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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 $^{1}H-^{15}N$ heteronuclear single-quantum correlation (HSQC) experiments, we were also able to examine the interaction between Apaf-1 and ^{15}N-labeled ProT α. Furthermore, we were 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 an imbalance of anti-apoptotic and pro-apoptotic effectors has 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 of apoptosis results in uncontrolled cell proliferation, such as certain cancers (*2*, *3*). One central player in apoptosis, apoptotic protease activating factor 1 (Apaf-1),[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 that 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 exchange, the Apaf-1−cytochrome *c* complex forms inactive aggregates (*12*). The central ring of the 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 (molecular mass of 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 strongly 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.

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[1]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-4*H*-1,4-benzothiazine-2-carboxylate; PPT, 5-pyrimidinecarboxylic acid, 1,2,3,4-tetrahydro-6-methyl-4-(2-phenylethenyl)-2-thioxo-, 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(2*H*)-isoquinolinone, 1-(4-bromophenyl)-1,4-dihydro-; BCP, 2-(1*H*-benzimidazol-1-yl)ethyl 2-chlorophenyl ether; MCC, methyl 9*H*-β-carboline-3-carboxylate.

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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 aggregation of the inactive Apaf-1−cytochrome *c* complex (*26*). Screening small molecules for their ability to perturb a cellular pathway, a strategy called forward chemical genetics, can yield new information for identifying 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 ~200000 compounds, 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 the mitochondrion-initiated death regulatory pathway.

MATERIALS AND METHODS

Materials. *Spodoptera frugiperda* (Sf-21) insect cells, CellFECTIN reagent, IPL-41 insect medium, all medium 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 baculovirus expression vector pFastBacI (GIBCO/Life Technologies) and transformed into *Escherichia coli* DH10Bac 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 medium supplemented with 10% FCS, 2.6 g/L tryptose phosphate broth, 4 g/L yeastolate, 0.1% Pluronic F-68, and 1× antibiotic/antimycotic. The viral stock was amplified to 50 mL, which was used to infect 1.0 L 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 5000 rpm and then resuspended in buffer A (pH 8.0) containing 1 M ethanol (buffer A consists of 20 mM Hepes, 10 mM KCl, 1.5 mM $MgCl_2$, and 1 mM Na_4EDTA). For all purification steps, buffer A was supplemented with 1 mM DTT, 0.1 mM PMSF, and 0.1 μM aprotinin. Cells were incubated on ice for 15 min and then lysed by homogenization with a tissue grinder. Lysed cells were centrifuged in a Beckman ultracentrifuge at 19000 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. The eluted protein was stored at −70 °C in buffer A with 20% glycerol. The protein concentration was determined by using a quantitative Bradford assay (Bio-Rad).

Preparation of Extracts. We obtained human HeLa S3 cells from the 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 $MgCl_2$, 1 mM sodium EDTA, 1 mM sodium EGTA, 1 mM DTT, and 0.1 mM PMSF]. The cells were homogenized and centrifuged at 1000*g* for 10 min at 4 °C. The supernatant was further centrifuged at 100000*g* for 1 h 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 a small molecule (such as Biotin-BETT) binding protein.

In Vitro Binding Studies. The Steptavidin Dynabeads (Invitrogen/Dynal Biotech, product 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 a level of 3 nmol/50 μL of beads for 30 min at 4 °C. The coated beads were washed three times using a magnet to remove nonbound material. The beads were then incubated overnight at 4 °C with 1 mL of HeLa S-100, which had been dialyzed against buffer B (buffer A containing 150 mM NaCl and supplemented with 0.1% NP40). After being incubated, the beads were washed with buffer B (500 μL each time) three times. Bound proteins were separated via 12% SDS−PAGE. Binding of recombinant proteins to compounds was performed essentially as described 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). Anti-FLAG M2−agarose beads (50 μL) (Sigma A2220, 5 mL) were washed with 500 μL of buffer A three times. A mixture of recombinant proteins, including FLAG-Apaf-1 (82 nM), dATP (5 μM), cyt *c* (100 nM), His-ProT (1.6 μM), and Hsp70 (50 nM), in the presence and absence of BETT was incubated at 30 °C for 1 h.

The mixtures of recombinant proteins were incubated with the washed beads overnight at 4 °C. The beads were centrifuged for 1 min at the maximum speed. The supernatants were saved as a reference. The beads were washed three times with 500 μL of buffer A. Protein bound to the beads were eluted with 50 μL of 3× FLAG peptide (0.5 μg/μL) with gentle shaking for 30 min at 4 °C. The beads were centrifuged for 1 min at the maximum 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 an Invitrogen silver stain kit.

ProT Western Blot. The membrane was presoaked for 1 h in 20 mM NaOAc (pH 5.2) transfer buffer (Bio-Rad Trans-Blot Transfer Medium, 0.2 μm nitrocellulose membrane). Transfer was performed at 30 V and 140 mA for 5 h in a cold room. The membrane was subsequently fixed with 0.5% glutaraldehyde (25% stock solution in H_2O, Sigma catalog no. G5882) for 1 h.

Determination of the ProT Level in ProT-Overexpressing U2OS Cells and Wild-Type U2OS Cells. All cell lysis extracts were normalized to the same concentration (1.2 mg/mL); 750 μL of a phenol/chloroform/isoamyl alcohol mixture (49.5:49.5:1) was added to each 250 μL cell lysis extract. The mixture was centrifuged at 14000 rpm for 10 min. The upper aqueous layer with ProT was 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 the pET 21b plasmid carrying the human His-tagged ProT cDNA. For uniformly ^{15}N- and ^{13}C-labeled protein samples, cells were grown in M9 minimal medium containing $^{15}NH_4Cl$ and [$^{13}C_6$]-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 conducted 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% D_2O (Cambridge Isotope Laboratories). The data were processed with NMRPipe and analyzed with NMRView.

In Vitro Fluorogenic Assay for Caspase-3 Activity. Caspase-3 activity was measured with an XFluor4 spectrometry reader (Tecan) in 384-well plates using 10 μM fluorogenic DEVD substrate (Calbiochem). In a typical experiment, 15 nM recombinant Apaf-1, 100 nM CAS, 50 nM Hsp70, 25 nM procaspase-9, 50 nM procaspase-3, 100 nM cyt *c*, 300 nM PHAPI, 5 μM dATP, 1.5 μM ProT α, and 10 μM fluorogenic DEVD substrate were used for the in vitro assay with buffer A [20 mM Hepes (pH 7.5), 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, and 0.1 mM PMSF] supplied with additional 2.5 mM $MgCl_2$. The assay was performed at 30 °C in a final volume of 20 μL.

For Figure 4B, titration of recombinant Apaf-1 was performed with varying Apaf-1 concentrations (0, 1, 2.5, 5, 10, 15, 20, 30, 50, 100, 150, and 300 nM); 100 nM CAS, 50 nM Hsp70, 25 nM procaspase-9, 50 nM procaspase-3, 100 nM cyt *c*, 300 nM PHAPI, 5 μM dATP, and 10 μM fluorogenic DEVD substrate were used here for the in vitro assay with buffer A supplied with additional 2.5 mM $MgCl_2$.

For Figure 4C, titration of recombinant dATP was performed with varying dATP concentrations (0, 0.001, 0.01, 0.1, 1, 2, 5, 10, 20, 50, 250, and 1000 μM); 15 nM recombinant Apaf-1, 100 nM CAS, 50 nM Hsp70, 25 nM procaspase-9, 50 nM procaspase-3, 100 nM cyt *c*, 300 nM PHAPI, and 10 μM fluorogenic DEVD substrate were used here for the in vitro assay with buffer A supplied with additional 2.5 mM $MgCl_2$.

For Figure 4D and Figure 4E, in a typical experiment, each small molecule at 50 μM, 15 nM recombinant Apaf-1, 100 nM CAS, 50 nM Hsp70, 25 nM procaspase-9, 50 nM procaspase-3, 100 nM cyt *c*, 300 nM PHAPI, 5 μM dATP, 1.5 μM ProT α, and 10 μM fluorogenic DEVD substrate were used for the in vitro assay with buffer A supplied with additional 2.5 mM $MgCl_2$. Figure 4E is the maximum activation at the 290 min time point of Figure 4D.

Direct ELISA for the Prothymosin α−Apaf-1 Interaction in the Presence of Small Molecules. (*i*) *Coating of the ELISA Microwells with ProT*. The ELISA microwells were coated with 200 μL of ProT solution (1 μg/mL) per well 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 washing buffer [PBS (pH 7.4) containing 0.05% (v/v) Tween 20]. Afterward, the microwells were incubated with a blocking solution [2% BSA solution (200 μL/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.

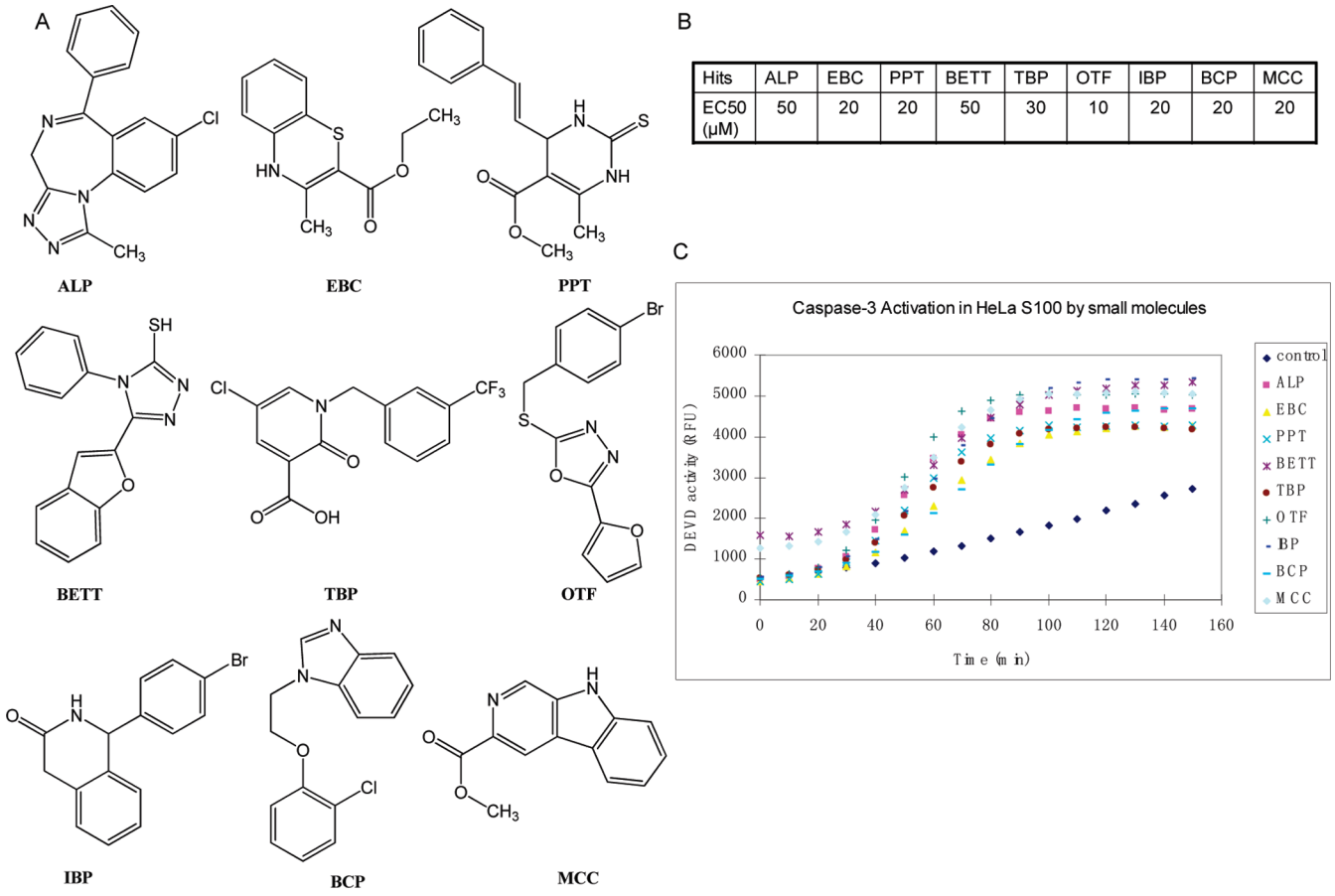
(*ii*) *ELISA Measurement*. Four microliters of a small molecule solution in DMSO at a final concentration of 4 μM to 2 mM was mixed with 96 μL of a C-FLAG-Apaf-1 solution (80 nM). The mixture was vortexed and then immediately pipetted into ProT α-coated microwells (200 μL/well) and incubated for 2 h at 37 °C. Each incubation step was followed by three rinses (200 μL of washing buffer/well). Mouse monoclonal ANTI-FLAG M2-Peroxidase (HRP) antibody (Sigma, A8592) was diluted at 1:10000 dilution in dilution buffer, added to a level of 200 μL/well, and incubated for 1 h at room temperature. The incubation step was followed by three rinses with washing buffer (200 μL/well). Color was developed by the addition of 200 μL of 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 mitochondrion-initiated caspase activation was first identified using a small molecule α-(trichloromethyl)-4-pyridineethanol (PETCM) that 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 ~200000 compounds. A panel of potent hits was identified from this large-scale screen to activate caspase-3 in a manner similar to that of PETCM but with a variety of chemical structures present (Figure 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 EC_{50} values ranging from 10 to 50 μM (Figure 1B). Caspase-3 in HeLa cell extracts was activated by the addition of 200 μM small molecule hits in a time course study, which showed significant stimulation after incubation for 40 min compared to the no-drug control (Figure 1C).

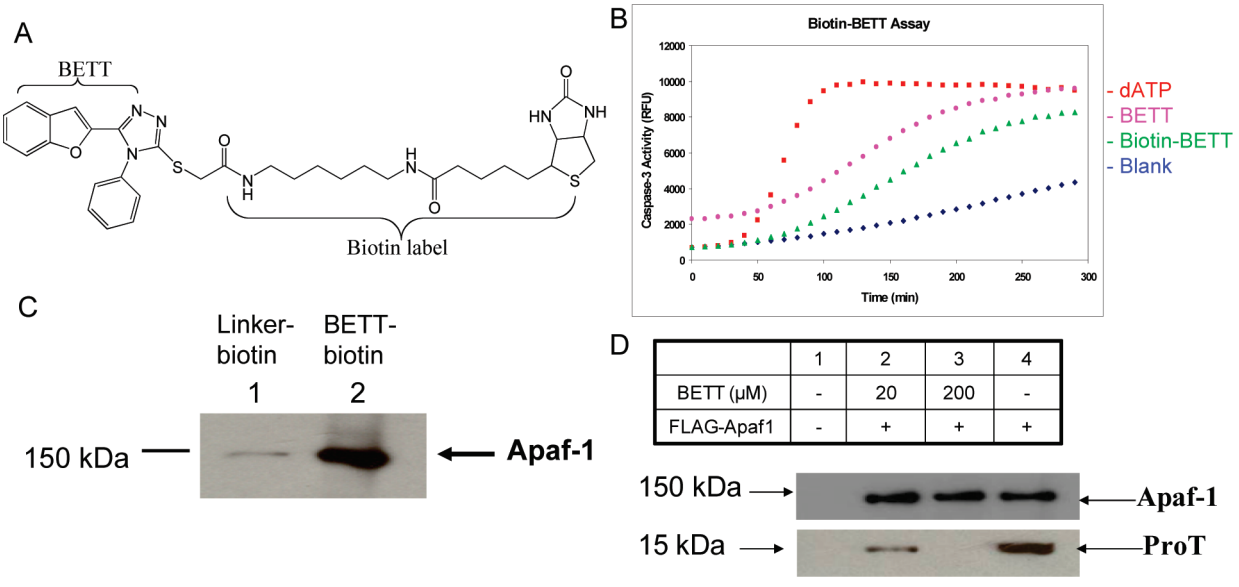
BETT Targets Apaf-1 To Stimulate Apoptosome Formation. To improve our understanding of the regulatory mechanisms of these small molecules, we wanted to identify their protein targets using a pull-down strategy. The pull-down strategy, although straightforward, depends on several factors to be successful. First, we have to find a compound among these good hits that is suitable for biotin labeling, such as compounds with a side chain that can be modified. Second, the affinity has to be sufficiently high to allow us to distinguish the real target from nonspecific binding. We focused on BETT because this compound was proven to be particularly amenable to biotin labeling (Figure 2A) without altering its function as an activator of caspase-3 (Figure 2B). We incubated the biotin-tagged BETT (Figure 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 via SDS−PAGE, identified by mass spectrometry, and confirmed by Western blotting as essential protein Apaf-1 (Figure 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 an increasing amount of BETT present in the system (Figure 2D). Our findings here indicate that BETT does not directly interact with caspase-3 and caspase-9, therefore likely functioning upstream of apoptosome formation by blocking the interactions between Apaf-1 and ProT.

NMR Analysis of the Interaction between Apaf-1 and ^{15}N-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 but does not adopt a well-defined three-dimensional structure under



Hits	ALP	EBC	PPT	BETT	TBP	OTF	IBP	BCP	MCC
EC50 (μM)	50	20	20	50	30	10	20	20	20

FIGURE 1: Small molecules stimulate caspase-3 activation in HeLa cell cytosol. (A) Structures of small molecule hits (for full chemical names, see Abbreviations). (B) EC_{50} values of these small molecules to activate caspase-3. (C) Time course comparison of the stimulatory effects of small molecules (200 μM) in HeLa S-100.



	1	2	3	4
BETT (μM)	-	20	200	-
FLAG-Apaf1	-	+	+	+

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 S-100. (D) Direct interaction between FLAG-tagged Apaf-1 and His-ProT in an assay system including dATP, cyt *c*, Hsp70, and different amounts of BETT.

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 $^{1}H-^{15}N$ heteronuclear single-quantum correlation (HSQC) experiments with ^{15}N-labeled ProT α alone (Figure 3, black) and in the presence of Apaf-1 (Figure 3, red) were conducted. 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.

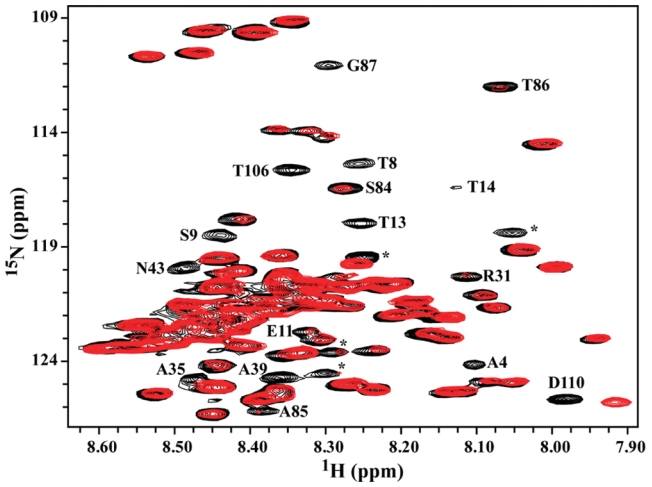


FIGURE 3: Interaction of ^{15}N labeled-ProT with Apaf-1 assessed by ^{1}H−^{15}N HSQC NMR spectra. Two-dimensional ^{1}H−^{15}N HSQC spectra of 25 μM ProT (black) and 5 μM ProT in the presence of 5 μM Apaf-1 (red). Residues whose peak intensities are one standard deviation below the mean (only fully resolved peaks were used for the analysis) in the red spectrum or have been broadened beyond detection are labeled. The peaks with an asterisk are unassigned residues most likely derived from the His_6 tag and linker region present in the protein construct used for the NMR experiments.

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, exhibit large chemical shift changes. Because of 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 suggest 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 capase-3 activation system. This reconstituted system includes Apaf-1 (15 nM), procaspase-9 (25 nM), procaspase-3 (50 nM), cyt *c* (50 nM), PHAPI (300 nM), CAS (100 nM), Hsp70 (50 nM), and ProT (1.5 μM), with other components dATP (5 μM), $MgCl_2$ (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 via affinity chromatography (Figure 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 (Figure 4B), titration of ATP level (Figure 4C), and caspase-3 activity (Figure 4D). In mammalian cells, ProT is an oncoprotein required for cell proliferation, with a function consistent with the anti-apoptotic 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 the intracellular ATP level is too low, cells are unable to activate caspase-9 even in the presence of apoptotic stimuli that induce cyt *c* release (*28*). In accordance, our ATP level titration experiment indicated that 5 μM dATP was required in our reconstituted system to activate caspase-3 (Figure 4C). We also tested the functional consequence of a small molecule binding to its target by examining the increase in the level of caspase-3 activation in the presence of small molecules (Figure 4D,E). The results indicate that the small molecules antagonize the inhibitory function of ProT during 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 targets 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 an ELISA 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 a small molecule solution from 4 μM to 2 mM in DMSO were applied into the ProT α-coated microwells. Monoclonal ANTI-FLAG M2-peroxidase (HRP) antibody produced in mouse was added; color was developed by addition of 200 μL of ABTS/well, and the optical absorbance was measured after 30 min at 410 nm (Figure 4F). With an increasing level of small molecule BETT applied, reductions in the level of Apaf-1 were detected via an ELISA. It is also worth noting that the concentration threshold needed to efficiently block prothymosin α−Apaf-1 interaction is highly consistent with an EC_{50} for BETT of ~50 μM. 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 Exhibit 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 wild-type (WT) cells were compared with U2OS cells overexpressing ProT under apoptosis-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-overexpessing U2OS cells showed apoptotic morphology, whereas control U2OS WT cells showed more than 70% cell death (Figure 5A). Cell death also correlated with the caspase-3 activation as a lower level of caspase-3 activation was also observed in the ProT-overexpressing U2OS cells (Figure 5B,C).

In summary, our study 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 the Apaf-1 ATP binding domain and inhibits binding of ATP to Apaf-1. Alternatively, ProT binds to some other site and causes the Apaf-1 ATP binding domain conformation change, thus inhibiting binding of ATP to Apaf-1. Small molecules interact with Apaf-1 at a site similar to that of ProT and prohibit binding of ProT to Apaf-1; on the other hand,

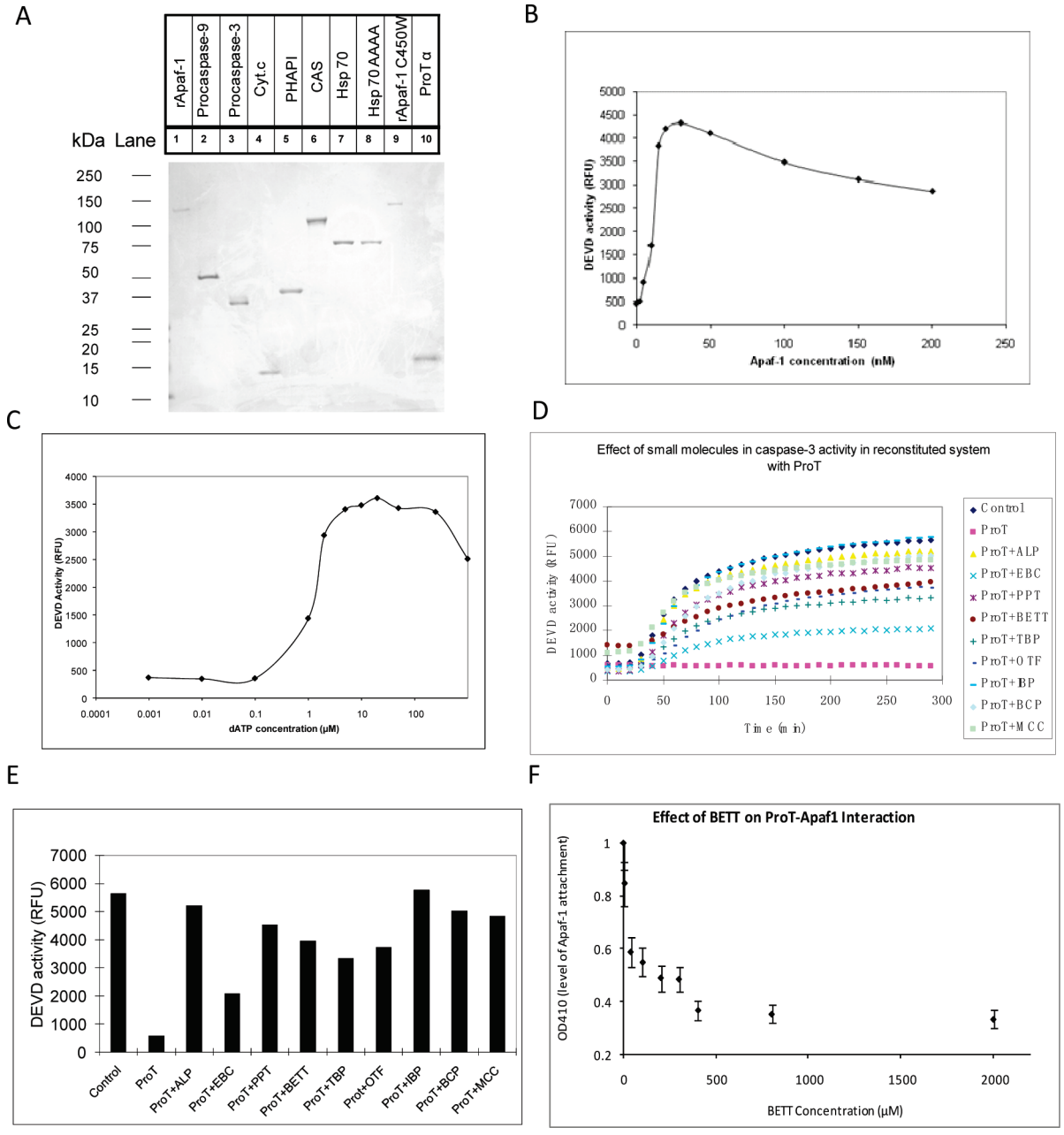


FIGURE 4: Regulatory function of small molecule BETT in a reconstituted system in vitro. Total reconstitution of the entire caspase-3 activation pathway was conducted using purified Apaf-1, procaspase-9, procaspase-3, cyt *c*, PHAPI, CAS, Hsp70, ProT, and regulatory compound BETT to mimic stress-induced 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 a recombinant system. (C) Titration of dATP for caspase-3 activity in a recombinant system. (D) Time course comparison of the stimulatory effects of small molecules in a reconstituted system. (E) Histogram comparison of the stimulatory effects of small molecules in a reconstituted system at maximum activation (290 min from Figure 3D). (F) Interaction of ProT and Apaf-1 is blocked by BETT probed by an ELISA. All the experiments were conducted at least three times with similar results. Detailed information for all the experiments is in Materials and Methods (In Vitro Fluorogenic Assay for Caspase-3 Activity).

they are small enough not to interfere with binding of ATP to Apaf-1. Therefore, those small molecules are able to release the inhibitory function of ProT to apoptosome formation. Currently, we are creating several Apaf-1 mutants to further test our hypothesis (Figure 4A). Specificity studies are being conducted using domain mutants corresponding to a 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 EC_{50} analysis for BETT and related compounds that gives a 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 EC_{50} assay. The cell culture and in vitro studies can be extended into comprehensive in vivo studies. This can be conducted 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

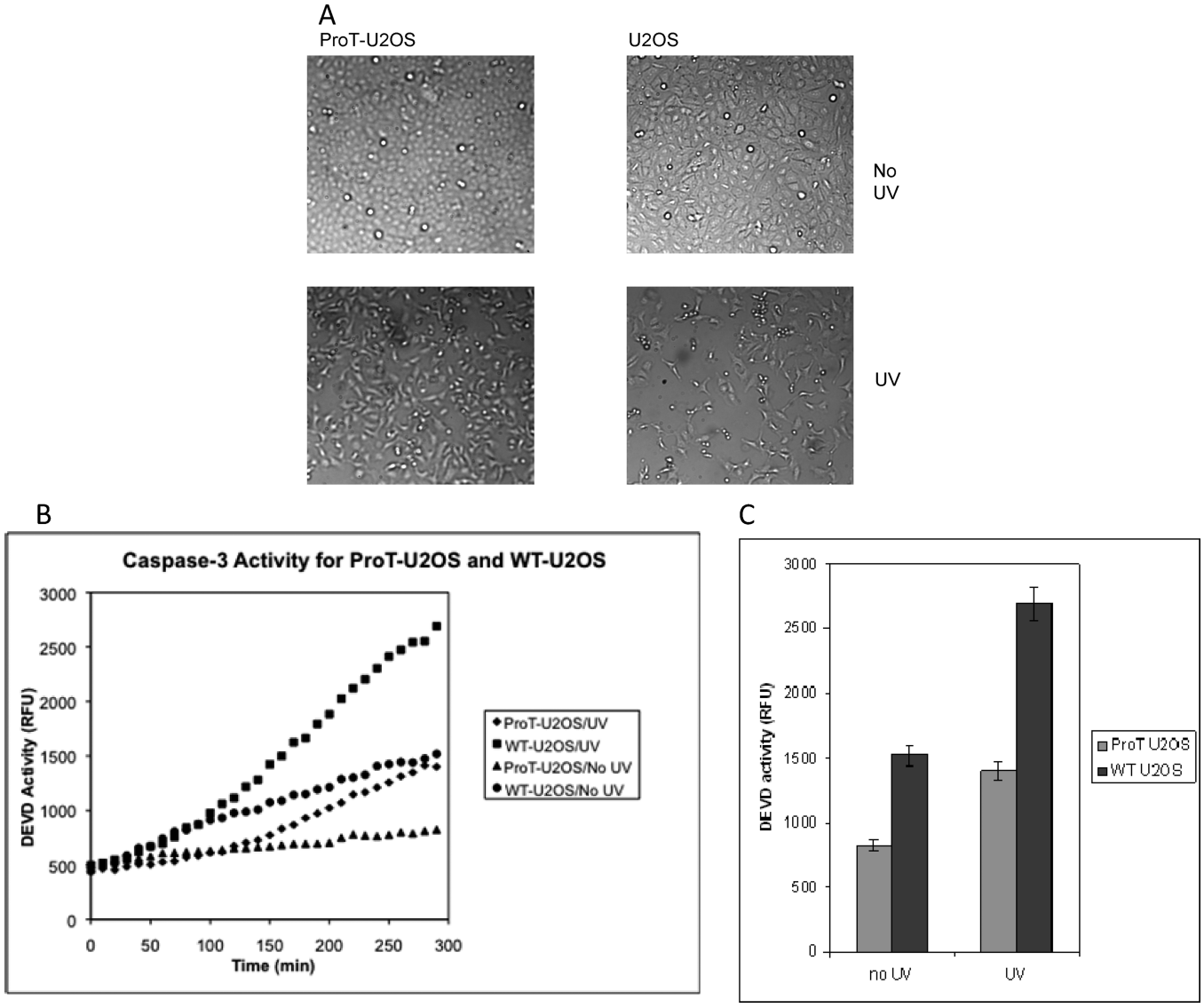


FIGURE 5: ProT-overexpressing U2OS cells exhibit resistance to an apoptotic stimulus. (A) ProT-overexpressing cells block UV-induced cell death. Micrographs of ProT-overexpressing U2OS cells and WT U2OS cells without UV treatment compared with 12 h after UV irradiation. (B) Time course comparison of ProT-overexpressing U2OS and WT U2OS cell lysates for caspase-3 activation. (C) ProT-overexpressing U2OS cells block caspase-3 activation by maximum activation comparison. All the experiments were conducted at least three times with similar results.

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 that can be excited with visible light and shown as green color under a microscope. We synthesized IAF-labeled BETT and applied it to cell media. By comparing the Hoescht-stained blue nucleus and MitoTracker Red-stained mitochondrion, we find the 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 may identify new apoptosis regulators and tumor suppressors that may uncover new drug leads for cancer treatment.

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REFERENCES

1. Thompson, C. B. (1995) Apoptosis in the Pathogenesis and Treatment of Disease. *Science 267*, 1456–1462.
2. Salvesen, G. S., and Dixit, V. M. (1997) Caspases: Intracellular signaling by proteolysis. *Cell 91*, 443–446.
3. Song, Z. W., and Steller, H. (1999) Death by design: Mechanism and control of apoptosis. *Trends Biochem. Sci. 24*, M49–M52.
4. Acehan, D., Jiang, X. J., Morgan, D. G., Heuser, J. E., Wang, X. D., and Akey, C. W. (2002) Three-dimensional structure of the apoptosome: Implications for assembly, procaspase-9 binding, and activation. *Mol. Cell 9*, 423–432.
5. Zou, H., Henzel, W. J., Liu, X. S., Lutschg, A., and Wang, X. D. (1997) Apaf-1, a human protein homologous to *C. elegans* CED-4, participates in cytochrome c-dependent activation of caspase-3. *Cell 90*, 405–413.
6. Li, P., Nijhawan, D., Budihardjo, I., Srinivasula, S. M., Ahmad, M., Alnemri, E. S., and Wang, X. D. (1997) Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. *Cell 91*, 479–489.
7. Hu, Y. M., Ding, L. Y., Spencer, D. M., and Nunez, G. (1998) WD-40 repeat region regulates Apaf-1 self-association and procaspase-9 activation. *J. Biol. Chem. 273*, 33489–33494.
8. Zou, H., Li, Y. C., Liu, H. S., and Wang, X. D. (1999) An APAF-1 center dot cytochrome c multimeric complex is a functional apoptosome that activates procaspase-9. *J. Biol. Chem. 274*, 11549–11556.

9. Goldstein, J. C., Waterhouse, N. J., Juin, P., Evan, G. I., and Green, D. R. (2000) The coordinate release of cytochrome c during apoptosis is rapid, complete and kinetically invariant. *Nat. Cell Biol. 2*, 156–162.
10. Wang, X. D. (2001) The expanding role of mitochondria in apoptosis. *Genes Dev. 15*, 2922–2933.
11. Jiang, X. J., and Wang, X. D. (2000) Cytochrome c promotes caspase-9 activation by inducing nucleotide binding to Apaf-1. *J. Biol. Chem. 275*, 31199–31203.
12. Kim, H. E., Du, F. H., Fang, M., and Wang, X. D. (2005) Formation of apoptosome is initiated by cytochrome c-induced dATP hydrolysis and subsequent nucleotide exchange on Apaf-1. *Proc. Natl. Acad. Sci. U.S.A. 102*, 17545–17550.
13. Rodriguez, J., and Lazebnik, Y. (1999) Caspase-9 and APAF-1 form an active holoenzyme. *Genes Dev. 13*, 3179–3184.
14. Thornberry, N. A., and Lazebnik, Y. (1998) Caspases: Enemies within. *Science 281*, 1312–1316.
15. Pineiro, A., Cordero, O. J., and Nogueira, M. (2000) Fifteen years of prothymosin α: Contradictory past and new horizons. *Peptides 21*, 1433–1446.
16. Gast, K., Damaschun, H., Eckert, K., Schulzeforster, K., Maurer, H. R., Mullerfrohne, M., Zirwer, D., Czarnecki, J., and Damaschun, G. (1995) Prothymosin-α: A Biologically-Active Protein with Random Coil Conformation. *Biochemistry 34*, 13211–13218.
17. Gomezmarquez, J., Segade, F., Dosil, M., Pichel, J. G., Bustelo, X. R., and Freire, M. (1989) The Expression of Prothymosin α-Gene in Lymphocytes-T and Leukemic Lymphoid-Cells Is Tied to Lymphocyte-Proliferation. *J. Biol. Chem. 264*, 8451–8454.
18. Tsitsiloni, O. E., Stiakakis, J., Koutselinis, A., Gogas, J., Markopoulos, C., Yialouris, P., Bekris, S., Panoussopoulos, D., Kiortsis, V., Voelter, W., and Haritos, A. A. (1993) Expression of α-Thymosins in Human Tissues in Normal and Abnormal Growth. *Proc. Natl. Acad. Sci. U.S.A. 90*, 9504–9507.
19. Cotter, M. A., and Robertson, E. S. (2000) Modulation of histone acetyltransferase activity through interaction of Epstein-Barr nuclear antigen 3C with prothymosin α. *Mol. Cell. Biol. 20*, 5722–5735.
20. Karetsou, Z., Kretsovali, A., Murphy, C., Tsolas, O., and Papamarcaki, T. (2002) Prothymosin α interacts with the CREB-binding protein and potentiates transcription. *EMBO Rep. 3*, 361–366.
21. Karetsou, Z., Sandaltzopoulos, R., Frangou-Lazaridis, M., Lai, C. Y., Tsolas, O., Becker, P. B., and Papamarcaki, T. (1998) Prothymosin α modulates the interaction of histone H1 with chromatin. *Nucleic Acids Res. 26*, 3111–3118.
22. Karetsou, Z., Martic, G., Tavoulari, S., Christoforidis, S., Wilm, M., Gruss, C., and Papamarcaki, T. (2004) Prothymosin α associates with the oncoprotein SET and is involved in chromatin decondensation. *FEBS Lett. 577*, 496–500.
23. Karapetian, R. N., Evstafieva, A. G., Abaeva, I. S., Chichkova, N. V., Filonov, G. S., Rubtsov, Y. P., Sukhacheva, E. A., Melnikov, S. V., Schneider, U., Wanker, E. E., and Vartapetian, A. B. (2005) Nuclear oncoprotein prothymosin α is a partner of Keap1: Implications for expression of oxidative stress-protecting genes. *Mol. Cell. Biol. 25*, 1089–1099.
24. Malicet, C., Dagorn, J. C., Neira, J. L., and Iovanna, J. L. (2006) p8 and prothymosin α: Unity is strength. *Cell Cycle 5*, 829–830.
25. Jiang, X. J., Kim, H. E., Shu, H. J., Zhao, Y. M., Zhang, H. C., Kofron, J., Donnelly, J., Burns, D., Ng, S. C., Rosenberg, S., and Wang, X. D. (2003) Distinctive roles of PHAP proteins and prothymosin-α in a death regulatory pathway. *Science 299*, 223–226.
26. Kim, H. E., Jiang, X. J., Du, F. H., and Wang, X. D. (2008) PHAPI, CAS, and Hsp70 promote apoptosome formation by preventing Apaf-1 aggregation and enhancing nucleotide exchange on Apaf-1. *Mol. Cell 30*, 239–247.
27. Yi, S. L., Boys, B. L., Brickenden, A., Konermann, L., and Choy, W. Y. (2007) Effects of zinc binding on the structure and dynamics of the intrinsically disordered protein prothymosin α: Evidence for metalation as an entropic switch. *Biochemistry 46*, 13120–13130.
28. Liu, X. S., Kim, C. N., Yang, J., Jemmerson, R., and Wang, X. D. (1996) Induction of apoptotic program in cell-free extracts: Requirement for dATP and cytochrome c. *Cell 86*, 147–157.
29. Vaesen, M., Barnikolwatanabe, S., Gotz, H., Awni, L. A., Cole, T., Zimmermann, B., Kratzin, H. D., and Hilschmann, N. (1994) Purification and Characterization of 2 Putative Hla Class-II Associated Proteins: Phapi and Phapii. *Biol. Chem. Hoppe-Seyler 375*, 113–126.
30. Chen, T. H., Brody, J. R., Romantsev, R. E., Yu, J. G., Kayler, A. E., Voneiff, E., Kuhajda, F. P., and Pasternack, G. R. (1996) Structure of pp32, an acidic nuclear protein which inhibits oncogene-induced formation of transformed foci. *Mol. Biol. Cell 7*, 2045–2056.
31. Matilla, A., Koshy, B. T., Cummings, C., Isobe, T., Orr, H. T., and Zoghbi, H. Y. (1997) The cerebellar leucine-rich acidic nuclear protein interacts with ataxin-1. *Nature 389*, 974–978.
32. Zhu, L. J., Perlaky, L., Henning, D., and Valdez, B. C. (1997) Cloning and characterization of a new silver-stainable protein SSP29, a member of the LRR family. *Bio. Mol. Biol. Int. 42*, 927–935.
33. Mencinger, M., Panagopoulos, I., Contreras, J. A., Mitelman, F., and Aman, P. (1998) Expression analysis and chromosomal mapping of a novel human gene, APRIL, encoding an acidic protein rich in leucines. *Biochim. Biophys. Acta 1395*, 176–180.
34. Brody, J. R., Kadkol, S. S., Mahmoud, M. A., Rebel, J. M. J., and Pasternack, G. R. (1999) Identification of sequences required for inhibition of oncogene-mediated transformation by pp32. *J. Biol. Chem. 274*, 20053–20055.
35. Li, M., Makkinje, A., and Damuni, Z. (1996) Molecular identification of I-1(PP2A), a novel potent heat-stable inhibitor protein of protein phosphatase 2A. *Biochemistry 35*, 6998–7002.
36. Seo, S. B., McNamara, P., Heo, S., Turner, A., Lane, W. S., and Chakravarti, D. (2001) Regulation of histone acetylation and transcription by INHAT, a human cellular complex containing the set oncoprotein. *Cell 104*, 119–130.
37. Mutai, H., Toyoshima, Y., Sun, W. Y., Hattori, N., Tanaka, S., and Shiota, K. (2000) PAL31, a novel nuclear protein, expressed in the developing brain. *Biochem. Biophys. Res. Commun. 274*, 427–433.
38. Wu, C. G., Habib, N. A., Mitry, R. R., Reitsma, P. H., vanDeventer, S. J. H., and Chamuleau, R. A. F. M. (1997) Overexpression of hepatic prothymosin α, a novel marker for human hepatocellular carcinoma. *Br. J. Cancer 76*, 1199–1204.
39. Eilers, M., Schirm, S., and Bishop, J. M. (1991) The Myc Protein Activates Transcription of the α-Prothymosin Gene. *EMBO J. 10*, 133–141.