Bioorganic Chemistry

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An Apoptosis-Inducing Small Molecule That Binds to Heat Shock Protein 70**

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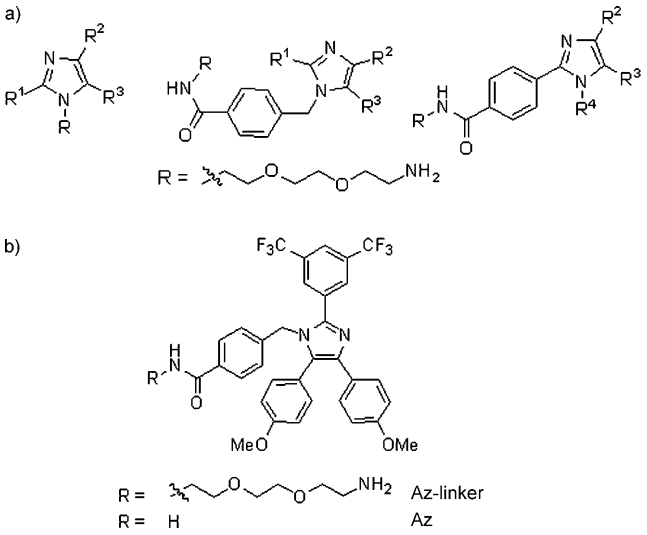
Apoptosis (or programmed cell death) is a fundamental biological process that regulates a variety of normal physiological processes, ranging from development to aging.[1] Damaged or unwanted cells in organisms are removed by the intrinsic and/or extrinsic apoptotic pathways. The intrinsic apoptotic pathway occurs by the release of cytochrome c from mitochondria. The extrinsic apoptotic pathway is caused by the binding of death ligands, such as TNF (tumor necrosis factor), Fas, and TRAIL (TNF-related-apoptosis-inducing ligand), to their corresponding receptors. Although programmed cell death is involved in a number of key biological phenomena, aberrant apoptosis results in diverse human diseases.[2] For example, the dysregulation of apoptosis disrupts tissue homeostasis by prolonging cell survival and contributes to the progression of diverse human tumors. In addition, retarded apoptosis causes the elimination of autoreactive lymphocytes to fail, leading to autoimmunity. Moreover, excessive apoptosis results in cell-loss disorders such as neurodegenerative (Alzheimer's and Parkinson's diseases) and cardiovascular diseases. Since apoptosis is involved in both normal physiology and various human diseases, research on apoptosis has become a central area in basic biological studies and in the development of therapeutic agents.

Small molecules that either induce or prevent apoptotic cell death have significant potential as therapeutic agents to treat apoptosis-related diseases.[3] In addition, these agents could also be employed to understand the roles that apoptotic regulatory proteins play in biological processes. Herein we describe a novel apoptosis-inducing small molecule which interacts with Hsc70 and Hsp70.

Cell-based screening with a small molecule library is an attractive approach to identify bioactive compounds that regulate protein functions in cells or affect processes such as cell differentiation or morphology.[4] We applied this approach to select molecules with apoptosis-inducing activity, using a recently prepared imidazole library on a solid support to identify bioactive compounds that induce interesting cellular events (Scheme 1 a).[5] The amine-conjugated diethylene glycol linker was introduced into the library for facile solid-phase synthesis and the identification of target protein(s) by affinity chromatography.



Scheme 1. Structures of a) an imidazole library (see the Supporting Information for substituents R^1–R^4) and b) apoptozole (Az).

To search for molecules that induce apoptosis in cells, 216 imidazole derivatives (1 μM) were incubated with the highly proliferative P19 embryonic carcinoma cell line for 3 h and subsequently treated with a mixture of annexin V-fluorescein (0.5 μg mL^{-1}) and propidium iodide (PI, 2 μg mL^{-1}) to rapidly screen for apoptosis inducers.[6] The exposure of phosphatidylserine on the outer leaflet of the cell plasma membrane is a key feature of the early stages (2–4 h) of apoptosis. Phosphatidylserine can be detected fluorescently by using annexin V-fluorescein. Propidium iodide (PI) can be used to monitor membrane-perturbed cells which result from the plasma membrane becoming permeable (a feature of necrosis) or late-stage apoptosis. Therefore, the combined use of annexin V-fluorescein and PI allows for the rapid evaluation of apoptosis in cells treated with the compound library. In our screen, compounds that exhibited positive annexin V and negative PI staining in P19 cells after 3 h incubation were selected as inducers of apoptosis. However, compounds that showed positive annexin V and positive PI staining in the cells were not selected as "hits" because it is possible that the treated cells underwent necrosis rather than apoptosis. One compound apoptozole-linker (Az-linker) showed a high level of positive annexin V and negative PI staining in P19 cells (Scheme 1 b). For further studies, Az (without linker) was resynthesized and purified (see the Supporting Information).

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To confirm that Az indeed induces apoptotic cell death, P19 cells and A549 cells (lung cancer cells) were incubated with 1 μM Az and then treated with a mixture of annexin V-fluorescein and PI. Analysis by microscopy showed that both cells incubated with Az for 3 h exhibited positive annexin V staining on the outer cell membrane and negative PI staining, thus indicating the early stages of apoptosis (Figure 1a).

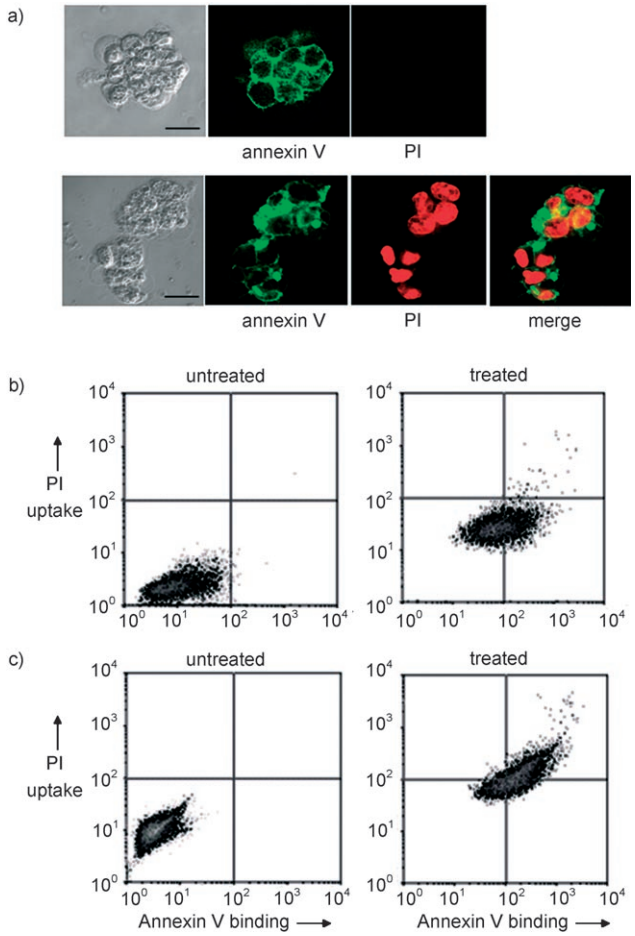

Figure 1. Induction of apoptosis by Az. a) P19 cells incubated with 1 μM Az for 3 h (top) and 12 h (bottom) followed by treatment with a mixture of annexin V and PI (scale bar = 20 μm). Cytograms of annexin V binding versus PI uptake in b) P19 cells and c) A549 cells treated with 1 μM Az for 12 h. Untreated cells are shown as a negative control.

However, cells treated for a longer time period (12 h) showed both positive annexin V and PI staining, thus indicating later stage apoptosis. Flow cytometric analysis indicates that about 90% of the cell population undergoes apoptotic death after 12 h incubation with 1 μM Az (as quantified from Figure 1b and c).

Further experiments were performed to confirm that cell death by Az was caused by apoptosis rather than necrosis. The loss of mitochondrial membrane potential is a hallmark of apoptosis. This phenomenon can be assessed by flow cytometry after staining with a JC-1 probe which is sensitive to membrane potential.[7] The intensity of the red fluorescence in P19 cells and A549 cells treated with 1 μM Az was significantly decreased, which indicates a loss of the mitochondrial membrane potential as a result of apoptotic cell death (Figure 2a and b). Apoptosis is also associated with

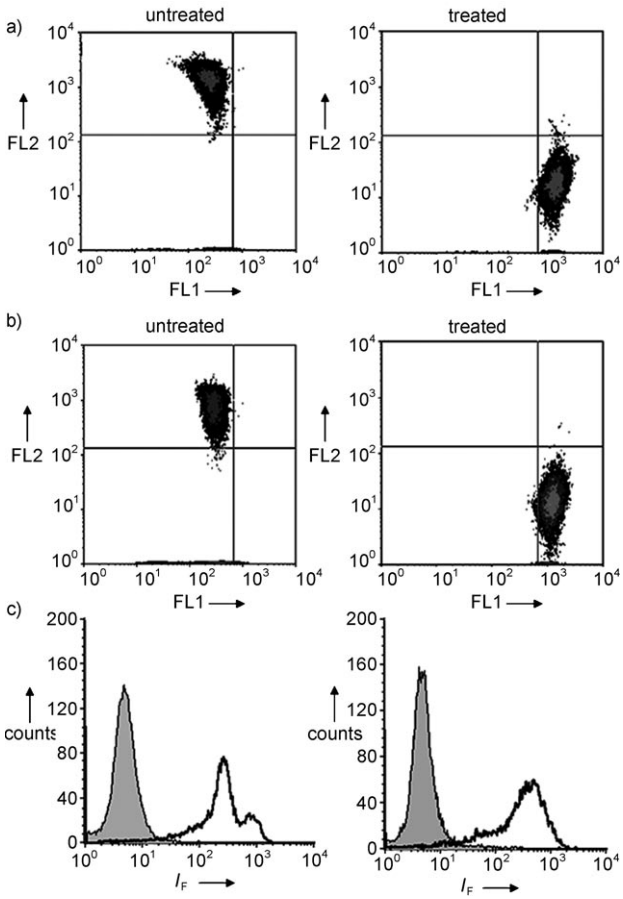

Figure 2. Induction of apoptosis by Az. Flow cytometry of: a) P19 cells and b) A549 cells treated with 1 μM Az for 12 h and stained with JC-1. Untreated cells are shown as a negative control (a dot plot of red fluorescence (FL2, JC-1 aggregate) versus green fluorescence (FL1, JC-1 monomer)). c) TUNEL assay of the apoptotic death of P19 cells (left) and A549 cells (right) treated with 1 μM Az for 12 h. The treated cells were analyzed by flow cytometry. The gray peak represents the negative control.

DNA fragmentation which can be detected by the terminal deoxynucleotidyl transferase (TUNEL) assay.[8] This assay showed an increase in TUNEL-positive P19 cells and A549 cells, thus indicating an increase in DNA fragmentation (Figure 2c). All the results clearly reveal that Az indeed has apoptosis-inducing activity.

To understand the molecular basis for apoptosis induced by Az, we made an attempt to identify the cellular target protein(s) of Az by affinity chromatography.[9] An Az-conjugated agarose matrix (Az matrix) was prepared by coupling Az-linker to carbonylimidazole-activated agarose. Protein extracts from P19 cells were incubated with the Az matrix for 1 h and bound proteins were analyzed by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS/PAGE). One heavy band at about 70 kDa was observed, but

was rarely seen in the presence of external Az (0.25 and 0.50 mM) or a matrix linked by an inactive imidazole derivative (L8), thus demonstrating the specificity for Az (Figure 3a). The protein in this band was identified as heat shock cognate 70 (Hsc70) by nanoLC-MS/MS.

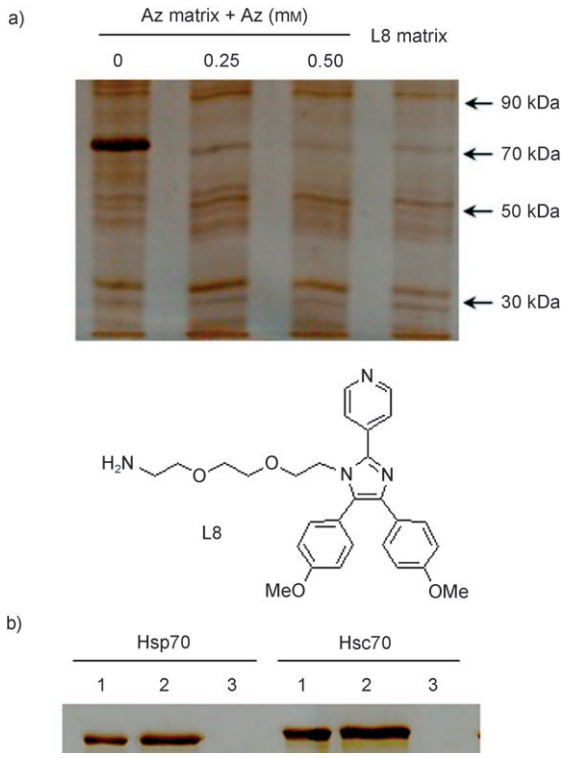

Figure 3. Identification of a cellular target protein that interacts with Az. a) Az matrix was incubated with protein extract from P19 cells in the absence of external Az (lane 1) and in the presence of external Az (lanes 2 and 3). As a negative control, inactive L8 matrix was incubated with protein extract (lane 4). b) Az matrix was incubated with recombinant Hsp70 and Hsc70, and protein bound to Az matrix was immunostained using anti-Hsp70 and anti-Hsc70 antibodies (lane 1: protein only, lane 2: bound protein in the absence of external Az, lane 3: bound protein in the presence of external Az (0.3 mM)).

To further verify whether Hsc70 is a target of Az, the Az matrix was incubated with recombinant Hsc70 and Hsp70 (an inducible Hsc70 homologue protein) in the presence and absence of external Az (0.3 mM). The bound protein was assessed by Western blot analysis using anti-Hsc70 and anti-Hsp70 antibodies. Both proteins interacted with the Az matrix in the absence of Az and did not bind to the matrix in the presence of external Az (Figure 3b). Since the constitutive Hsc70 is abundantly expressed in cells, whereas inducible Hsp70 is expressed at a very low level in cells, it is likely that only Hsc70 in the protein extract was detected by affinity chromatography. The binding affinities of Az to Hsc70 and Hsp70 were measured by surface plasmon resonance (SPR) spectroscopy (Figure 4). For these studies, an Az–biotin conjugate was immobilized on the neutravidin-coated gold surface. The dissociation constants (K_d values) for Az-Hsc70 and Az-Hsp70 interactions were determined to be 0.21 and 0.14 μM, respectively.

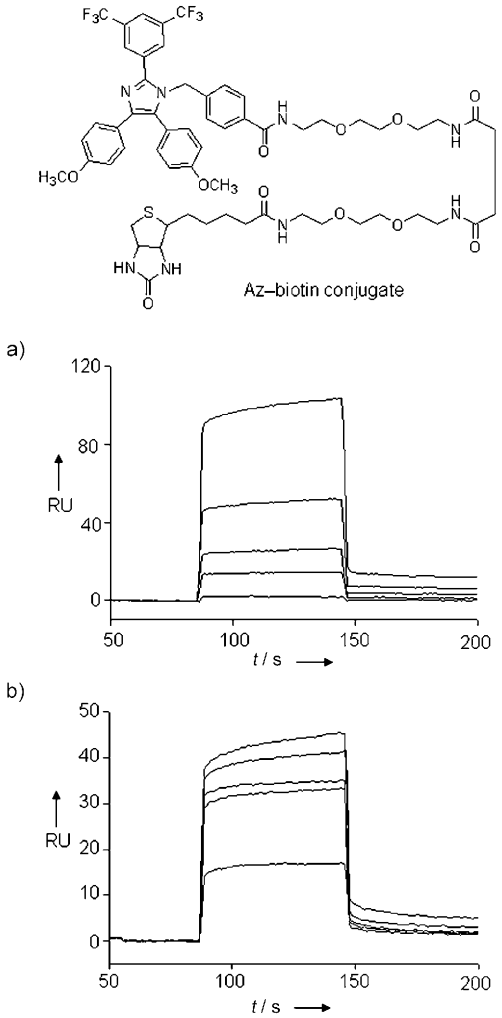

Figure 4. Determination of K_d values for: a) Az-Hsc70 and b) Az-Hsp70 interactions by using SPR spectroscopy (RU: response unit). Az–biotin conjugate was immobilized on the neutravidin-coated gold surface (protein concentrations: 500, 250, 125, 61.5, and 31.5 nM).

Finally, the antitumor activities of Az were investigated since the Hsp70 family (Hsc70 and Hsp70) are anti-apoptotic chaperone proteins and are known to be involved in the development of cancers.[10] To this end, we assessed the ability of this compound to induce cell death in several cancer cell lines, including SK-OV-3 (ovarian cancer cells), HCT-15 (colon cancer cells), and A549 (lung cancer cells). Az had IC_{50} values of 0.22, 0.25, and 0.13 μM in SK-OV-3, HCT-15, and A549 cells, respectively (see the Supporting Information). As expected, the cancer cells used for this study expressed Hsc70 and Hsp70 at higher levels than did normal cells (data not shown). The results demonstrate the potency of Az as an antitumor agent, which possibly inhibits the function of Hsc70 and/or Hsp70.

In conclusion, we have identified from a cell-based assay a small molecule that interacts with Hsc70 and Hsp70.[11,12] It is likely that this compound induces apoptotic cell death by inhibiting the function of Hsp70 and/or Hsc70, which antagonize apoptosis by interfering with multiple checkpoints in the apoptosis pathways. As an apoptotic inhibitor Az holds

considerable potential as a cancer therapeutic and can also be used to further understand the molecular basis of Hsp70-related apoptotic process.

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