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Novel Small Molecules Relieve Prothymosin α -Mediated Inhibition of Apoptosome Formation by Blocking Its Interaction with Apaf-1

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Abstract

Structurally diverse small molecules, including 5-(2-Benzofuryl)-4-phenyl-1,2,4-triazole-3-thiol (BETT), have been identified via high-throughput screening as activators of caspase-3 in HeLa cell extracts. However, little is known about their mechanism of action. In this study, we investigate how BETT regulates Prothymosin α (ProT), a nuclear protein previously shown to play essential roles in apoptosis. We first showed that Apaf-1 is the direct target protein of BETT. We further demonstrated that BETT relieved ProT-mediated inhibition of apoptosome formation by blocking the interaction between Apaf-1 and ProT. Using Two-dimensional ¹H/¹⁵N-Heteronuclear Single Quantum Correlation (HSQC) experiments, we were also able to examine the interaction between Apaf-1 and ¹⁵N-labeled ProT α . Furthermore, we are able to reconstitute the entire caspase-3 activation pathway using purified ProT, Apaf-1, procaspase-9, procaspase-3, Hsp70, cytochrome c, PHAPI, CAS, and regulatory compounds to mimic stress induced apoptosis *in vitro*. Together, these studies would lead to novel and specific methods for the prevention, diagnosis and treatment of human cancer.

Apoptosis is the dominant form of programmed cell death during embryonic development, normal tissue turnover and cellular response to stress. Apoptosis is characterized by a variety of morphological changes, including blebbing, loss of membrane asymmetry and attachment, cell shrinkage, nuclear fragmentation, chromatin condensation, and chromosomal DNA fragmentation. Dysregulated apoptosis as a result of imbalance of anti-apoptotic and pro-apoptotic effectors have been implicated in an extensive variety of diseases. Apoptosis is upregulated in diseases such as AIDS, neurodegenerative disorders and ischemic stroke (1), whereas an insufficient amount apoptosis results in uncontrolled cell proliferation, such as certain cancers (2–3). One central player in apoptosis, apoptotic protease activating factor 1 (Apaf-1), consists of three functional domains: the N-terminal caspase recruitment domain (CARD), the middle nucleotide-binding and oligomerization domain (NOD), and the C-terminal regulatory region which comprises 13 WD-40 repeats (4–8) and normally keeps Apaf-1 in an autoinhibitory state. During apoptosis, cytochrome c is released from mitochondria to the cytosol causing caspase activation (9–10). The released cytochrome c readily binds to Apaf-1 and induces a conformational change that drives the formation of a heptamer Apaf-1-cytochrome c complex called the apoptosome (4,11). The formation of this complex depends on the availability of exogenous nucleotide dATP/ATP; without nucleotide

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exchange, Apaf-1/cytochrome c form inactive aggregates (12). The central ring of apoptosome is formed by the conjugation of seven CARD and NOD domains of Apaf-1, and each of the seven spokes extending from the central ring is composed of 13 WD-40 repeats bound to one cytochrome c. The apoptosome recruits and activates procaspase-9, which in turn activates a cascade of caspases (6,13). These caspases cleave a variety of protein targets and eventually lead to cell death (14).

Prothymosin α (ProT α) is a small, highly acidic protein found in the nuclei of virtually all mammalian tissues (15). The human form has 110 amino acid residues (M.W. 12.1 kDa; PI~3.5). Uniquely, this protein is observed to be biologically active while intrinsically disordered with a random coil conformation (16). ProT α has been shown to play essential roles in cell proliferation (17–18), transcriptional regulation (19–20), chromatin remodeling (21–22), oxidative stress-response (23) and apoptosis (24–25). Furthermore, this protein is highly expressed in certain tumor tissues and used as a cancer prognostic marker; although the exact mechanism for its oncogenic property is not quite clear yet. Previously, we identified a death regulatory pathway by using a combined high-throughput chemical screen and biochemical fractionation approach. The pathway consists of oncoprotein ProT and tumor suppressor putative HLA-DR-associated proteins (PHAP), each playing a distinctive role in regulating apoptosome formation and activity (25). ProT negatively regulates caspase-9 activation by inhibiting apoptosome formation, although the underlying mechanisms remain elusive. Further study demonstrated that PHAP, cellular apoptosis susceptibility protein (CAS), and heat shock protein (Hsp70) function together to accelerate nucleotide exchange on Apaf-1 and prevent inactive Apaf-1/cytochrome c aggregation (26). Screening small molecules for their ability to perturb a cellular pathway, a strategy called forward chemical genetics, can yield new information to identify other related pathways. In this study, we utilized forward chemical genetics and identified a panel of novel small-molecule activators of apoptosis from a library of ~200,000 compounds screening, which inhibits the function of ProT in the regulation of the mitochondrial cell death pathway. Furthermore, we demonstrated that the small molecule BETT relieved ProT inhibition of apoptosome formation by blocking the interaction between Apaf-1 and ProT. Together, this work contributed to the thorough understanding of mitochondria-initiated death regulatory pathway.

Materials and Methods

Materials

Spodoptera frugiperda (Sf-21) insect cells, Cell-FECTIN reagent, IPL-41 insect medium, all media supplements, and protease inhibitors were obtained from GIBCO/Life Technologies, Grand Island, NY. dATP was obtained from Amersham Pharmacia Biotech. Enzyme grade Hepes, imidazole, MgCl_2 , Na_4EDTA , KCl, NaCl, and DTT were obtained from Sigma. ECL reagents were obtained from Amersham Pharmacia Biotech. Acrylamide (99.9%) and all gel electrophoresis chemicals were obtained from Bio-Rad.

Production of Recombinant Apaf-1

Full-length Apaf-1 cDNA was subcloned into the baculovirus expression vector pFastBacI (GIBCO/Life Technologies) and transformed into DH10Bac *Escherichia coli* cells. The recombinant viral DNA was purified according to the Bac-to-Bac Baculovirus Expression System procedure (GIBCO/Life Technologies) and used to transfect Sf-21 insect cells with CellFECTIN reagent. The insect cells were grown in IPL-41 insect media supplemented with 10% FCS, 2.6 g/l tryptose phosphate broth, 4 g/l yeastolate, 0.1% Pluronic F-68, and $1 \times$ antibiotic/antimycotic. The viral stock was amplified to 50 ml, which was used to infect 1.0 liter of Sf-21 cells at a density of 2×10^6 cells/ml. Infected cells were harvested 40 h postinfection. Cells were centrifuged for 20 min at 5,000 rpm and then resuspended in buffer

A, pH 8.0 containing 1 M ethanol (buffer A = 20 mM Hepes/10 mM KCl/1.5 mM MgCl₂/1 mM Na₄EDTA). For all purification steps, buffer A was supplemented with 1 mM DTT, 0.1 mM PMSF, and 0.1 μ M aprotinin. Cells were incubated on ice for 15 min then lysed by homogenization with a tissue grinder. Lysed cells were centrifuged in a Beckman ultracentrifuge at 19,000 rpm for 1 h. The cell supernatant was loaded onto a 4 ml Ni-nitrilotriacetic acid column, and the column was washed with 300 ml of buffer A, pH 7.5 containing 1 M NaCl and 20 mM imidazole. Apaf-1 then was eluted by using 8 ml of buffer A, pH 7.5 containing 250 mM imidazole. Eluted protein was stored at -70°C in buffer A with 20% glycerol. Protein concentration was determined by using a quantitative Bradford assay (Bio-Rad).

Preparation of Extracts

We obtained human HeLa S3 cells from National Cell Culture Center. The cell pellets were resuspended in 5 volumes of buffer A (20 mM HEPES-KOH [pH 7.5], 10 mM KCl, 1.5 mM MgCl₂, 1 mM sodium EDTA, 1 mM sodium EGTA, 1 mM DTT, and 0.1 mM PMSF). The cells were homogenized, and centrifuged at $1,000 \times g$ for 10 min at 4°C . The supernatant was further centrifuged at $100,000 \times g$ for 1 hr in a Beckman SW 28 rotor. The resulting supernatant (S-100 fraction) was stored at -80°C and used as the starting material for the purification of small molecule (such as Biotin-BETT) binding protein.

In vitro Binding Studies

The Streptavidin dynabeads (Invitrogen/Dynal Biotech, Prod. No. 112.05, 2 ml at 10 mg/ml) were washed three times with Buffer A before use. Biotinylated small molecules were precoupled to Streptavidin dynabeads at 3 nmol/50 μ l of beads for 30 min at 4°C . The coated beads were washed three times using a magnet to remove non-bound material.

The beads were then incubated for O/N at 4°C with 1 ml of HeLa S100, which had been dialyzed against buffer B (buffer A containing 150 mM NaCl and supplemented with 0.1% NP40). After incubation, the beads were washed with buffer B (500 μ l each time) for 3 times. Bound proteins were separated on the 12% SDS-PAGE gel. Binding of recombinant proteins to compounds was performed essentially as above, except that streptavidin beads were blocked with 5 mg/ml BSA before coupling with compounds.

FLAG-Apaf-1 Immunoprecipitation with Recombinant N-His Prothymosin α (ProT)

50 μ l Anti-FLAG M2 –Agarose bead (Sigma A2220-5 mL) were washed with 500 μ l Buffer A for three times. A mixture of recombinant proteins including Flag-Apaf-1 (82 nM), dATP (5 μ M), cyt.c (100 nM), His-ProT (1.6 μ M) and Hsp70 (50 nM) in the presence and absence of BETT were incubated at 30°C for 1h.

The mixtures of recombinant proteins were incubated with the washed beads overnight at 4°C . The beads were centrifuged for 1 min at max speed. The supernatants were saved as reference. The beads were washed three times with 500 μ l buffer A. Protein bound to the beads were eluted with 50 μ l $3 \times$ FLAG peptide (0.5 μ g/ μ l) with gentle shaking for 30 min at 4°C . The beads were centrifuged for 1 min at max speed and the supernatants were transferred to fresh tubes. The eluted proteins were separated on a 15% SDS PAGE gel, which was then stained with Invitrogen silver stain kit.

ProT Western Blot

The membrane was presoaked for 1h in 20 mM NaOAc, pH 5.2 transfer buffer. (BIO-RAD Trans-Blot Transfer Medium-0.2 μ m nitrocellulose membrane). Transfer was performed at 30

V, 140 mA for 5 h in a cold room. The membrane was subsequently fixed with 0.5% Glutaldehyde (stock 25% solution in H₂O, Sigma # G5882) for 1 h.

Determination of ProT level in ProT overexpressing U2OS cells and WT U2OS cells

All cell lysis extracts were normalized to the same concentration (1.2 mg/ml). 750 µl phenol: chloroform: isoamyl alcohol (49.5:49.5:1) were added to each 250 µl cell lysis extract. The mixture were centrifuged at 14,000 rpm for 10 min. The upper aqueous layer with ProT were dried with a speedvac down to 20 µl, and then run on a 15% gel and stained with Coomassie blue.

Expression and purification of recombinant ProT

Briefly, the protein was expressed in *E. coli* BL21 (DE3) cells using pET 21b plasmid carrying the human His-tagged ProT cDNA. For uniformly ¹⁵N/¹³C-labeled protein samples, cells were grown in M9 minimal medium containing ¹⁵NH₄-Cl and ¹³C₆-D-glucose (Cambridge Isotope Laboratories) as the sole nitrogen and carbon sources, respectively. Recombinant ProT was purified by successive Ni column and anion exchange chromatography.

NMR Spectroscopy

All NMR experiments were recorded at 25° C on a Varian NMR spectrometer operating at a proton frequency of 600 MHz and equipped with a cryogenic triple resonance probe. The NMR buffer contained 40 mM HEPES, pH 7.0, and 10% D₂O (Cambridge Isotope Laboratories). The data was processed with NMRPipe and analyzed with NMRView.

In vitro Fluorogenic Assay for Caspase-3 Activity

Caspase-3 activity was measured by an XFluor4 spectrometry reader (Tecan) in 384-well plates using 10 µM of fluorogenic DEVD substrate (Calbiochem). In a typical experiment, 15 nM of recombinant Apaf-1, 100 nM of CAS, 50 nM of Hsp70, 25 nM of procaspase-9, 50 nM of procaspase-3, 100 nM of cytochrome c, 300 nM of PHAPI, 5 µM of dATP, 1.5 µM of ProT α, and 10 µM of fluorogenic DEVD substrate were used for *in vitro* assay with buffer A (20 mM Hepes, pH 7.5, 10 mM KCl, 1.5 mM MgCl₂, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, and 0.1 mM PMSF) supplied with additional 2.5 mM of MgCl₂. The assay was performed at 30 °C in 20 µL final volume.

For Figure 4B: Titration of recombinant Apaf-1 is performed with vary Apaf-1 concentrations (0, 1, 2.5, 5, 10, 15, 20, 30, 50, 100, 150 and 300 nM). 100 nM of CAS, 50 nM of Hsp70, 25 nM of procaspase-9, 50 nM of procaspase-3, 100 nM of cytochrome c, 300 nM of PHAPI, 5 µM of dATP, and 10 µM of fluorogenic DEVD substrate were used here for *in vitro* assay with buffer A supplied with additional 2.5 mM of MgCl₂.

For Figure 4C: Titration of recombinant dATP is performed with vary dATP concentrations (0, 0.001, 0.01, 0.1, 1, 2, 5, 10, 20, 50, 250 and 1000 µM). 15 nM of recombinant Apaf-1, 100 nM of CAS, 50 nM of Hsp70, 25 nM of procaspase-9, 50 nM of procaspase-3, 100 nM of cytochrome c, 300 nM of PHAPI, and 10 µM of fluorogenic DEVD substrate were used here for *in vitro* assay with buffer A supplied with additional 2.5 mM of MgCl₂.

For Figure 4D and 4E: In a typical experiment, 50 µM of each small molecule, 15 nM of recombinant Apaf-1, 100 nM of CAS, 50 nM of Hsp70, 25 nM of procaspase-9, 50 nM of procaspase-3, 100 nM of cytochrome c, 300 nM of PHAPI, 5 µM of dATP, 1.5 µM of ProT α, and 10 µM of fluorogenic DEVD substrate were used for *in vitro* assay with buffer A supplied with additional 2.5 mM of MgCl₂. 4E is the maximum activation at the time point 290 min of 4D.

Direct ELISA for the Prothymosin α and Apaf-1 interaction in the presence of small molecules

1. Coating of the ELISA microwells with ProT—The ELISA microwells were coated with 200 μ l/well ProT solution (1 μ g/ml) in coating buffer (citrate buffer: 0.15 M, pH 5.0) and incubated overnight at 37 °C. The coating buffer was discarded and the microwells were rinsed twice with PBS pH 7.4 washing buffer (PBS pH 7.4 containing 0.05% v/v Tween 20). Afterwards, the microwells were incubated with a blocking solution of 2% BSA solution (200 μ l/well) for 1 h at 37 °C. After incubation, the blocking solution was discarded and the microwells were rinsed three times with washing buffer. The prepared ProT α coated microwells were ready for use in titer determination, displacement curve or ELISA measurement experiments.

2. ELISA measurement—4 μ l of small molecule solution in DMSO with the final concentration of 4 μ M to 2 mM were mixed with 96 μ l of C-FLAG-Apaf 1 solution (80 nM). The mixture was vortexed and then immediately pipetted into ProT α coated microwells (200 μ l/well) and incubated for 2 h at 37 °C. Each incubation step was followed by three rinses at 200 μ l washing buffer/well. Mouse monoclonal ANTI-FLAG M2-Peroxidase (HRP) antibody (Sigma, A8592) was diluted at 1:10,000 dilution in dilution buffer, added to 200 μ l/well and incubated for 1 h at room temperature. The incubation step was followed by three rinses 200 μ l/well with washing buffer. Color was developed by the addition of 200 μ l ABTS/well and optical absorbance was measured after 30 min at 410 nm.

RESULTS AND DISCUSSION

Novel small molecules stimulate apoptosome formation and caspase-3 activation in HeLa cell extracts

The death pathway that regulates mitochondria-initiated caspase activation was first identified using a small molecule α -(trichloromethyl)-4-pyridineethanol (PETCM) which relieves ProT inhibition of apoptosome formation. However, the structure of PETCM does not show any chemical group other than the hydroxyl group that can be easily tagged (25). Furthermore, modification of the hydroxyl group inhibits PETCM activity. To overcome these limitations, we conducted a more extensive chemical screen using a library of ~200,000 compounds. A panel of potent hits was identified from this large-scale screen to activate caspase-3 in a similar manner to PETCM but with a variety of chemical structures present (Fig.1A). Addition of these small molecules to the HeLa S-100 fraction in the absence of exogenous dATP activated caspase -3 in a dose-dependent manner as measured by the liberation of fluorogenic artificial caspase-3 substrate with EC50 ranging from 10–50 μ M (Fig.1B). Caspase-3 in HeLa cell extracts was activated by the addition of 200 μ M small molecule hits in a time course study, which showed significant stimulation after 40 minutes of incubation compared to no-drug control (Fig.1C).

BETT targets Apaf-1 to stimulate apoptosome formation

To further understand the regulatory mechanisms of these small molecules, we wanted to identify their protein targets using a pull-down strategy. The pull down strategy, although straightforward, depends on several factors to be successful. First, we have to find a compound among these good hits that is suitable for biotin labeling, such as compounds with side chain which can be modified. Second, the affinity has to be high enough to allow us to distinguish the real target from non-specific binding. We focused on BETT because this compound was proven to be particularly amenable for biotin labeling (Fig 2A) without altering its function as activator of caspase-3 (Fig 2B). We incubated the biotin-tagged BETT (Fig 2A) with HeLa cell extracts and performed pull-down experiments with streptavidin-coated beads. The supernatant was checked for regulatory molecule-induced caspase activation for the disappearance of its target. The pellet was washed and the bound proteins were separated on

SDS-PAGE, identified by mass spectrometry and confirmed by Western blot as essential protein Apaf-1 (Fig. 2C). We next demonstrated the direct interaction of ProT with Apaf-1 using an *in vitro* system including Flag-Apaf-1, dATP, cyt.c, His-ProT and Hsp70. Also less ProT was detected with increasing amount of BETT present in the system (Fig. 2D). Our findings here indicate that BETT does not directly interact with caspase 3 and caspase 9, therefore likely functioning upstream of apoptosome formation through blocking the interactions between Apaf-1 and ProT.

NMR analysis of the interaction between Apaf-1 and ¹⁵N-labeled ProT α

Many proteins have been identified as being intrinsically unstructured, yet still biologically functional. ProT plays an essential role in cell proliferation and apoptosis, however does not adopt a well-defined three-dimensional structure under physiological conditions (15). Since intrinsically disordered proteins cannot be easily crystallized, we therefore performed nuclear magnetic resonance (NMR) spectroscopy to analyze the interaction between ProT α and Apaf-1 at the atomic level in solution. Two-dimensional ¹H/¹⁵N-Heteronuclear Single Quantum Correlation (HSQC) experiments of ¹⁵N-labeled ProT α alone (Figure 3, black) and in the presence of Apaf-1 (Figure 3, red) were recorded. Using the chemical shift assignments previously reported by Yi *et al* (27) for ProT α , we were able to identify several residues directly involved in the interaction with Apaf-1. As shown in figure 3, the peaks corresponding to residues A4, T8, S9, E11, T13, T14, R31, A35, N43, S84, T86, G87, and T106 in the protein complex have reduced intensity (one standard deviation below the mean) when compared to the rest of the peaks or have been broadened beyond detection. In addition, the peaks for residues in the C-termini, E108 and D110 show large chemical shift changes. Due to the narrow chemical shift dispersion and the large peak overlap present in the spectra, a full analysis of the ProT α binding interface is challenging. However, our data suggests that the regions delineated by ProT α residues 4–14, 31–43, 84–87, and 106–110 strongly and specifically bind to Apaf-1.

ProT inhibits caspase-3 activation and small molecules antagonize the inhibitory function of ProT in a recombinant system

Next we investigated the biochemical details of the function of small molecules including BETT using a reconstituted caspase 3 activation system. This reconstituted system include Apaf-1 (15 nM), procaspase 9 (25 nM), procaspase 3 (50 nM), cytochrome c (50 nM), PHAPI (300 nM), CAS (100 nM), Hsp70 (50 nM), ProT (1.5 μ M) with other components dATP (5 μ M), MgCl₂ (2.5 mM) and regulatory compounds to mimic stress induced apoptosis *in vitro*. All recombinant proteins were expressed in bacteria or baculovirus and the tagged recombinant proteins were purified through affinity chromatography (Fig. 4A). Using this system, we performed a systematic analysis of the biochemical cascade leading to caspase-3 activation. The measurable steps in this cascade include: titration of Apaf-1 concentration (Fig 4B), titration of ATP level (Fig. 4C), and caspase-3 activity (Fig. 4D). In mammalian cells, ProT is an oncoprotein required for cell proliferation, with a function consistent with the antiapoptotic activity identified here. Since apoptosome formation requires nucleotide hydrolysis and exchange, apoptosis is regulated by the cellular ATP level. During apoptosis, Apaf-1 has been shown to use dATP as a cofactor. Cytochrome c binding to Apaf-1 induces hydrolysis of dATP to dADP, which is subsequently replaced by exogenous dATP. Indeed, when intracellular ATP is too low, cells are unable to activate caspase-9 even in the presence of apoptotic stimuli that induce cytochrome c release (28). In accordance, our ATP level titration experiment indicated that 5 μ M dATP was required in our reconstituted system to activate caspase 3 (Fig. 4C). We also tested the functional consequence of small molecule binding to its target by examining the increase of caspase-3 activation in the presence of small molecules (Fig. 4D, E). The results indicate that the small molecules antagonize the inhibitory function of ProT in apoptosome formation *in vitro*. PHAP (29–37), CAS and Hsp70 promote apoptosome formation by

preventing Apaf-1 aggregation and enhancing nucleotide exchange on Apaf-1 (26) when ProT (38,39) is suppressed by small molecules. Considering that ProT and Apaf-1 directly interact, and BETT target Apaf-1, we wanted to test whether BETT relieves ProT inhibition of apoptosome formation by directly blocking the interactions between ProT and Apaf-1. We used ELISA assay to probe the interaction between ProT and Apaf-1. ProT was precoated on the ELISA microwells, and the mixtures of C-FLAG-Apaf 1 solutions (80 nM) and small molecule solution from 4 μ M to 2 mM in DMSO were applied into the ProT α coated microwells. Monoclonal ANTI-FLAG M2-Peroxidase (HRP) antibody produced in mouse were added and color was developed by addition of 200 μ l/well ABTS and the optical absorbance was measured after 30 min at 410 nm (Fig. 4F). With increasing level of small molecule BETT applied, reductions in Apaf-1 were detected by ELISA assay. It is also worth noting that the concentration threshold needed to efficiently block Prothymosin α and Apaf-1 interaction is highly consistent with EC₅₀ of BETT at around 50 μ M. Taken together, these observations support the notion that small molecule BETT relieves ProT-mediated inhibition of apoptosome formation by blocking the interaction between Apaf-1 and ProT.

ProT overexpressing U2OS cells show resistance to an apoptotic stimulus

Cell division and programmed cell death (apoptosis), although seemingly opposing, are tightly linked. Prothymosin α is a small highly acidic protein found in the nuclei of virtually all mammalian tissues. It is highly conserved in mammals and widely expressed in various tissues. While the exact mechanism of Prothymosin α function remains elusive, it has been associated with cell growth and survival. To examine a putative anti-apoptotic role of ProT *in vivo*, U2OS WT cells were compared with U2OS cells overexpressing ProT under apoptotic-inducing conditions. When irradiated with ultraviolet (UV) light, the ProT overexpressing cells showed a lower rate of apoptosis. Twelve hours after UV irradiation, only 20% of the ProT overexpressing U2OS cells showed apoptotic morphology, whereas control U2OS WT cells showed more than 70% cell death (Fig. 5A). Cell death also correlated with the caspase-3 activation as lower caspase-3 activation was also observed in the ProT overexpressing U2OS cells (Fig. 5B and 5C).

In summary, our study herein demonstrates that the small molecule BETT pulls down Apaf-1 but not caspase 3 and caspase 9, indicative of regulatory functions prior to apoptosome formation. Considering that ProT and Apaf-1 directly interact *in vitro*, it is possible that ProT binds to Apaf-1 ATP binding domain and inhibits ATP binding to Apaf-1. Alternatively, ProT binds to some other site and causes the Apaf-1 ATP binding domain conformation change, thus inhibiting ATP binding to Apaf-1. Small molecules interact with Apaf-1 at the similar site as ProT and prohibit ProT binding to Apaf-1; on the other hand, they are small enough so not interfere with ATP binding to Apaf-1. Therefore, those small molecules are able to release the inhibitory function of ProT to apoptosome formation. Currently we are making several Apaf-1 mutants to further test our hypothesis (Fig. 4A). Specificity studies are being conducted using domain mutants corresponding to loss or gain of function. Detailed structural determinations will be initiated and performed when ligand-protein complexes are fully characterized.

As mentioned above, we have conducted a quantitative EC₅₀ analysis for BETT and related compounds that gives good signal over 4 orders of magnitude in drug concentration. For small molecule derivatives, these assays measure how readily small molecules interact with their target proteins. We will construct several synthetic variants of our best hits. The efficacy of these compounds will then be tested using our EC₅₀ assay. The cell culture and *in vitro* studies can be extended into comprehensive *in vivo* studies. This can be carried out by analyzing these regulatory molecules in a variety of cancer cell lines in response to a number of apoptotic stimuli. In addition, these compounds will be tested for their ability to permeate the cell membrane using photogenic tagged compounds. It is likely that some of these compounds

function in a highly cell type- and stimulus-specific manner and play essential roles in tumor suppression. It is very important to study the compound uptake for potential drug development. 6-iodoacetamidofluorescein (6-IAF) is a common thiol-reactive probe which can be excited with visible light and shown as green color under microscope. We synthesized IAF labeled BETT and applied to cell media. By comparing the Hoescht stained blue nucleus and MitoTracker Red stained mitochondrion, BETT-IAF conjugate is likely localized in cell cytosol by live cell imaging. A better understanding of the mechanisms of apoptosis pathway is essential for designing more specific and efficient methods for prevention, diagnosis and treatment of human cancer. Our study herein may identify new apoptosis regulators and tumor suppressors which may uncover new drug leads for cancer treatment.

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Abbreviations

Apaf-1	Apoptotic protease activating factor 1
Hsp70	Heat shock protein 70
cyt. c	cytochrome c
PHAPI	Putative HLA-DR- associated protein I
CAS	Cellular apoptosis susceptibility protein

Chemical Names of Small Molecule Hits

ALP	Alprazolam
EBC	Ethyl 3-methyl-4H-1,4-benzothiazine-2-carboxylate
PPT	5-Pyrimidinecarboxylic acid, 1,2,3,4-tetrahydro-6-methyl-4-(2-phenylethenyl)-2-thio-, methyl ester
BETT	5-(2-Benzofuryl)-4-phenyl-1,2,4-triazole-3-thiol
TBP	5-chloro-2-oxo-1-[3-(trifluoromethyl)benzyl]-1,2-dihydro-3-pyridinecarboxylic acid
OTF	1,3,4-Oxadiazole, 2-[[[(4-bromophenyl)methyl]thio]-5-(2-furanyl)-
IBP	3(2H)-Isoquinolinone, 1-(4-bromophenyl)-1,4-dihydro-
BCP	2-(1H-benzimidazol-1-yl)ethyl 2-chlorophenyl ether
MCC	Methyl 9H- β -carboline-3-carboxylate

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Figure 1. Small molecules stimulate caspase-3 activation in HeLa cell cytosol. (A) Structures of small molecule hits (For full chemical names, see Materials and Methods). (B) EC50 of these small molecules to activate caspase-3. (C) Time course comparison of the stimulatory effects of small molecules (200 μ M) in HeLa S100.

Figure 2. BETT stimulates caspase-3 activation in HeLa cell cytosol. (A) Structure of Biotin-labeled BETT. (B) Time course comparison of the stimulatory effects of Biotin-BETT and BETT without exogenous dATP. (C) BETT-Biotin can pull down Apaf-I in HeLa cell S100. (D) Direct interaction between FLAG-tagged Apaf-1 and His-ProT in an assay system also including dATP, cyt. c, Hsp70 and different amount of BETT.

Figure 3.

Interaction of ^{15}N labeled-ProT with Apaf-1 by ^1H - ^{15}N HSQC NMR spectra. Two-dimensional $^1\text{H}/^{15}\text{N}$ -HSQC experiment of 25 μM ProT (black) and 5 μM ProT in the presence of 5 μM Apaf-1 (Red). Residues whose peak intensities are one Std. Dev. below the mean (only fully resolved peaks were used for the analysis) in the red spectrum or have been broadened beyond detection are labeled. The peaks with a * are unassigned residues most likely derived from the His₆ tag and linker region present in the protein construct used for the NMR experiments.

Figure 4.

The regulatory function of small molecule BETT in a reconstituted system *in vitro*. Total reconstitution of the entire caspase-3 activation pathway was conducted using purified Apaf-1, procaspase-9, procaspase-3, cytochrome c, PHAPI, CAS, Hsp70, ProT, regulatory compound BETT to mimic stress induced apoptosis *in vitro*. (A) An aliquot of each purified recombinant protein was applied to 4–20% Tris-HCl followed by Coomassie Blue staining. (B) Titration of recombinant Apaf-1 for caspase-3 activity in recombinant system. (C) Titration of dATP for caspase-3 activity in recombinant system. (D) Time course comparison of the stimulatory effects of small molecules in reconstituted system. (E) Histogram comparison of the stimulatory effects of small molecules in reconstituted system at maximum activation (290 min

from 3D). (F) Interaction of ProT and Apaf-1 is blocked by BETT probed by ELISA assay. All the experiments were done at least three times with similar results. Detailed information for all the experiments is in Materials and Methods section- *in vitro* fluorogenic assay for caspase 3 activity.

Figure 5.

ProT overexpressed U2OS cells show resistance to an apoptotic stimulus. (A) ProT overexpressed cells block UV-induced cell death. Micrographs of ProT overexpressed U2OS cells and WT U2OS cells without UV treatment compared with 12 hours after UV irradiation. (B) Time course comparison of ProT overexpressed U2OS and WT U2OS cell lysates for caspase-3 activation. (C) ProT overexpressed U2OS cells block caspase-3 activation by maximum activation comparison. All the experiments were done at least three times with similar results.