Prion-like Nanofibrils of Small Molecules (PriSM) Selectively Inhibit Cancer Cells by Impeding Cytoskeleton Dynamics*†‡

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Emerging evidence reveals that prion-like structures play important roles to maintain the well-being of cells. Although self-assembly of small molecules also affords prion-like nanofibrils (PriSM), little is known about the functions and mechanisms of PriSM. Previous works demonstrated that PriSM formed by a dipeptide derivative selectively inhibiting the growth of glioblastoma cells over neuronal cells and effectively inhibiting xenograft tumor in animal models. Here we examine the protein targets, the internalization, and the cytotoxicity pathway of the PriSM. The results show that the PriSM selectively accumulate in cancer cells via macropinocytosis to impede the dynamics of cytoskeletal filaments via promiscuous interactions with cytoskeletal proteins, thus inducing apoptosis.

Self-propagation of certain proteins affords aggregated oligomers with β-sheet structure, known as prions (1). In mammals, about a dozen proteins form prions that cause neurodegenerative diseases when they accumulate over a critical threshold (2). Despite being neurotoxic in prion form, these prion proteins exhibit beneficial functions in their monomeric forms (e.g. PrP is neuroprotective) (3). Interestingly, recent studies also identified prions that have beneficial or even essential functions in cells (4). Such nonpathogenic prions include the cytoplasmic polyadenylation element-binding protein (CPEB) (5), the mitochondrial antiviral signaling protein (MAVS) (6), and the T-cell-restricted intracellular antigen 1 (TIA-1) (7) (2). Although the prion form and the monomeric form of TIA-1 have different functions (formation of stress granules by the prion TIA-1 and posttranscriptional regulator of gene expression by the monomeric TIA-1) (8), the only biologically active species of cytoplasmic polyadenylation element-binding protein and mitochondrial antiviral signaling protein, intriguingly, are their prion-like forms. Moreover, many non-pathogenic prions also exist in fungi (9), such as yeast (10) and Podospora anserine (11). Interestingly, protein aggregates formed by mixing with fatty acid (HAMLET) also exhibit beneficial function (12, 13). These facts strongly imply that, similar to the self-organization of proteins to generate dynamic protein assemblies (e.g. microtubules) (14), self-propagation of proteins to form prions is also an inherent property of proteins for maintaining the well-being of cells.

Like the self-propagated prions, small molecules also form prion-like nanofibrils, which grow by self-assembly and adopt the β-sheet structures (15). These prion-like nanofibrils of small molecules (termed as PriSM in this study) possess distinctive activities. One recent study found that the nanofibrils formed by low complexity sequences of RNA-binding proteins can recruit and retain mRNAs to form cell-free RNA granules (16), an action similar to that of TIA-1. Another study observed the assembly of phenylalanine in high concentration to form fibrils, which exhibit neurototoxicity similar to pathologic prions (17). Recent research on small molecule activators of a proenzyme also unintendedly discovered small molecules, which aggregate to form prion-like nanofibrils that selectively activate pro-caspase 3 to induce cell death (18). These intriguing results of prion-like nanofibrils with a primary bioactivity imply that it is possible to engineer PriSM to exhibit beneficial functions, similar to that of the non-pathologic prions.

Engineering PriSM has several distinct advantages. (i) Reversibility: Although prions self-propagate irreversibly, the formation of PriSM is reversible. Such reversibility renders the functions of PriSM to be transient, a useful feature that is essential for spatio-temporal control of the desired functions. (ii) Tunability: Proper molecular design allows the formation of the PriSM to be regul-
The mechanism of the selective cytotoxicity of PriSM of 1 toward cancer cell. PriSM of 1 enter the cell by macropinocytosis and impede the cytoskeletal proteins. The disruption of the dynamics of cytoskeletal filaments activates BAD and p53, which consequently arrest G1/G0 cell cycle, activate caspase cascade and downstream poly(ADP-ribose) polymerase, and initiate apoptosis.

Here we report the studies aimed to delineate the cellular mechanism of the selective cytotoxicity of PriSM of 1 against cancer cells (Scheme 1). Our results show that PriSM of 1 enter cell via macropinocytosis and accumulate in the cytosol. Unlike most monomeric small molecule inhibitors, PriSM of 1 promiscuously interact with proteins, especially the cytoskeletal proteins (e.g. α/β-tubulins, actins, and vimentin). PriSM of 1 significantly disrupt the formation of microtubules in both cell-free assays and cancer cells. Also, PriSM of 1, at high concentration, inhibit the assembly of actin filament in polymerization assays and disrupt the formation of intermediate filaments formed by vimentin inside cells. In contrast to the malfunction of Tau proteins, presumably triggered by amyloid Aβ and causing neuron death (22), the high level expression of Tau has a protective role against the action of the PriSM of 1. Moreover, PriSM of 1 not only decrease the proliferation of cancer cells (e.g. T98G, Capan-2, and HepG2) derived from high mortality tumors, but also selectively inhibit the growth of cancer cells (HeLa) in the presence of stromal cells (e.g. HS-5) during co-culture, which underscores the promises of the use of PriSM to target cancer cells in tumor microenvironment (23). This work illustrates the possibility of molecular engineering of other PriSM, as a new class of dynamic, supramolecular entities, for carrying out a diverse range of biological functions, including acting as a fundamentally new type of anticancer agents.

EXPERIMENTAL PROCEDURES

Materials and Methods—All cell lines (HeLa CCL2, PC-12 Adh CRTL-1721.1, HS-5 CRTL-11882, MCF7 HTB-22, Capan-2 HTB-80, MES-SA CRTL-1976, and HepG2 HB-8065) were obtained from ATCC. All antibodies were obtained from Abcam. Tubulin and actin polymerization assay, tubulins, and biotinylated tubulins were obtained from Cytoskeleton. Tubulin tracker and all culture media were from Invitrogen, LentiBrite™ RFP-vimentin lentiviral biosensor was from Millipore, PathScan apoptosis multi-target sandwich ELISA kit was from Cell Signaling Technology, and anti-Tau siRNA and Tau expression plasmid were from Santa Cruz Biotechnology. All other chemicals and reagents were obtained from Fisher Scientific. Circular dichroism was performed on a JASCO J-810 spectrometer, transmission electron microscopy was performed on a Morgagni 268 transmission electron microscope, MTT viability assay and tubulin polymerization assay were performed on a DTX 880 multimode detector, flow cytometry was performed on a FACSCalibur flow cytometer, isothermal titration calorimetry was performed on a TA Instruments low-vol- nane NANO ITC, fluorescence spectra were examined on an RF-5301PC spectrofluorophotometer, and confocal images were examined on a Leica SP2 microscope. In all experiments, PriSM of 1 refers to solution of 1 at 192 µg/ml and monomers of 1 refers to solution of 1 at 144 µg/ml or lower, unless otherwise stated.

Thioflavin T (ThT) Binding Assay—ThT stock solution was prepared on the day of the experiment by adding 5 mg of thioflavin T into 10 ml of PBS buffer (pH 7.6) and filtering through a 0.22-µm PVDF syringe filter. After diluting 20 µl of the stock solution into a total volume of 1.5 ml of PBS buffer, the fluorescence of the solution (λex = 440 nm) was measured as the background. For as-prepared 1, freshly prepared stock solution of 1 (1.44 mg in 300 µl in double-distilled water; dissolved by the addition of 3 µl of 1 N NaOH and sonication) was immediately diluted into PBS buffer and mixed by pipetting. 20 µl of ThT stock solution was then added to make a final volume of 1.5 ml. For filtered 1, prior to the addition of ThT stock solution, the solutions of 1 were filtered through a 0.22-µm nylon filter, and then 1.48 ml of the filtered solution was taken out to mix with 20 µl of the ThT stock solution. For gel-diluted 1, instead of using the freshly prepared stock solution of 1, a hydrogel of 1 (5 mg/ml; PBS (pH 7.4), prepared 4 h before the experiment) was used. A spectrofluorophotometer was used to measure the fluorescence intensity of the solutions (λex = 440 nm), and the
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peak value of the emission at ~482 nm was recorded. Data represent the mean ± S.D. of three independent experiments.

Congo Red Staining—Cells in exponential growth phase were seeded in a glass-bottomed culture chamber at 1 × 10^6 cells/well. The cells were allowed to adhere for 24 h at 37 °C, 5% CO₂. The culture medium was removed, and new culture medium containing 1 at 192 μg/ml was added. After 24 h of incubation, cells were washed with PBS buffer three times and stained with 0.1 mg/ml Congo red in PBS buffer for 30 min at 37 °C in dark. The cells were rinsed three times in PBS and then kept in the PBS for imaging.

Hydrogel-based Protein Pulldown Assay—4 × 10^7 of HeLa cells were scrapped from Petri dish and wash with PBS buffer for three times and then centrifuged at 300 × g for 5 min. The collected cell pellet was mixed with phosphate buffer (100 mM) and then snap-frozen and thawed for three cycles to lyse the cells. The cell lysate was centrifuged at 12,000 × g for 20 min at 4 °C to remove the whole cells, nuclei, and large mitochondria. 50 μl of the supernatant lysate was gently mixed with 30 μl of hydrogel of 1 (4.8 mg/ml in PBS buffer, pH 7.6) on rotator at room temperature for 30 min. The hydrogel was collected by centrifugation (12,000 × g, 5 min, 4 °C), and the supernatant was collected and placed on ice before analysis. The hydrogel was washed three times by gently mixing with 50 μl of the washing buffer (50 mM phosphate buffer, pH 7.6, supplemented with 150 mM NaCl) on rotator at room temperature for 10 min followed by separation on a centrifuge (12,000 × g, 5 min, 4 °C). Supernatants were collected and placed on ice before analysis. Finally, the remaining hydrogel was dissolved using 1:1 washing buffer and 5× Laemmli buffer. All other samples were mixed with 5× Laemmli buffer (final concentration 2×) before SDS-PAGE.

Tubulin Polymerization Assay—1.53 mg of 1 was dissolved in 197 μl of water via sonication and the addition of 3 μl of 1 N NaOH. The resulting solution (7.65 mg/ml) was immediately diluted in the general tubulin buffer (80 mM PIPES, 2 mM MgCl₂, 0.5 mM EGTA; pH 6.9) to form a series of stock solutions at 2.4, 1.92, 1.44, and 0.96 mg/ml 1. Mixing 40 μl of the stock solutions or just the general tubulin buffer with 280 μl of cold TP buffer (750 μl of general tubulin buffer; 250 μl of glycercol buffer (80 mM PIPES, 2 mM MgCl₂, 0.5 mM EGTA, 60% v/v glycerol; pH 6.9); 10 μl of 100 mM GTP) created the polymerization cocktails. 100 μl of the as-prepared reaction mixture was added to half-area of a 96-well plate. The plate was warmed to 37 °C for 15 min. Tubulin solution (10 mg/ml in general tubulin buffer supplemented with 1 mM GTP) was quickly thawed and then placed on ice. 37 μl of the tubulin solution was quickly added to each well and mixed well. Aς360 nm of the plate was then measured for 60 min at 37 °C. For the filtered experiment, the reaction mixture containing 1 was gently passed through a 0.22-μm nylon filter, and then 100 μl of the filtered reaction mixture was mixed with 37 μl of the tubulin solution. Data represent the mean ± S.D. of three independent experiments.

Congo Red Staining—Cells in exponential growth phase were seeded in a glass-bottomed culture chamber at 1 × 10⁶ cells/ml. The cells were allowed to adhere for 24 h at 37 °C, 5% CO₂. The culture medium was removed, and new culture medium containing 1 at 0 or 192 μg/ml was added. After 24 h of incubation, cells were washed with PBS buffer three times and stained with Tubulin Tracker™ Green at 100 nM and DAPI 300 nM in PBS buffer for 30 min at 37 °C in dark. The cells were rinsed three times in PBS and then kept in the PBS buffer for imaging.

In Vivo Tubulin/Microtubule Assay—Cells in exponential growth phase were seeded in a 6-well plate at 4 × 10⁶ cells/well. Four wells were labeled as the test well, the control well, the positive control well, and the negative control well, respectively. The cells were allowed to adhere for 24 h at 37 °C, 5% CO₂. The culture medium was removed. 2 ml of new culture medium containing 1 at 192 μg/ml was added to the test well, and just the culture medium was added to the other three wells. After 24 h of incubation, cells in each well were collected and lysed in 200 μl of prewarmed lysis and microtubule stabilization buffer from cytoskeleton (100 mM PIPES; 5 mM MgCl₂; 1 mM EGTA; 30% v/v glycerol; 0.1% Nonidet P40; 0.1% Triton X-100; 0.1% Tween 20; 0.1% β-mercaptoethanol; 0.001% Antifoam; 1 mM ATP; 0.1 mM GTP; 1% protease inhibitor mixture, pH 6.9). 2 μl of taxol at 2 mM was added into the lysates prepared from the positive control well, and 2 μl of CaCl₂ at 200 mM was added into the lysates prepared from the negative control well. 3 μl of the lysates from each well was taken out for quantification of total protein concentration. The remaining lysates were centrifuged at 100,000 × g for 30 min at 37 °C. The supernatants, containing free tubulins, were quickly removed. The pellets, containing microtubules, were lysed again by incubation on ice for 15 min with 200 μl of ice-cold water supplemented with CaCl₂ at 2 mM. Based on the total protein concentration of each lysate, the volume required to get 10 μg of total protein was calculated. The same volume of the supernatant and the pellet samples produced from each lysate were taken for SDS-PAGE and Western blot analysis.

Actin Polymerization Assay—I was dissolved in water via sonication to get 9.6 mg/ml stock. The stock solution was immediately diluted by the general actin buffer (5 mM Tris-HCl, 0.2 mM CaCl₂, 0.2 mM ATP; pH 8.0) to form a series of 14.2× solutions of 1. 200 μl of ice-cold pyrene-actin monomer (20 mg/ml) was added to each well. Then, 20 μl of just the general actin buffer or the 14.2× 1 solutions was added. Immediately, 20 μl of actin polymerization buffer (0.5 mM KCl, 20 mM MaCl₂, 50 mM guanidine carbonate, 10 mM ATP, 100 mM Tris-HCl, pH 7.5) was added to each well. The plate was then monitored for 70 min at 25 °C. For the filtered experiment, the 14.2× solutions of 1 were gently passed through a 0.22-μm nylon filter before adding to the well. For the aging experiment, the 9.6 mg/ml solutions of 1 were formed 1 day prior to experiment. Data represent the mean ± S.D. of three independent experiments.

Actin Staining—Cells in exponential growth phase were seeded in glass-bottomed culture chambers at 1 × 10⁶ cells/ml. The cells were allowed to attach for 24 h at 37 °C, 5% CO₂. The culture medium was removed, and new culture medium containing 1 at 0 or 192 μg/ml was added. After 24 h of incubation, cells were washed with PBS buffer three times and fixed by 3% formaldehyde in PBS for 10 min. After removal of solution and washing the cells with PBS two times, the cells were incubated with 0.1% Triton X-100 for 10 min. After removal of solution and washing the cells with PBS two times, the cells were incubated with Alexa Flou® 647 phalloidin at 0.033 μM and
Vimentin-RFP Transfection—Cells in exponential growth phase were seeded in glass-bottomed culture chambers at $1 \times 10^4$ cells/ml. The cells were allowed to adhere for 24 h at 37 °C, 5% CO$_2$. After removing the culture medium, 1 ml of new culture medium containing LentiBrite™ RFP-vimentin lentiviral biosensor at 20 multiplicity of infection (20 lentiviral particles per cell) was added in each dish and incubated at 37 °C, 5% CO$_2$ for 24 h. Fresh medium was added to replace the lentivirus-containing medium, and the cells were incubated for another 48 h. After incubation, new culture medium containing I at 0 or 240 μg/ml (gel-diluted solution) was added, and the cells were incubated for 24 h. At the end of incubation, the cells were washed with PBS buffer three times and stained by Hoechst at 1 μM in PBS buffer for 30 min at 37 °C in dark. The cells were rinsed three times in PBS and then kept in PBS buffer for imaging.

Tau Silencing and Tau Expression—Both Tau silencing and Tau expression were achieved using TurboFectin™ 8.0. Cells in exponential growth phase were seeded in glass-bottomed culture chambers at $1 \times 10^4$ cells/ml. The cells were allowed to adhere for 24 h at 37 °C, 5% CO$_2$. For complex formation, 4 μl of TurboFectin 8.0 was first mixed with 100 μl of serum-free medium in a sterile tube and incubated at room temperature for 5 min. Then, 2 μg of Tau plasmid or Tau siRNA was mixed with the solution and incubated at room temperature for 20 min. After removing the culture medium and adding 1 ml of fresh medium into each dish, the complex solution was added into the medium drop by drop. Afterward the dish was gently rocked to mix the complex, and the cells were incubated at 37 °C, 5% CO$_2$ for 48 h before experiment.

Co-administration with Taxol or Nocodazole—HeLa cells in exponential growth phase were seeded in a 96-well plate at a concentration of 50,000 cells/well. The cells were allowed to attach to the wells for 24 h at 37 °C, 5% CO$_2$. For co-administration with taxol, the cells were treated with 50 nM taxol with monomeric or PriSM of I for 24 h. For co-administration with nocodazole, the cells were first treated with 20 μM of nocodazole for 1 h, and then the media were replaced with medium containing PriSM of I. The nocodazole-treated cells were only treated with 20 μM of nocodazole for 1 h, and then the media were replaced with fresh medium. Viability of the cells was measured after another 23 h of incubation.

RESULTS

PriSM of I and Their Selective Cytotoxicity on Cancer Cells—We have found that a small dipeptidic molecule (I) self-assembles, via multiple intermolecular hydrogen bonds and aromatic-aromatic interactions, to form cross-β like structures at concentration ≥192 μg/ml (Fig. 1) (15, 20). We also found that I (at 192 μg/ml and above) effectively inhibits the growth of glioblastoma cells, with the cell death window between 38 and 44 h of incubation (20). To correlate the cytotoxicity to the formation of PriSM of I, we stained the solutions of as-prepared I at various concentrations with ThT (24). As shown Fig. 2a, the emission of ThT starts to increase in a near linear manner with the concentrations of I at and above 163 μg/ml, indicating that the critical concentration for forming PriSM of I is at about 163 μg/ml.

The addition of I above the critical concentration significantly decreases the viability of HeLa cells (Fig. 2a), but addition of I below the critical concentration is innocuous to the cells. The cytotoxicity of I toward these cancer cells significantly deviates from the sigmoidal dose-response law (25) and coincides with the formation of PriSM of I. Considering that PriSM of I and monomeric I co-exist in the solutions at concentrations above the critical aggregation concentration of I (163 μg/ml), we partially removed PriSM of I from the solutions by filtering the solutions through a 0.22-μm nylon filter ($C_{filter}$) (Table 1), as the average length of PriSM of I is about 0.2 ± 0.09 μm with I at 192 μg/ml (measured from TEM images; data not shown). The filtration largely abrogates both the fluorescence of ThT induced by I and the cytotoxicity of I on HeLa cells at the concentrations of I at 192 and 240 μg/ml (Fig. 2b). Moreover, Congo red staining of the HeLa cells incubated with I at 192 μg/ml for 24 h shows the fluorescence of Congo red in the cytosol (Fig. 2c), indicating that PriSM of I exist inside cells. These results confirm that (i) PriSM of I are cytotoxic; and (ii) monomers of I are innocuous.

We have assessed the inhibitory effect of PriSM of I on xenograft HeLa tumor on nude mice and found that peritumoral injection of PriSM of I effectively inhibits the tumor progression without eliciting noticeable inflammation on the animals (21). To further evaluate the selectivity of the cytotoxicity of PriSM of I, we monitored the cytotoxicity of PriSM of I and monomers of I on several types of cancer cells (i.e. MES-SA, Capan-2, MCF-7, HepG2) of different tissue origins and on a stromal cell (i.e. HS-5). As shown in Fig. 2d, although monomers of I (I at 144 μg/ml) are innocuous to all cells tested,
PriSM of 1 effectively inhibit the growth of cancer cells at 48 h of incubation, but exhibit little toxicity to HS-5 cells up to 72 h of incubation. This result agrees with the previous observation that PriSM of 1 show little toxicity to a neuronal cell PC12 even for 7 days of treatment (20).

To further confirm the selective toxicity of PriSM of 1 toward cancer cells, we incubated HS-5- and GFP-expressing HeLa cells (HeLa-GFP), co-cultured at a 1:1 ratio, with or without PriSM of 1 for 60 h. As shown in Fig. 2e, most of the treated cells emit no noticeable green fluorescence, indicating that the living cells are mostly HS-5 cells. On the contrary, about two-thirds of the cells in the untreated co-culture give green fluorescence at the end of incubation, agreeing with the fact that HeLa cells proliferate faster than HS-5 cells (Fig. 2f). These results confirm that PriSM of 1 selectively inhibit cancer cells in the co-culture that mimics the tumor microenvironment (23, 26).

PriSM of 1 (1 at 192 μg/ml) effectively inhibit the growth of the four types of cancer cells at 48 h of incubation, but exhibit little toxicity to HS-5 cells up to 72 h of incubation. This result agrees with the previous observation that PriSM of 1 show little toxicity to a neuronal cell PC12 even for 7 days of treatment (20). To further confirm the selective toxicity of PriSM of 1 toward cancer cells, we incubated HS-5- and GFP-expressing HeLa cells (HeLa-GFP), co-cultured at a 1:1 ratio, with or without PriSM of 1 for 60 h. As shown in Fig. 2e, most of the treated cells emit no noticeable green fluorescence, indicating that the living cells are mostly HS-5 cells. On the contrary, about two-thirds of the cells in the untreated co-culture give green fluorescence at the end of incubation, agreeing with the fact that HeLa cells proliferate faster than HS-5 cells (Fig. 2f). These results confirm that PriSM of 1 selectively inhibit cancer cells in the co-culture that mimics the tumor microenvironment (23, 26).

PriSM of 1 Induces Apoptosis—Apoptosis/necrosis assay (Fig. 3a) shows that FITC-annexin V stains the membrane of some of the HeLa cells treated by PriSM of 1 for 24 h, but propidium iodide hardly stains any of the cells, suggesting that those cells are in early apoptosis (27). Then, we monitored the concentration change of several key signaling molecules of the survival and apoptosis pathways in the HeLa cells incubated by PriSM of 1 using PathScan apoptosis multi-target sandwich ELISA (28) over time. As shown in Fig. 3b, the concentration of Bad increases after 6 h of incubation, and the amount remains unchanged afterward. Later, the amount of tumor suppressor p53 starts to increase at 12 h, peaks at 24 h, and decreases at 36 h of incubation. The increase of p53 is commonly observed in cells with microtubule dysfunction (29). Finally, the amounts of active poly(ADP-ribose) polymerase and active caspase 3 start to increase at 24 h and continue increasing to 36 h of incubation. The change of the amounts of these key signal molecules over time suggests that PriSM of 1 initiate the activation of Bad and p53, which later activate the caspase cascade and downstream poly(ADP-ribose) polymerase to eventually induce apoptosis in HeLa cells. We also find that HeLa cells treated by PriSM of 1 have a lower number of cells entering mitosis (G2/M phase: 8.96%) compared with untreated cells (G2/M phase: 22.9%) (Fig. 3c). This result agrees with the observation that accumulation of p53 induced by dysfunction of microtubules (see below) leads to the arrest of cell cycle at G0/G1 phase (31, 32).

Endocytosis of PriSM of 1—By measuring the intracellular concentrations of 1 in the lysate of the HeLa cells incubated
with PriSM of 1 and monomeric 1 for 24 h at 37 or 4 °C, we found that the concentrations of 1 inside the cells increase with the increase of temperature for both the case of PriSM of 1 and the case of monomeric 1 (Fig. 3d). Thus, we used inhibitors of different endocytotic processes to determine the pathways of the cell entry of PriSM of 1 and monomeric 1: filipin III for caveolae-mediated endocytosis (33); chlorpromazine for clathrin-mediated endocytosis (34); and ethylisopropylamiloride for macropinocytosis (35). To reduce the cytotoxicity of the inhibitors, the time of co-incubation of the inhibitors with PriSM of 1 and monomeric 1 was shortened to 2 h. As shown in Fig. 3e, although the addition of filipin III or chlorpromazine has little effect on the intracellular amount of 1, the presence of ethylisopropylamiloride effectively decreases the intracellular amount of 1, suggesting that both PriSM of 1 and monomers of 1 enter the cells via macropinocytosis. Lysotracker and Congo red co-staining of HeLa cells treated by PriSM of 1 for 24 h reveals that the cell barely shows co-localized fluorescence (Fig. 3f), suggesting that PriSM of 1 can escape from lysosome to accumulate in cytosol.

PriSM of 1 Promiscuously Interact with Cellular Proteins—

We utilized the previously established molecular hydrogel protein pulldown assay (36), tailored for evaluating the interactions between PriSM and proteins, to separate the protein targets of PriSM of 1 from cytosolic extract of HeLa cells. Silver stain shows three bands between 40 and 70 kDa (Fig. 4a) (36). Mass protein spectrometry reveals the proteins in the bands (supplemental Table S1), mainly including 16 cytoskeleton-related proteins, 16 RNA-associated proteins, 14 metabolic enzymes, 7 DNA-associated proteins, 5 proteases, and 5 chaperones. The functions and the structures of these proteins have no universal similarity, suggesting that PriSM of 1 promiscuously interact with these proteins in multiple pathways. Among these proteins, the building blocks of cytoskeletal filaments (tubulins, vimentin, and actins) all appear with relatively high protein coverage (Table 2) in the result of MS profiling. Western blot confirms the cytoskeletal proteins in the pulldown (Fig. 4b, lane B). Specifically, α-tubulin exists in a high amount in lane B and is absent from all three wash-off lanes, suggesting strong interaction between PriSM of 1 and tubulins. Unlike tubulins, β-actin and vimentin appear in the first two wash-off lanes and lane B, indicating weaker interactions between PriSM of 1 and the two proteins. Moreover, several of the metabolic enzymes and chaperones associated with tubulins and actins are also present, such as T-complex protein 1 subunit β and enolase 1, which further supports the interaction of PriSM of 1 with the cytoskeleton proteins. GAPDH is absent in lane B, confirming that the washing steps effectively remove the nonspecific or weak binding proteins from PriSM of 1.

PriSM of 1 Impede Dynamics of Cytoskeletal Proteins—
The well established assays for evaluating the functions of cytoskeletal filaments allow us to examine the interactions between PriSM of 1 and the cytoskeletal proteins as a demonstration of the effect of PriSM of 1 on cellular proteins. We first used in vitro tubulin polymerization assays to measure the influence of PriSM of 1 to the polymerization of tubulins to form microtubule. As shown in Fig. 4c, in the presence of PriSM of 1, the polymerization curves deviate from the control curve, with significantly lower polymerization rate and final amount of microtubule. However, in the presence of monomers of 1, the polymerization curves appear essentially the same as those of control (i.e. reaction mixture without 1). Partial removal of the PriSM of 1 from the reaction mixture via filtration (prior to the addition of tubulins) results in a similar polymerization curve to that of control. Negatively
stained TEM images of the PriSM of 1 containing reaction mixture at the end of the polymerization reveal both long and short microtubules, with the microtubules in contact with and being impeded by PriSM of 1 (Fig. 4e). The control, on the other hand, exclusively displays long microtubules (Fig. 4d).

TABLE 2
List of PriSM-interacting proteins identified from protein mass spectrometry

| Protein                  | Gene symbol               | No. of total peptides | Protein coverage (%) |
|-------------------------|---------------------------|-----------------------|----------------------|
| β-Tubulin 2C            | TUBB2C_IPH00007752.1      | 68                    | 54.2                 |
| α-Tubulin 4A            | TUBA4A_IPH00007750.1      | 62                    | 56.9                 |
| T-complex protein 1 subunit β' | CCT2_IPH00297779.7       | 50                    | 63.4                 |
| β-Actin                 | ACTB_IPH00021439.1        | 44                    | 36.5                 |
| Vimentin                | VIM_IPH00418471.6         | 37                    | 51.1                 |
| Protein disulfide isomerase family A, member 3 | PDIA3_IPH00025252.1 | 35                    | 47.5                 |
| Enolase 1, (α)"        | ENO1_IPH00465248.5        | 35                    | 50.7                 |
| FK506-binding protein 4 | FKBP4_IPH000219005.3      | 34                    | 50.2                 |
| β-Tubulin 1             | TUBB_IPH001011654.2       | 31                    | 23.2                 |
| Glucose-6-phosphate dehydrogenase | G6PD_IPH000216008.4  | 31                    | 45.8                 |
| Eukaryotic translation elongation factor 1, α 2 | EEF1A2_IPH00014424.1 | 29                    | 30.2                 |

" Proteins that associate with tubulin or actin.
We stained HeLa cells by a taxol-based fluorescent tracker (37) to visualize the distribution of microtubules in cells. Although untreated HeLa cells display microtubules as long, smooth filaments that stretch through the cell body (Fig. 4f), the HeLa cells treated with PriSM of 1 display dots of fluorescence (clusters of short microtubules), along with scattered short microtubule filaments (Fig. 4g). In vivo tubulin/microtubule assays (38) reveal that cells incubated with PriSM of 1 exhibit a similar ratio of free tubulins to microtubules with that of the untreated cells (Fig. 4h), further suggesting that the dots of fluorescence are from clusters of short microtubules.

We then examined the effect of PriSM of 1 on the formation of microfilament of actins. Alexa Fluor-conjugated phalloidin staining of F-actin (39) reveals that HeLa cells treated with PriSM of 1 contain F-actins as shorter filaments and dots (Fig. 5e and f).
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4j). Such short filament and dot morphologies are significantly different from the normal F-actins, which have long filaments that extend throughout the cytoplasm to form networks (Fig. 4i). We also examined the formation of intermediate filaments by vimentin in the HeLa cells transfected with vimentin-RFP (40). HeLa cells treated with a high amount of PriSM of 1 exhibit less vimentin in fibrillar structures than untreated cells (Fig. 4, k and l), suggesting that PriSM of 1 are able to inhibit the formation of intermediate filaments in cells.

Antagonistic Effect of Tau to PriSM of 1—We transfected PC12 cells with Tau siRNA to silence Tau protein (41) (Fig. 5a). Tau-silenced PC12 cells have evidently decreased viability upon the treatment of PriSM of 1 when compared with control PC12 cells that remain viable (Fig. 5b). The addition of PriSM of 1 also induces clustering of microtubules in the Tau-silenced PC12 cells (Fig. 5, c and d). On the other hand, after being transfected with Tau cDNA clone (Fig. 5e), theTau-overexpressing HeLa cells, being incubated with PriSM of 1, exhibit noticeably higher viabilities than the control HeLa cells (Fig. 5f). Upon the treatment with PriSM of 1 for 24 h, the Tau-overexpressing HeLa cells show fewer clusters of short microtubules and still exhibit some long microtubules (Fig. 5, g and h). As the function of Tau is to stabilize microtubules and to promote the growth of microtubules (42), the antagonistic effect observed in these results not only implies that the distinct mechanism of PriSM of 1 can impede microtubule growth, but also suggests that administration of PriSM of 1 will induce little side effect on neuronal cells.

Conclusions—The selective inhibition of cancer cells by PriSM of 1 shown in this study is a primary outcome synthesized from several independent mechanisms. (i) PriSM of 1, like taxol and other anticancer cytoskeletal drugs, prevent mitosis and inhibit cell proliferation. (ii) PriSM of 1 enter cells via macropinocytosis. The elevated macropinocytosis of cancer cells (43), as a part of the Warburg effect, enables selective accumulation of PriSM of 1 in the cancer cells, which differs from PriSM of 1 with the current anticancer cytoskeletal drugs. Because the PriSM of 1 only form above the critical concentration, stromal cells and neuronal cells, having low uptake of 1, hardly build up PriSM of 1 inside cells, thus remaining viable. (iii) Also, although not explored in this study, PriSM of 1 promiscuously interact with proteins/enzymes (e.g. protein disulfide isomerase and glucose-6-phosphate dehydrogenase) in metabolic pathways. Such interactions may synergistically induce the apoptosis of cancer cells, including drug-resistant cancer cells (e.g. T98G). Besides the above mechanisms, the peptidic nature of 1 not only allows the degradation of 1 by aminopeptidase (21) to prohibit long term accumulation and chronic toxicity of 1, but also offers fast and low cost production of 1. These insights, together with the observation that PriSM of 1 have synergic effect with the cytoskeletal drugs taxol and nocodazole (Fig. 6), provide evidence for the promise of PriSM as a new paradigm of anticancer agents. Moreover, the diversity of small molecular nanofibrils (15, 19, 44) offers abundant opportunity for generating other PriSM as functional entities.

PriSM of 1 shares many properties with prions, such as possessing β-sheet-like assembly; environment dependent self-as-
The selective cytotoxicity of PriSM of 1 to cancer cells provocatively implies that cytotoxic prions might contribute to the inverse comorbidity between cancers and neurodegenerative diseases, an intriguing observation discovered from epidemiological and clinical studies (50). Although they mostly exist in and affect the central nervous system, pathogenic prions can also exist in all tissues, including blood (51). It is reasonable to speculate that the pathogenic prions inhibit the proliferation of cancer cells, as is true of the PriSM of 1, to result in the observed lower incidence rate of cancers in patients with neurodegenerative diseases. Such an assumption, however, still lacks an explanation for the lowered incident rate of neurodegenerative disease in cancer patients, pointing to a new mechanism, which is not defined at the genetic level, for understanding the inverse comorbidity between cancers and neurodegenerative diseases.

Acknowledgments—We thank Prof. G. A. Petsko and Prof. Nikolaius Grigorieff for helpful discussions and the EM facilities of Brandeis University for TEM.

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