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A Simplified Thermal Proteome Profiling Approach to Screen Protein Targets of a Ligand

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Thermal proteome profiling is a powerful energetic-based chemical proteomics method to reveal the ligand-protein interaction. However, the costly multiplexed isotopic labeling reagent, mainly Multiplexed isobaric tandem mass tag (TMT), and the long mass spectrometric time limits the wide application of this method. Here a simple and cost-effective strategy by using dimethyl labeling technique instead of TMT labeling is reported to quantify proteins and by using the peptides derived from the same protein to determine significantly changed proteins in one LC-MS run. This method is validated by identifying the known targets of methotrexate and geldanamycin. In addition, several potential off-targets involved in detoxification of reactive oxygen species pathway are also discovered for geldanamycin. This method is further applied to map the interactome of adenosine triphosphate (ATP) in the 293T cell lysate by using ATP analogue, adenylyl imidodiphosphate (AMP-PNP), as the ligand. As a result, a total of 123 AMP-PNP-sensitive proteins are found, of which 59 proteins are stabilized by AMP-PNP. Approximately 53% and 20% of these stabilized candidate protein targets are known as ATP and RNA binding proteins. Overall, above results demonstrated that this approach could be a valuable platform for the unbiased target proteins identification with reduced reagent cost and mass spectrometric time.

Introduction

Characterization of interaction between drugs and protein targets is critical for the development of new drugs and understanding the mode of action of drugs.[1,2] Energetics-based chemical proteomics methods such as cellular thermal shift assay (CETSA),[3,4] thermal proteome profiling (TPP),[5–7] the stability of proteins from rates of oxidation (SPROX),[8,9] drug affinity responsive target stability assay[10–12] and limited proteolysis (LiP)[13–15] provide important platforms for the discovery of post-translational of modification functional protein and novel interactions between drugs and protein targets.[16,17] The advantage of this type of method is that the ligand does not require modification or derivatization. This feature is significantly different with other affinity purification based chemical proteomics methods.[18]

CETSA and TPP are more attractive for the screening of the protein targets binding to a ligand. These two methods are both based on the principle of ligand-induced thermal stabilization. However, the limitation of CETSA is that only particular proteins with available antibodies can be validated as targets by immunoblotting readout.[3] TPP is developed by coupling CETSA with multiplexed quantitative mass spectrometry, which allows for the high-throughput monitoring of cellular protein engagement by small molecules or drugs in the proteome scale.[19,20] In this approach, the supernatants with and without ligand treatment from 10 temperatures were labeled with Multiplexed isobaric tandem mass tag (TMT) reagents and analyzed by liquid chromatography/tandem mass spectrometric (LC-MS/MS) to yield the melting curves and determine the thermal shifts. Because of the high complexity of the sample, fractionation before LC-MS/MS analysis is required. And to reduce the false-positive target identifications, two biological replicates are typically performed. Therefore, this strategy will consume a lot of mass spectrometric time. Furthermore, the multiplexed labelling reagent, TMT, is also very expensive, which limits the wide application of this method.

In this study we aimed to develop a cost-effective, fast, and simplified thermal proteome profiling (STPP) approach for the initial screening of proteins bound to a ligand. The isotopical dimethyl labeling strategy has the advances of rapid labeling, accurate quantification, and cost-effective.[21,22] In this study, it was used to quantify the thermal shift induced by the binding of a

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ligand for each temperature to reduce cost. Unlike in the conventional TPP where 10 temperatures covering the whole melting curving are analyzed, only 2–3 temperatures with significant precipitation were analyzed in STPP approach. To further reduce cost and time, only 1D LC-MS/MS was applied to analyze the labeled sample for each temperature. The significantly stabilized proteins were determined from single LC-MS/MS run by the multiple peptide measurements corresponding to the same proteins. This approach can not only identify the ligand binding protein targets, but also can effectively reduce reagent cost and mass spectrometric time, which is suitable for the initial screening of the protein targets of ligands.

1. Results

1.1. STPP Approach for Proteome-Wide Protein Target Identification

The workflow for this simplified TPP (STPP) approach is outlined in **Figure 1**. The cell lysate was exposed to a ligand or vehicle, and then was heated for 3 min to a temperature to induce thermal denaturation and precipitation. The soluble proteins in the supernatant were separated from precipitated proteins through centrifugation. Equal volume of supernatants in the ligand and vehicle groups at the selected temperature were subjected to filter-aided sample preparation (FASP), protein digestion, and stable isotopic dimethyl labeling in parallel. The differentially labeled peptides in the ligand and vehicle groups at the same temperature were combined as a single sample for quantitative proteomics analysis with 1D LC-MS/MS run. The above procedure is quite similar to the conventional TPP where costing 10-plex neutron-encoded isobaric mass tagging reagents (TMT10) are used to label the supernatants from 10 temperatures, which allows the generation of melting curves to determine the thermal shifts. However, the cost-effective dimethyl labeling reagent has maximum of three channels. In this study, we use two channels to label a pair of supernatants for the samples treated with and without ligand at one elevated temperature. To find the significantly changed proteins in thermal stability experiment between the two supernatants, at least three LC-MS/MS measurements must be performed, which consuming a lot of mass spectrometric time. Taking advantage that protein quantification is achieved by quantification of multiple peptides in shotgun proteomics, we determine the *p*-value for each protein based on the multiple measurements for multiple peptides. This allows determine *p*-values at protein level for a single LC-MS/MS run. In this study, the proteins with p-value < 0.05 and abundance fold change > 1.74 or < -1.74 were considered as the direct or indirect targets of a ligand. As different proteins may have different melting temperatures, above procedure could be applied to different temperatures to determine the corresponding proteins with significant thermal shifts. And these potential candidates were combined together as the final list.

1.2. Validation of the Approach with Model Drugs

First, we used the well-characterized model drug, methotrexate (MTX), to validate the feasibility of our approach. This model drug was chosen because the pronounced thermal stability shift was observed for the binding of MTX with its known protein target dihydrofolate reductase (DHFR) in the energetics-based approaches.[4,20] After the aliquots of 293T cell lysate treated with either MTX or dimethyl sulfoxide (DMSO) were heated to a range of temperatures, the supernatants were separated from the precipitates through centrifugation. One part of the supernatant was analyzed by western blotting by using DHFR antibody, and the remaining part was reserved for quantitative proteomics analysis. According to western blotting (**Figure 2**A), the known target protein DHFR was resistant to precipitate with the presence of MTX, whereas the amount of DHFR without MTX decreased rapidly in the supernatant with the increasing of temperature. Next, 53 and 56 °C samples with obvious thermal shift were selected for quantitative proteomics analysis. Each sample at different temperaments was analyzed by two LC-MS/MS runs to verify the reliability of our approach. After database searching, only proteins with at least three quantitative peptides were kept to facilitate the determination of *p*-value for each protein, about 2000 proteins were quantified with *p*-values in each LC-MS/MS run (Table S1, Supporting Information). As shown in the volcano figures, the known target DHFR protein was identified as the top hit in both replicate runs at 53 °C (replicate 1: fold change = 2.31, *p*-value = 0.0026; replicate 2: fold change = 2.29, *p*-value = 0.0338) (Figure 2B and Table S1, Supporting Information). At 56 °C, DHFR and m7GpppX diphosphatase (DCPS) were found to be the significant hits (Figure 2C). DCPS could be an off-target of MTX, which is not reported by previous datasets and requires further verification. Above results indicated that the protein target DHFR of MTX could be reproducibly identified by STPP approach.

This STPP approach is then applied to screen the protein targets of geldanamycin which was well known to interact with adenosine triphosphate (ATP) domain of Heat Shock Protein 90 (HSP90) proteins.[23] In this experiment, PBS with 1% EDTA-free cocktail and 0.2% NP-40 was used to lysis 293T cells and

Significance Statement

A simplified thermal proteome profiling approach (STPP) was developed to screen protein targets of a ligand. This approach was performed to quantify proteins by using dimethyl labeling technique instead of TMT labeling and determined significantly changed proteins in one LC-MS run by using the peptides derived from the same protein, which allowed the effective reduction of reagent cost and mass spectrometric time. STPP approach could identify both the known targets of drugs and also the off-target proteins. Furthermore, we also found some ATP-binding proteins that have not been reported before, which provided the supplementary information for the map of ATP-binding proteins in 293T cell lysate. STPP approach was more suitable for the rapid screening of drug targets and verification of candidate targets screened by other methods such as affinity chromatography and other energetics-based chemical proteomics methods for drug target identification. It is a simple and convenient tool for the unbiased target proteins identification and thereby facilitates the understanding of the mode of action of drugs.

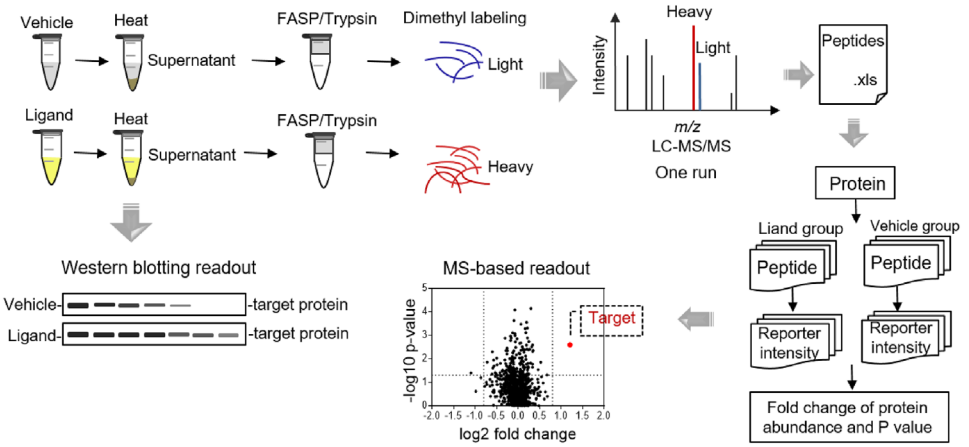

Figure 1. Workflow of the STPP for the identification of target proteins of drugs or small molecules. The cell lysate was exposed to ligands or vehicle and briefly heated to different temperatures, after which the soluble proteins were separated from denatured proteins through centrifugation. The equal volume of supernatants for the ligand and vehicle groups with the same heated temperature were subjected to filter-aided sample preparation, trypsin digestion, and stable isotopic dimethyl labeling. After combining samples from ligand and vehicle-treated groups at each temperature into one sample, they were analyzed by one LC-MS/MS run. The potential protein targets were determined by fold change of protein abundance and *p*-value calculated by reporter intensities of multiple peptides from the same protein.

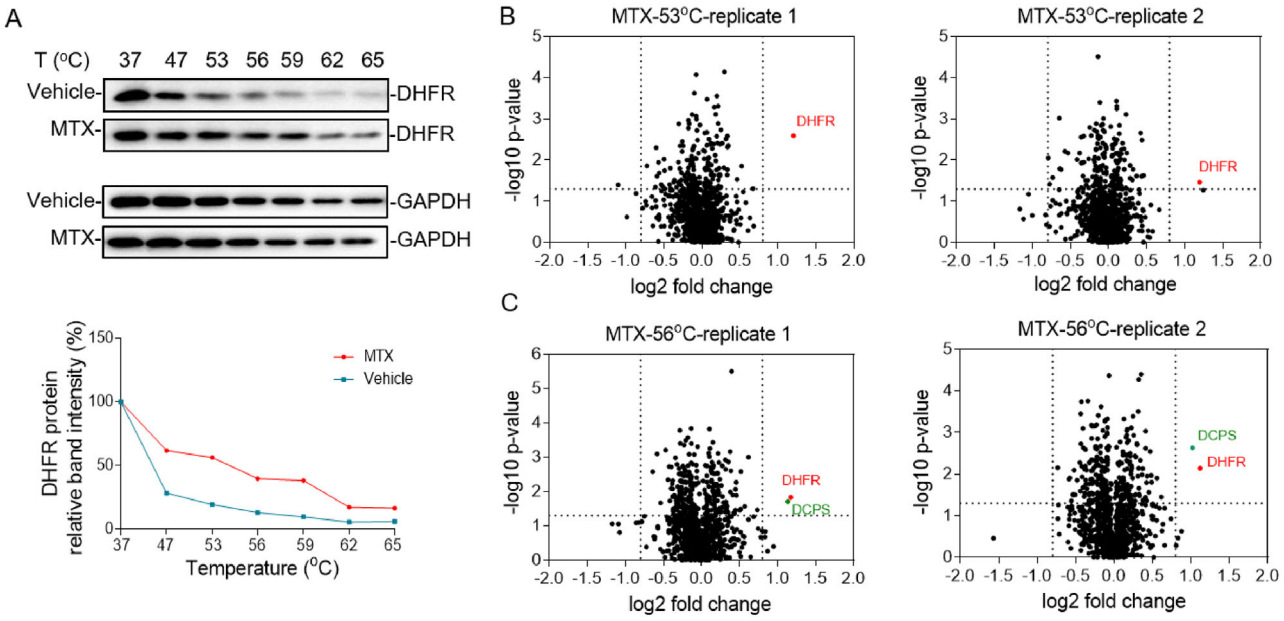

Figure 2. The identification of known target of MTX. A) Drug MTX stabilized the known target DHFR in 293T cell lysate as evidenced by western blotting. The relative band intensity in curve fitting was normalized by the protein intensity of sample at 37 °C. The known target DHFR of MTX was identified as a significant hit both at B) 53 °C and C) 56 °C-treated samples by quantitative proteomics. Two repreticate runs were performed for each temperature. The *p*-value cutoff was set as 0.05 and fold change cutoff were set 1.74 and −1.74.

the resulted cell lysate was then incubated with 100 µM geldanamycin or vehicle for 20 min at room temperature. In our preliminary experiment, it was found that HSP90AB1 protein had a wide melting temperature window when binding with geldanamycin. There was a weak shift in protein thermal stability of HSP90AB1 after treatment with geldanamycin even at 65 °C from western blotting readout (data not shown). Moreover, it was reported that NP-40 is able to shift the melting point of a protein to higher temperature.[16] Thus, we performed the STPP approach with three relatively high temperatures, 66, 69, and 72 °C (Table S2, Supporting Information). It was found 52 stabilized proteins were significant hits (fold change > 1.74, *p*-value < 0.05) for the six LC-MS/MS runs of the three temperatures after removing the duplicated proteins (Table S3, Supporting Information). As expected, the well-known targets of geldanamycin, HSP90AA1 and HSP90AB1, were identified as hits in all the six runs, revealing HSP90 family proteins were stabilized by the geldanamycin (**Figure 3**A–C). In addition, HSP90B1 protein was identified as a hit

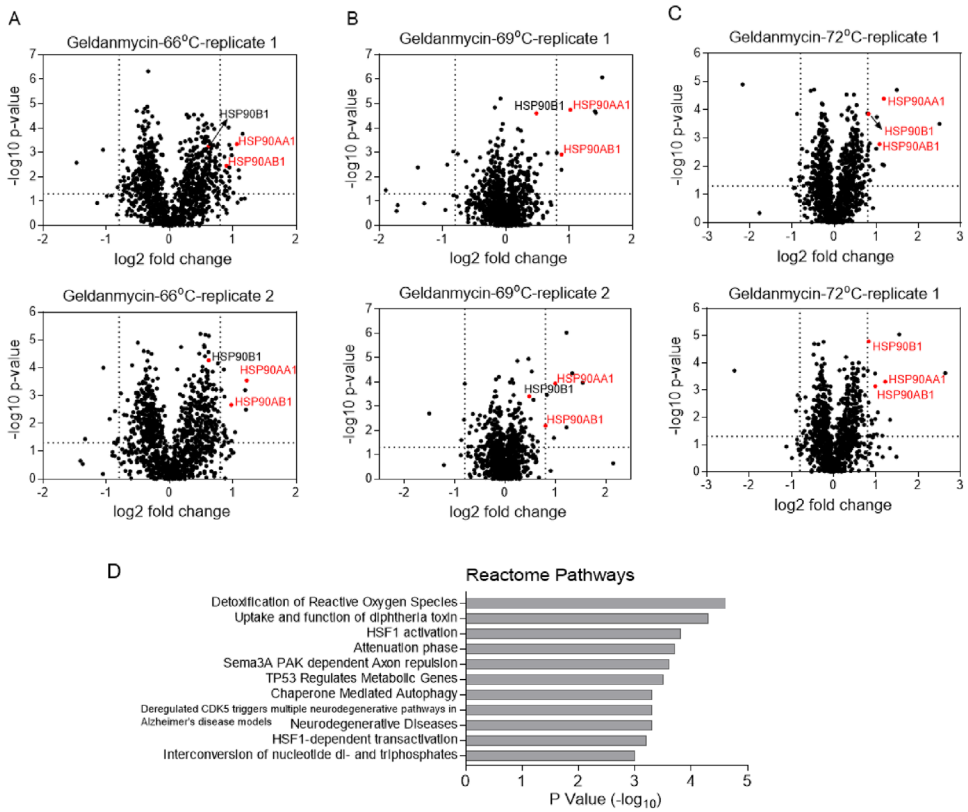

Figure 3. Identification of target proteins of geldanamycin. Volcano plots of results obtained in 293T cell lysate heated at A) 66 °C, B) 69 °C, and C) 72 °C. The two replicate MS runs were performed at each temperature. Red dots represented the known target proteins of geldanamycin. (D) The pathways analysis of candidate protein targets identified with significant thermal shift at least twice among the three temperatures to profile the possibility of hepatotoxicity induced by geldanamycin. The *p*-value cutoff was set as 0.05 and fold change cutoff were set 1.74 and −1.74.

only at 72 °C (Figure 3C), which might be due to the low affinity between HSP90B1 and geldanamycin.

In addition to these three known targets, 14 candidate protein targets were identified with significant thermal shift at least twice among the three temperatures (Table S3, Supporting Information). We conducted pathway analysis for these potential protein targets. Interestingly, the top enriched GO terms related to detoxification of reactive oxygen species (ROS) pathway (Figure 3D). This is consistence with the ROS-mediated hepatotoxicity induced by geldanamycin based on phenotypic study,[24] implying the high reliability of the identified targets.

1.3. The Thermal Profile of ATP-Binding Proteome

ATP (**Figure 4**A), an important cofactor, plays an important role in cellular energetic metabolism, protein folding, and protein phosphorylation.[25,26] Also, recent reports showed that ATP has the function as biological hydrotrope.[27,28] The interactions between ATP and proteins have pivotal function in many cellular processes. Among these interactions, only about 600 are known.[29–31] And much more ATP-binding proteins need to be discovered. We applied our approach to discover ATP binding proteins in 293T cell lysate using a non-hydrolyzable ATP analogue, adenylyl imidodiphosphate (AMP-PNP) (Figure 4A), as a ligand.

We heated the aliquots of 293T cell lysate treated with either AMP-PNP or vehicle to different temperatures and digested equal volume of supernatants after centrifugation. Cyclin-dependent kinase 9 (CDK9), a kinase, is an ATP binding protein.[32] The western blotting readout revealed that AMP-PNP protected CDK9 from unfolding, whereas vehicle-treated cell lysate showed a pronounced decrease in CDK9 abundance with the increasing temperature (Figure 4B). Based on the thermal stability shift of CDK9, the sample pairs from four temperatures, that is, 47, 52, 57, and 62 °C, were chosen for TPP (Table S4, Supporting Information). It was found that only a few proteins exerted thermal stabilization or destabilization changes at 47 °C, and more proteins showed increased stabilization with gradually increasing temperature (Figure 4C–F).

Stabilized proteins are more likely to be the direct targets of the ligand. It was found 7, 20, 33, and 23 proteins were significantly stabilized (fold change > 1.74, p-value < 0.05) at these four temperatures (**Figure 5**A). Among these stabilized protein targets, a set of 6, 9, 19, and 10 proteins were previously annotated as ATP-binding proteins by the GO analysis with DAVID online tool in the four temperatures, and the ratios of known ATP-binding

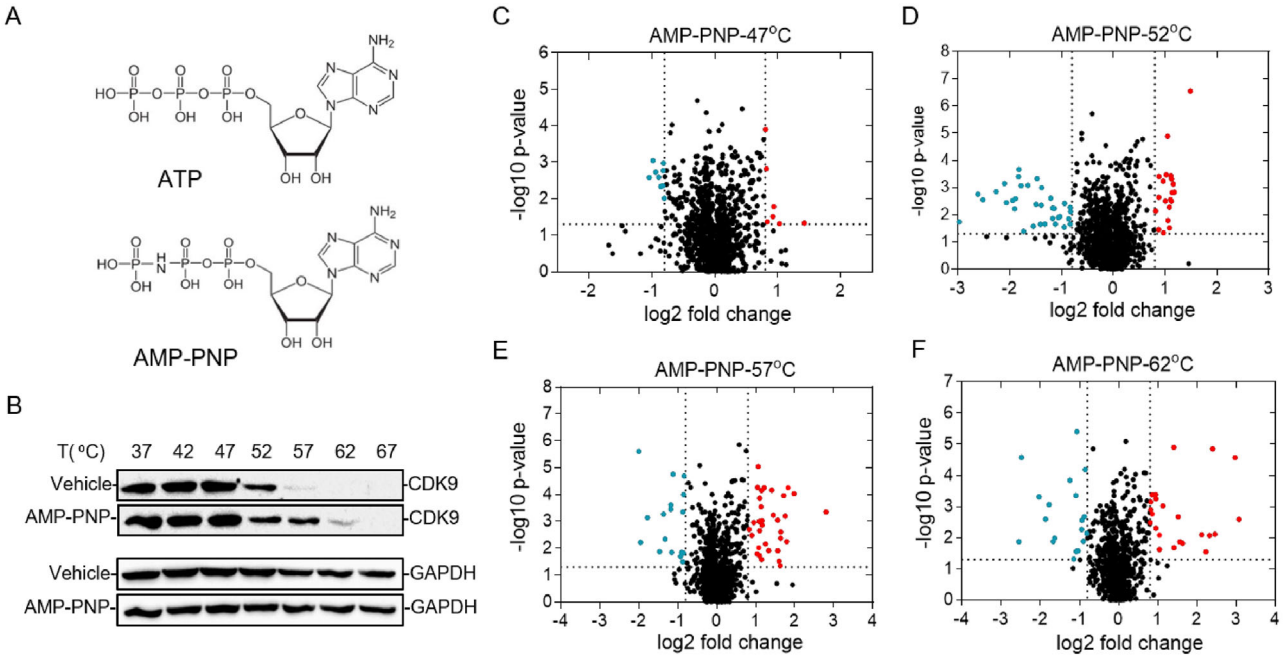

Figure 4. Identification of protein targets of ATP by using AMP-PNP. (A) The chemical structure of ATP and AMP-PNP. (B) The western blotting readout confirmed AMP-PNP stabilized CDK9 kinase. Volcano plots of results obtained from 293T cell lysate heated at C) 47 °C, D) 52 °C, E) 57 °C, and F) 62 °C. Red dots represented the proteins stabilized by AMP-PNP. Blue dots represented the proteins destabilized by AMP-PNP. The *p*-value cutoff was set as 0.05 and fold change cutoff were set 1.74 and −1.74.

proteins to the all ATP-stabilized protein targets were 85.71 %, 45.00%, 57.58%, and 43.48% (Figure 5A). A set of 21 proteins were identified at least twice (the overlap) as AMP-PNP-stabilized protein targets among the four temperatures (Figure 5B), indicating that the thermal stability windows of these proteins cover more than two of these four temperatures.

We combined the AMP-PNP-stabilized proteins derived from the four temperatures to form a final list. Totally, 59 stabilized candidate protein targets were obtained after removal of duplicated protein targets, of which 52.54% were annotated as ATP-binding proteins (Figure 5A, Tables S5 and S6, Supporting Information). The percentage of stabilized proteins which were previously known to bind ATP to total ATP-stabilized protein targets identified by this approach was higher than that identified by SILAC-SPROX approach in *Saccharomyces cerevisiae* (about 30%), LIP-SMap approach in *Escherichia coli* (about 40%), and TPP approach in Jurkat cell (less than 50%),[27,31,33] revealing the high specificity of our approach. In addition, 20.34% and 10.17% AMP-PNP stabilized proteins previously not annotated as ATP-binding proteins were known to bind with RNA and nucleotides based on GO term analysis (Figure 5C). The ATP is able to bind to nucleotides mainly due to the reason that they contain ATP binding pockets by analysis of structural data.[34] The high percentage of ATP, RNA, and nucleotides binding proteins to total AMP-PNP stabilized proteins demonstrated the reliability and robustness of this approach. In addition to the direct binding proteins, the proteins associated with ATP binding proteins may also be stabilized. A protein complex, 26S proteasome, was taken as an example. Its regulatory subunits including PSMC1, PSMC2, PSMC3, PSMC4, PSMC5, and PSMC6 identified in this study were ATP-binding proteins, whereas 10 non-ATPase regulatory subunits (PSMD) that previously was not annotated as ATP-binding proteins were also found to be stabilized in the presence of AMP-PNP (Table S6, Supporting Information). This phenomenon was in line with previous observation, and it was driven by so-called co-stabilization of subunits within complex.[27] This means non-ATPase regulatory subunits in complex do not directly bound with APM-PNP and their thermal stabilization can be propagated from complex contained at least of one ATP-binding subunit.

Comparison of proteins with stabilization shifts in this study and conventional TPP,[27] we found that 38 proteins were identified, both in this study and conventional TPP, 21 of them are ATP-binding proteins (Figure S1A,B and Table S7, Supporting Information). Moreover, 21 proteins including 10 annotated ATP binding proteins and 11 not annotated ATP binding were identified in this study but not in conventional TPP (Figure S1B and Table S7, Supporting Information). Most of these non-annotated ATP binding proteins were enriched for RNA binding proteins. The difference in stabilized proteins identification between these two approaches may be partly due to the different cell lines, 293T cell was investigated in our approach, while Jurkat cell was used in the literature. This approach is simple but effective, and has the advantage of reduction of reagent cost and mass spectrometric time.

1.4. AMP-PNP-Induced Destabilization of Proteins

The ligand binding not only can stabilize the proteins, but can also destabilize proteins. One of the major reasons for protein destabilization is that ligand binding to one region of a protein

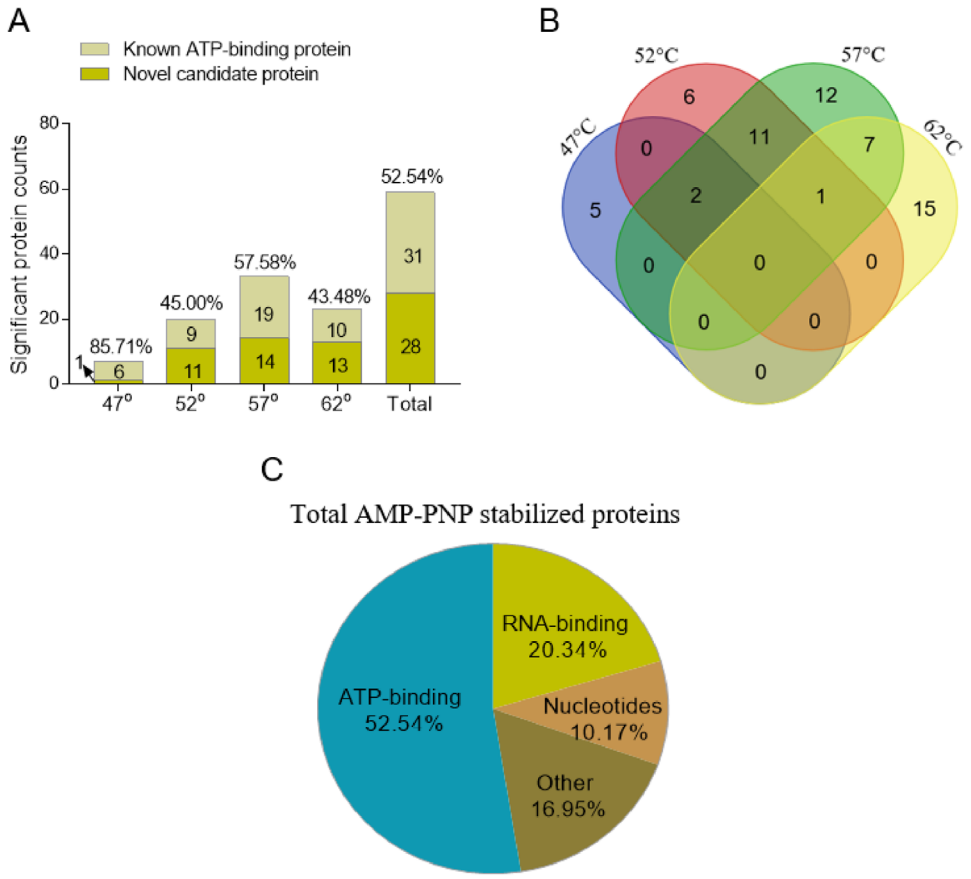

Figure 5. Characterization of the identified ATP binding proteins. (A) The ratios of known ATP-binding proteins to the all identified stabilized proteins. (B) Venn diagram showing overlap of stabilized proteins identified in different temperatures. (C) Distribution of the stabilized protein hits with different known ligands.

could induce conformational changes and disrupt the protein–protein interaction.[35,36] The destabilization of proteins induced by ATP was observed in many reports.[37,38] A total of 9, 34, 17, and 19 proteins were identified as the AMP-PNP induced destabilized proteins among the four temperatures (fold change < -1.74, $p < 0.05$), of which 15 proteins were repeatedly identified in two temperatures (Figure S2, Supporting Information). After combining these proteins from the four temperature points, a set of 64 destabilized proteins was obtained (Table S5, Supporting Information). Interestingly, no protein annotated with ATP-binding was found in these destabilized proteins according to GO term analysis (data not shown). Moreover, the largest affected group of these destabilized proteins are annotated RNA-binding proteins (60.90%) (Table S5, Supporting Information), which was in line with previous observation in Jurkat cell.[27]

AMP-PNP-induced protein destabilization is likely because the AMP-PNP occupies the binding pocket of subunit(s) of a complex, which results in the dissociation of others subunit(s) of a complex.[39] This hypothesis was supported by an analysis of the total 123 stabilized and destabilized proteins detected here by performing STRING online tool, which demonstrated that more than 90% destabilized proteins were interacted with at least one of the other AMP-PNP stabilized protein hits (Figure S3, Supporting Information). For example, the 60S ribosomal protein subunits including RPL12 and RPL22 identified in this study were stabilized upon AMP-PNP addition, whereas the other subunits such as RPL5, RPL9, RPL12, RPL29, RPL31, and RPL35 were detected to be destabilized. RPL family proteins were also observed as hits in the *S. cerevisiae* with iTRAQ-SPROX assay.[38] Therefore, this approach could be also used for the investigation of the networks of protein–protein interaction.

2. Discussion

In this simplified TPP approach, thermal shifts induced by addition of ligand were determined by quantitative proteomics using dimethyl labeling reagent instead of costing TMT labeling reagent for each temperature. The significant changes in protein abundance were assessed by analyzing the intensities of multiple peptides from the same protein in ligand and vehicle-treated groups, which allowed the determination of the *p*-values of proteins for the study with a single LC-MS/MS run. To improve proteome coverage, the screening could be performed with multiple temperatures.

The selection of temperatures used for quantitative proteome to screen the targets of a drug is the crucial part of this experiment. We need to ensure that the selected temperature

is within the melting temperature ranges of the proteins of interest, such that the significant difference in abundance between ligand-bound and unbound proteins can be achieved to enable unbiased assessment of the protein targets. Most previous reports are using the heating temperature within the range of 37–67 °C to precipitate proteins in TPP due to the large fraction of proteins that have a melting point in this temperature range.[19] For our MTX and AMP-PNP experiments, the temperatures (50–65 °C) that occurred significantly thermal shifts for known targets were within the above temperature range according to western blotting readouts, which was consistent with previous reports.[3,5,20] However, the temperatures (66, 69, and 72 °C) selected for quantitative proteome to validate known targets of geldanmycin exceeded the common used temperature range in TPP. The temperatures for TPP should not be limited to 37–67 °C range. It depends on the actual melting temperature of proteins of interest, it could be revealed by western blotting if there are known targets. Several studies also reported that some proteins showed significant thermal shifts at high temperatures, for example, thymidylate synthase (TS) (45–85 °C) in K562 cells, deoxycytidine kinase (60–84 °C) in K562 cells, and MetAP2 (60–85 °C) in mouse liver lysates.[40,41]

Since most proteins have a relatively wide melting temperature window, typically at least three temperatures with a wide range should cover the temperature windows for most proteins. Even so, because the temperatures selected to denature proteins in our STPP approach is less than that in the TPP method, the sensitivity of target proteins identification is likely to be reduced. This is a common shortcoming of this type of strategies with fewer temperatures[42] or pool different temperatures.[43] However, our method is more suitable for the rapid screening of drug targets and verification of candidate targets screened by other methods such as affinity chromatography and other energetics-based chemical proteomics methods for drug target identification.

It should be noted that one disadvantage of this approach is the low proteome coverage, typically about 2000 proteins were quantified at each temperature, because of the use of single 1D LC-MS/MS analysis. While about 5000–8000 proteins could be quantified in conventional TPP by using 2D LC-MS/MS analysis.[5] Proteins quantified with less than three peptides were removed in the data processing for *p*-value computation is also a reason for the low coverage. In addition, the samples we monitored for thermal stability shifts were processed with higher temperatures, which had less soluble proteins in the supernatant and so low number of protein identifications. Of course, the coverage of this approach can also be increased by fractionation of the labelled digest prior to MS analysis as in the conventional TPP approach.

In summary, we presented a STPP to be a general approach for unbiased identification of protein targets for drugs or small molecules. This approach identified both the known targets of drugs and also the off-target proteins, many of which were previously not known to interact with the drugs. Moreover, this approach features with low reagent cost and short mass spectrometric time. It is a simple and convenient tool for fast screening or validation of the protein targets of drugs and thereby facilitates our understanding of the mode of action of drugs.

3. Experimental Section

Material and Cell Culture: MTX was purchased from Sigma-Aldrich (St. Louis, MO), geldanamycin were from Selleck (Houston, TX), AMP-PNP was from Thermo Fisher (San Jose, CA). All above mentioned reagents were dissolved and diluted using DMSO except AMP-PNP that was dissolved and diluted in ddH2O. Complete (EDTA-free) protease inhibitor cocktail was from Sigma-Aldrich (St. Louis, MO, USA). Labeling reagents CH_2O, CD_2O, and CH_3BNN_a were from Sigma-Aldrich (St. Louis, MO).

293T cells were cultured in RPMI1640 (Gibco, Gaithersburg, MD) containing 10% fetal bovine serum (Gibco, NY) and 1% streptomysin (Beyond, Haimen, China) under the condition of 37 °C, 5% CO_2.

Preparation of Cell Extract for Stabilization Profiling: After washing with cold PBS three times, the cells were lysed using PBS (pH 7.4) containing 1% EDTA-free cocktail in the MTX and AMP-PNP experiment. For the geldanamycin experiment, 0.2% NP-40 was added in PBS with cocktail. The cell suspensions were freezed using liquid nitrogen, followed by thawed at 37 °C using water bath. After about 60% was thawed, the cell suspensions were transferred on ice to continue thawing and this procedure was repeated three times. The soluble proteins in the supernatant were separated from cell precipitation by centrifuging at 20 000 g for 20 min at 4 °C. The thermal shift experiments were performed similar to the procedure reported by Savitskis.[5] Briefly, the supernatants were divided into two equal aliquots, one aliquot was treated with drugs or small molecules dissolved in DMSO or ddH_2O, and the other aliquot was treated with an equivalent amount solvent alone as vehicle. After the incubation with ligand or vehicle for 20 min at room temperature, the lysates were divided into several aliquots of 50 µL in new 200 µL PCR tubes and heated individually at different temperatures for 3 min using thermal cycler, followed by cooling at room temperature for 3 min. The heated lysates were subjected to centrifugation at 20 000 g for 20 min at 4 °C to separate soluble proteins from precipitated proteins. One portion of the soluble proteins was used for western blotting, and the left portion was used for quantitative proteomics analysis. The protein concentration was determined by using BCA reagent.

Western Blotting for Detecting the Thermal Shift: The soluble proteins in supernatants were separated by means of SDS-PAGE and were transferred onto a polyvinylidence difluoride membrane. The membrane was blocked with 5% skim milk in TBS containing 1% Tween 20. Primary anti-DHFR, anti-CDK9 (Proteintech, Chicago, IL), and secondary goat anti-rabbit HRP-IgG antibodies (Abcam, Cambridge, UK) were used for immunoblotting according to the manufacturer's instructions. The chemiluminescence intensities were visualized and quantified by the ECL detection kit (Thermo Fisher Scientific, USA) and the images were obtained by using Fusion FX7 imaging system (Vilber Infinit, France).

Sample Preparation for MS Analysis: The equal volume supernatants in the ligand group and vehicle group were transferred into 10 k ultrafiltration units (Sartorius AG, Germany) and subjected to FASP processing. The supernatants were then reduced by addition of 20 mM dithiothreitol for 2 h at 37 °C and alkylated by reaction with 40 mM IAA in the dark environment for 40 min. Subsequently, the protein samples were digested with trypsin to protein ratio of 1: 20 at 37 °C overnight.

The digested peptides were subjected to dimethyl labeling. Briefly, the peptides in the vehicle group were labeled with 4% CH_2O and 0.6 M CH_3BNNa as light labeling, and the peptides in the ligand group were labeled with 4% CD_2O and 0.6 M CH_3BNNa as heavy labeling. After the reaction was performed for 1 h at 25 °C, ammonia was added to terminate the reaction. After being acidified by formic acid, the labeled peptides in the ligand and vehicle group at the same temperature were pooled together. Finally, the peptide mixtures were desalted with C18 SEP-PAK cartridges (Waters Associates, Milford, MA). Peptides were eluted with ACN/0.1% TFA (80/20, v/v) and lyophilized in a SpeedVac (Thermo Fisher Scientific, San Jose, CA, USA).

LC-MS/MS Analysis: The analysis of tryptic peptides was performed on an Ultimate 3000 RSLCnano system coupled with a Q-Exactive-HF mass spectrometer, controlled by Xcalibur software version 2.1.0 (Thermo Fisher Scientific, Waltham, MA, USA). The dimethyl-labeled peptides were dissolved in 0.1% formic acid/water, and the concentrations were deter-

mined by using NanoDrop 2000 (Thermo Fisher Scientific, USA). Briefly, 1 μg of the re-suspended peptides were automatically loaded onto a C18 trap column (200 m i.d) at a flow rate of 5 μL min^{-1}. The capillary analytical column (150 μm i.d.) was packed in-house with 1.9 μm C18 ReproSil particles (Dr. Maisch GmbH). Peptides were separated using a gradient of 7–70% acetonitrile with 0.1% formic acid over 120 min at a flow rate of 600 nL min^{-1}. The LC-MS/MS system was operated in data-dependent MS/MS acquisition mode. The full mass scan acquired in the Orbitrap mass analyzer was from m/z 350 to 1750 with a resolution of 60 000 (m/z 200). The MS/MS scans were also acquired by the Orbitrap with a 15 000 resolution (m/z 200), and the AGC target was set to 5×10^4. The spray voltage and the temperature of the ion transfer capillary were set to 2.6 KV and 275 °C, respectively. The normalized collision energy for HCD and dynamic exclusion were set as 27% and 20 s, respectively.

Protein Identification and Quantification: Raw files were searched by using MaxQuant software (version 1.5.3.30.) against the Uniprot human database containing 70 037 entries. Multiplicity was set to 2 with dimethLys0 and dimethNter0 as light labeling whereas dimethLys4 and dimethNter4 as heavy labeling. Carbamidomethylated cysteine was selected as a fixed modification, while oxidation of methionine and N-terminal protein acetylation were searched as variable modifications. Trypsin was set for enzymatic digestion with maximum two missed cleavages. The false discovery rate was set to 0.01 as a filter for both proteins and peptides. Precursor and fragment mass tolerances were set at 10 ppm and 0.02 Da, respectively. Moreover, the options of re-quantify and match between runs were required.

Statistical Processing: To analyze the thermal shift data statistically, code was written to automatically process the peptide output file from MaxQuant, to perform normalization, to calculate *p*-values and ratios of protein abundance, and to identify proteins with significant change in thermal stability. Briefly, the intensity of each peptide was normalized by the median value of the ratio of peptides in vehicle-treated and ligand-treated groups. The normalized intensities were then used for the calculation of peptide ratios between the two groups. Subsequently, the peptides with the median ratio and the two peptides closest to the median ratio, a total of three peptides among all peptides from each protein were selected and their corresponding reporter intensities in the vehicle and ligand-treated samples were used for protein *p*-value calculation by Student's *t*-test. Protein ratios were determined using the median of all peptide-feature ratios for each protein. The proteins with p-value < 0.05 and abundance fold change > 1.74 or < -1.74 were considered as the direct or indirect candidates of drugs. The direct candidates identified at different temperatures were combined as the final target list. The volcano plots were generated by Graphpad Prism 5 software (Graphpad Software, Inc, La Jolla, CA).

Pathway and Gene Ontology Analysis: Reactome online tool was used for pathway analysis of protein target hits in the geldanamycin experiment. DAVID online tool was used for functional enrichment analysis of all protein target hits for Gene Ontology (GO) in the AMP-PNP experiment. The protein–protein interaction analysis was conducted with STRING online tool in the AMP-PNP experiment. The minimum required interaction score was set high confidence (0.700).

MS Proteomics Data Availability: The raw data for mass spectrometry analysis can be downloaded via ftp://massive.ucsd.edu/MSV000085140/.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

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