, *J. Am. Chem. Soc.*, 2007, **129**, 9258-9259.

25. G.-H. Kim, D. Halder, J. Park, W. Namkung and I. Shin, *Angew. Chem. Int. Ed*., 2014, **53**, 9271-9274.

26. Z. M. Qian, H. Li, H. Sun and K. Ho, *Pharmacol. Rev*., 2002, **54**, 561-587.

27. M. H. Lee, T. V. Giap, S. H. Kim, Y. H. Lee, C. Kang and J. S. Kim, *Chem. Commun*., 2010, **46**, 1407-1409.

28. P. Aisen and I. Listowsky, *Annu. Rev. Biochem*., 1980, **49**, 357-393.

Figure and Figure legends

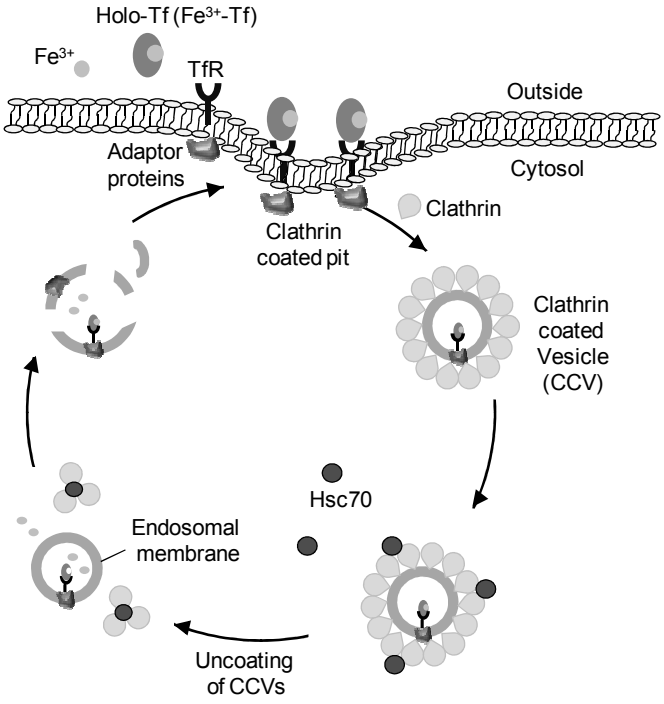

Fig. 1. Clathrin-mediated endocytosis and cellular uptake of iron via transferrin receptor-mediated endocytosis (Tf: transferrin, TfR: transferrin receptor). See text for details.

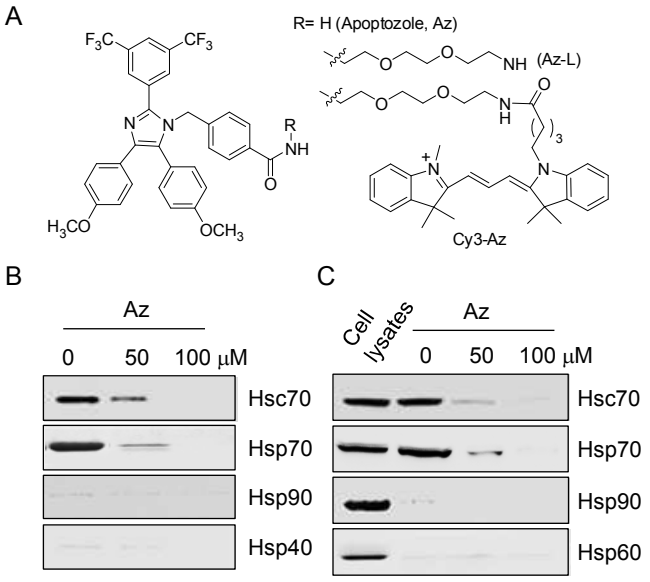

Fig. 2. Az binds to Hsc70 and Hsp70. (A) Structure of compounds. (B) The Az-linked resin was incubated with purified Hsp90, Hsc70, Hsp70 and Hsp40 in the absence and presence of Az. Proteins bound to the resin were visualized by silver staining. (C) The Az-linked resin was incubated with HeLa cell lysates which were treated with Az for 1 h. Bound proteins were analyzed by using western blots.

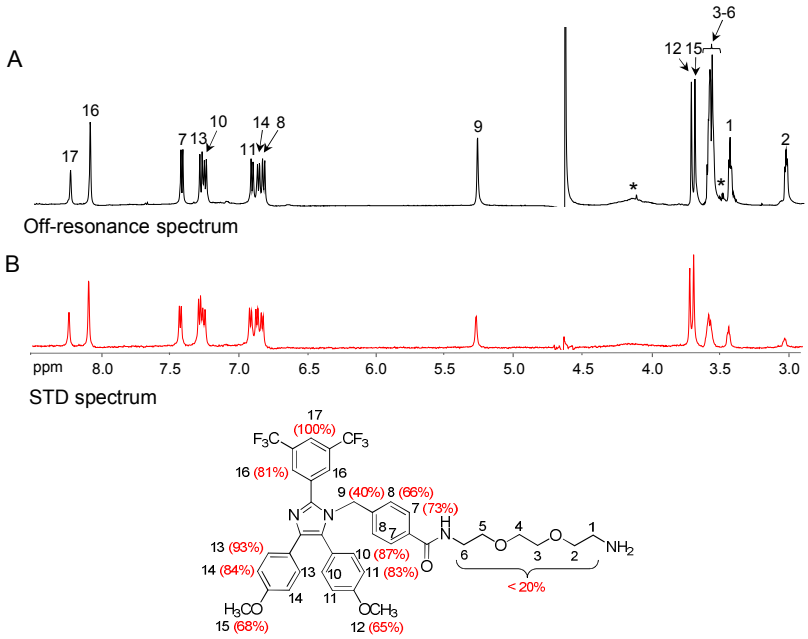

Fig. 3. STD NMR data. (A) Off-resonance spectrum of a solution of an ATPase domain (10 μM) of Hsc70 and Az-L (1 mM) in 20 mM deuterated Tris (pD = 7.0) containing 1% d_6-DMSO. The asterisk indicates impurities present in the protein sample. (B) Top: STD NMR spectrum. Bottom: The relative degree of saturation of the individual hydrogen is mapped into the structure of Az-L. The ratio of intensity I_{STD}/I_0 was normalized using the largest STD of H17 (100%) as a reference.

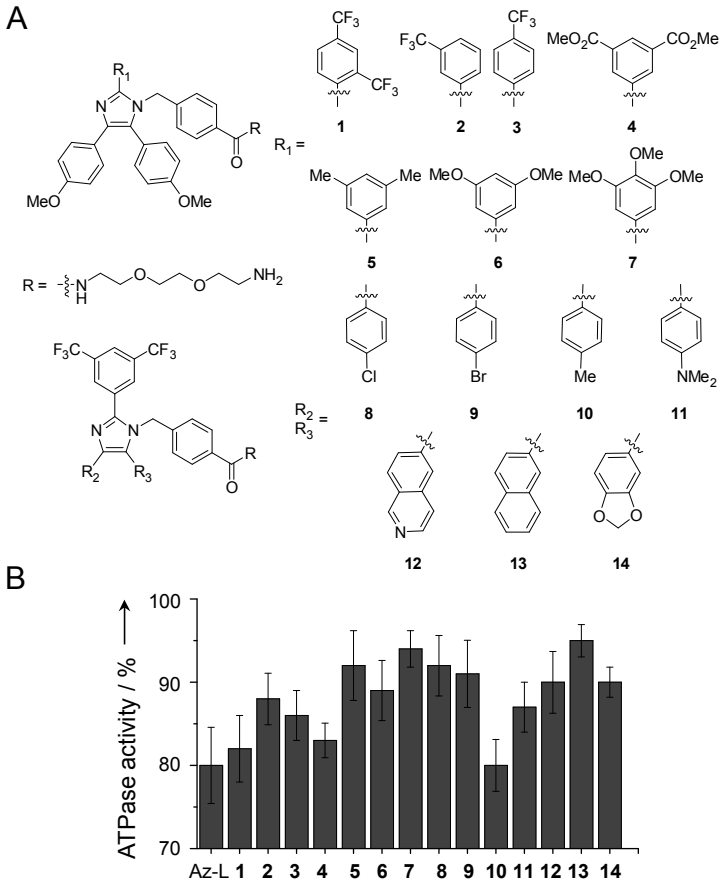

Fig. 4. Structure-activity relationships. (A) Structure of Az analogues **1**–**14**. (B) Inhibition of ATPase activity of an ATPase domain of Hsc70 by compounds. ATPase activities were measured using a Malachite green assay after incubation of an ATPase domain with 100 μM of each compound and 50 μM ATP (error bar = SD).

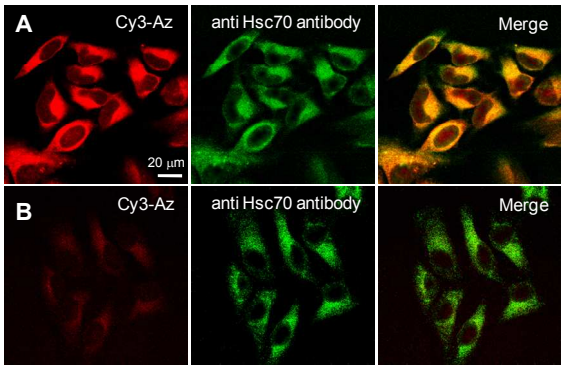

Fig. 5. Az binds to Hsc70 in cells. (A) Confocal fluorescence images of HeLa cells treated with 500 nM Cy3-Az for 1 h, and then treated with Hsc70 antibody and FITC-labeled secondary antibody. (B) Fluorescence images of HeLa cells pretreated with 5 μM Az for 1 h followed by sequential treatment with 500 nM Cy3-Az for 1 h, Hsc70 antibody and FITC-labeled secondary antibody.

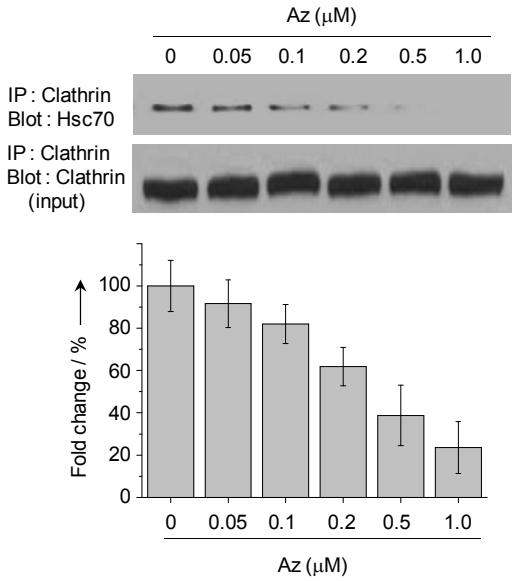

Fig. 6. Az blocks association of Hsc70 with clathrin in cells. Top: HeLa cells were treated with various concentrations of Az for 12 h at 37 ^{o}C and then lysed with NP-40 buffer. The cell lysates were incubated with clathrin antibody overnight at 4 ^{o}C. Immunocomplexes were captured with protein G beads for 0.5 h at room temperature. Co-immunoprecipitated Hsc70 with clathrin was determined using Hsc70 antibody. Bottom; Quantitative data of western blot analysis of Hsc70 (error bar = SD).

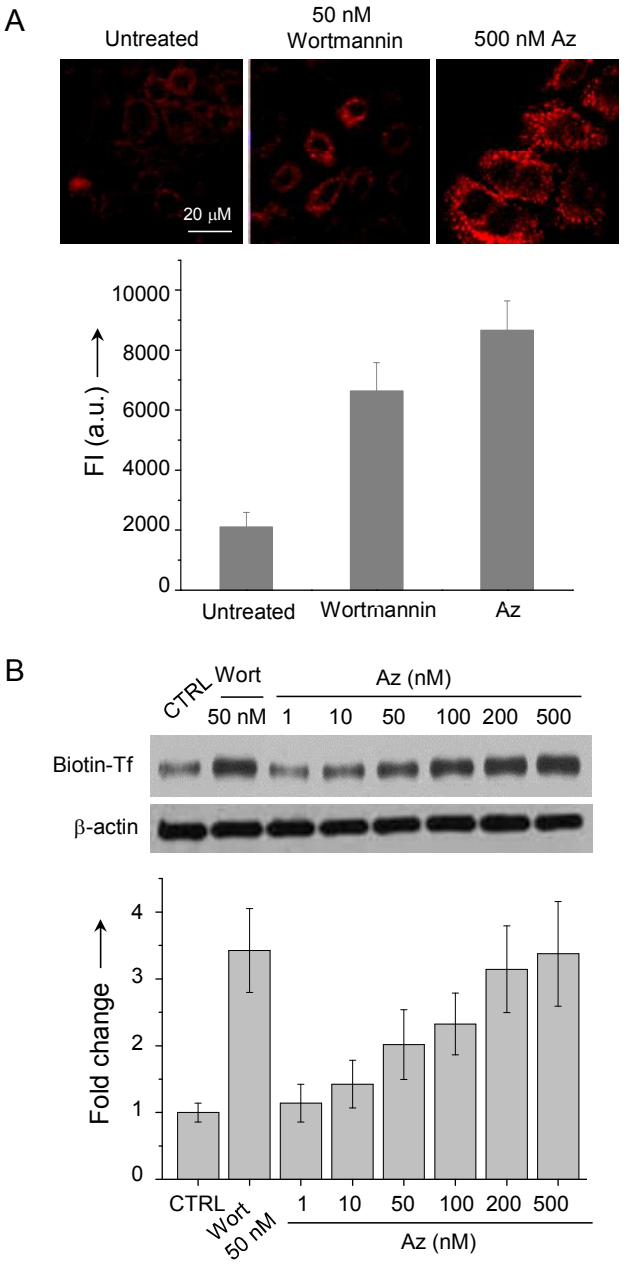

Fig. 7. Detection of Tf internalized into cells. (A) Top: HeLa cells treated with 500 nM Az or 50 nM wortmannin for 4 h were incubated with biotin-Tf for 0.5 h and then Cy3-labeled streptavidin. Bottom; Quantitative data of fluorescence intensity (error bar = SD). (B) Top: HeLa cells were treated with various concentrations of Az or 50 nM Wortmannin for 12 h and then incubated with biotin-Tf for 0.5 h. Protein was detected using HRP labeled streptavidin. Bottom: Quantitative data of Tf internalized into cells (error bar = SD).

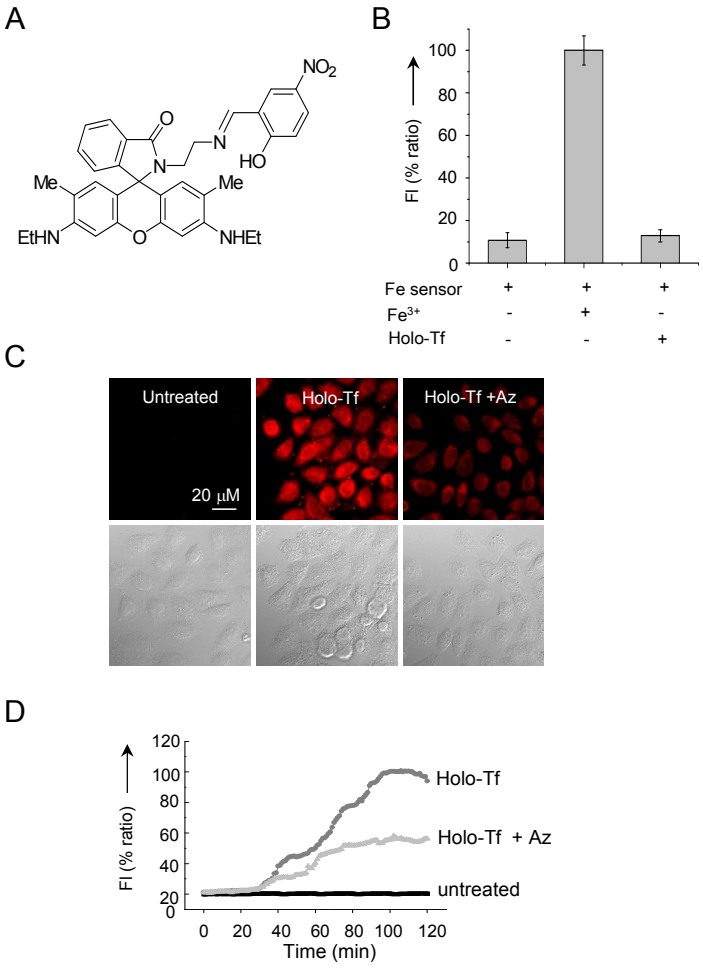

Fig. 8. Fluorescence detection of Fe^{3+}. (A) Structure of a Fe^{3+} fluorescent probe **FS1**. (B) Fluorescence intensity of **FS1** after addition to a solution containing free Fe^{3+} or holo-Tf. (C) Fluorescence images of cells after incubation of HeLa cells with 500 nM Az for 4 h followed by treatment with 50 μg/mL holo-Tf (Fe^{3+}-Tf) for 0.5 h at 37 ^{o}C. The cells were stained with 25 μM **FS1** for 2 h at 37 ^{o}C. (D) HeLa cells were treated with 500 nM Az for 4 h and then incubated with 50 μg/mL holo-Tf for 0.5 h at 37 ^{o}C. The cells were stained with 25 μM **FS1** and a change in fluorescence intensity was measured using a fluorescence microplate reader over 2 h.