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A semi-quantitative Drug Affinity Responsive Target Stability (DARTS) assay for studying Rapamycin/mTOR interaction

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Dear Editors,

Please find enclosed our manuscript entitled “A semi-quantitative Drug Affinity Responsive Target Stability (DARTS) assay for studying Rapamycin/mTOR interaction”, which we would like to submit for publication as an original research in *JOVE*.

Drug Affinity Responsive Target Stability (DARTS) is a simple and robust approach to identify potential protein targets for small molecules. It is particularly useful for the identification of unknown targets of small molecules, but can also be used to validate potential protein-ligand interactions predicted or identified by other means. In this study, we further enhanced the data analysis capabilities of the DARTS experiment by monitoring the changes in protein stability and estimating the affinity of protein-ligand interactions. The protein-ligand interactions can be plotted into two curves: proteolytic curve and dose dependence curve. This method is likely to be a powerful method for accurately identifying novel target proteins and for the optimization of drug target engagement.

We confirm that this manuscript has not been published elsewhere and is not under consideration by another journal. All authors have approved the manuscript and agree with submission to JOVE. The authors have no conflicts of interest to declare.

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We look forward to hearing from you at your earliest convenience.

Yours sincerely,

Chuanju Liu

TITLE:

A Semi-Quantitative Drug Affinity Responsive Target Stability (DARTS) assay for studying Rapamycin/mTOR interaction

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KEYWORDS:

DARTS, Semi-quantitative, mTOR, Rapamycin, Interactions, Target

SUMMARY:

In this study, we enhanced the data analysis capabilities of the DARTS experiment by monitoring the changes in protein stability and estimating the affinity of protein-ligand interactions. The interactions can be plotted into two curves: a proteolytic curve and a dose-dependence curve. We have used mTOR-rapamycin interaction as an exemplary case.

ABSTRACT:

Drug Affinity Responsive Target Stability (DARTS) is a robust method for detection of novel small molecule protein targets. It can be used to verify known small molecule-protein interactions and to find potential protein targets for natural products. Compared with other methods, DARTS uses native, unmodified, small molecules and is simple and easy to operate. In this study, we further enhanced the data analysis capabilities of the DARTS experiment by monitoring the changes in protein stability and estimating the affinity of protein-ligand interactions. The protein-ligand interactions can be plotted into two curves: a proteolytic curve and a dose-dependence curve. We have used the mTOR-rapamycin interaction as an exemplary case for establishment of our protocol. From the proteolytic curve we saw that the proteolysis of mTOR by pronase was inhibited by the presence of rapamycin. The dose-dependency curve allowed us to estimate the binding affinity of rapamycin and mTOR. This method is likely to be a powerful and simple method for accurately identifying novel target proteins and for the optimization of drug target engagement.

INTRODUCTION:

Identifying small molecule target proteins is essential to the mechanistic understanding and development of potential therapeutic drugs[1-3]. Affinity chromatography, as a classical method for identifying the target proteins of small molecules, has yielded good results[4,5]. However, this method has limitations, in that chemical modification of small molecules often results in reduced or altered binding specificity or affinity. To overcome these limitations, several new strategies have recently been developed and applied to identify the small molecule targets without chemical modification of the small molecules. These direct methods for target identification of label-free small molecules include drug affinity responsive target stability (DARTS)[6], stability of proteins from rates of oxidation (SPROX)[7], cellular thermal shift assay (CETSA)[8,9], and thermal proteome profiling (TPP)[10]. These methods are highly advantageous because they use natural, unmodified small molecules and rely only on direct binding interactions to find target proteins[11].

Among these new methods, DARTS is a comparatively simple methodology that can easily be adopted by most labs[12,13]. DARTS depends on the concept that ligand-bound proteins demonstrate modified susceptibility to enzymatic degradation relative to unbound proteins. The new target protein can be detected by examination of the altered band in SDS-PAGE gel through liquid chromatography-mass spectrometry (LC-MS/MS). This approach has been successfully implemented for identification of previously unknown targets of natural products and drugs[14-19]. It is also powerful as a means to screen or validate binding of compounds to a specific protein[20,21]. In this study, we present an improvement to the experiment by monitoring the changes in protein stability with small molecules and identifying protein-ligand binding affinities. We use mTOR-rapamycin interaction as an example to demonstrate our approach.

PROTOCOL:

1. Collect and lyse cells

1.1. Grow 293T cells using Dulbecco's modified Eagle medium (DMEM) with 10% fetal bovine serum, 2 mM glutamine and 1% antibiotics. Incubate cultures at 37 °C under 5% CO_2.

NOTE: The growth state of the cells may affect the stability of subsequent experiments.

1.2. Expand cells in culture until reaching 80–90% confluence.

1.3. Mix 345 µL of cell lysis reagent (see the **Table of Materials**) with 25 µL of 20x protease inhibitor cocktail, 25 µL of 1 M sodium fluoride, 50 µL of 100 mM β-glycerophosphate, 50 µL of 50 mM sodium pyrophosphate, and 5 µL of 200 mM sodium orthovanadate. Keep the lysis buffer chilled on ice.

NOTE: Other lysis buffers with various detergents (e.g., Triton X-100 or NP-40) can be used with DARTS as long as they are non-denaturing. Membrane proteins or nuclear proteins can be extracted by adding 0.4% Triton X-100 or 0.4% NP-40 to the cell lysate.

1.4. Wash the cells twice with cold phosphate-buffered saline (PBS).

1.5. Use a cell scraper to collect the cells in an appropriate amount of cell lysis buffer and transfer the lysing cells into a 1.5 mL tube.

NOTE: The number of cells needed for each DARTS experiment will vary based on how much protein can be extracted from various cell lines. In general, the protein concentration of the lysate used is between 4–6 µg/µL. One 10 cm plate of 293T cells at 85–90% confluency, lysed with 300 µL of lysis buffer typically results in a lysate with a protein concentration of ~5 µg/µL.

1.6. Mix the lysis buffer/lysing cells well and incubate the tube on ice for 10 min.

1.7. Centrifuge the tube at 18,000 × *g* for 10 min at 4 °C.

1.8. Transfer the supernatant into a new 1.5 mL tube and keep chilled on ice.

1.9. Perform a BCA assay to approximate the protein concentration of lysates.

2. Incubate protein lysates with the small molecule

2.1. Split 99 µL of the lysates into two 1.5 mL tubes.

2.2. Make a starting stock concentration of 10 mM small molecule. When performing DARTS, one may begin with a higher concentration of the small molecule (5–10x the EC50 value) to ensure optimal binding.

2.2.1. Add either 1 µL of solvent that the small molecule is soluble in or 1 µL of small molecule stock solutions to each aliquot of lysate. Incubate cell lysate with the solutions for 30–60 min at room temperature with shaking.

2.3. On ice, establish serial dilutions (1:200, 1:400, 1:800 and 1:1600) of freshly thawed pronase solution in 1x TNC (For 1 mL of 10x TNC buffer, mix 300 µL of ultrapure water with 100 µL of 5 M sodium chloride, 100 µL of 1 M calcium chloride, and 500 µL of 1 M Tris-HCl, pH 8.0).

2.4. Examine a wide-breadth of pronase:protein ratios (e.g., spanning from 1:100 to 1:2000) to guarantee observability of the step-wise effect of pronase on target(s).

NOTE: To calculate pronase concentrations (example): 5 µg/µL protein concentration x 20 µL sample = 100 µg protein. For a pronase:protein ratio of 1:100 we need 0.5 µg/µL (100 µg ÷ 100 ÷ 2 µL) pronase solution. This experiment may have to be repeated several times to obtain a suitable range of pronase:protein ratios.

3. Perform proteolysis

NOTE: For proteolysis, steps are carried out at room temperature unless otherwise noted

3.1. Following incubation with the small molecule, divide each aliquot into 20 µL samples.

3.2. Add 2 µL of the range of pronase solutions in each sample at specific intervals (every 30 s). Use an equal volume of 1x TNC buffer to establish an undigested control sample.

3.3. After 5–20 min, halt digestion via addition of 2 µL of cold 20x protease inhibitor cocktail every 30 s. Mix well and incubate on ice for 10 min.

3.4. Dilute samples with the appropriate volume of 5x SDS-PAGE loading buffer and boil at 95 °C for 5 min.

3.5. Carry out the next portion of the experiment or store the protein sample at −80 °C.

4. Quantification and analysis

4.1. After DARTS, perform Coomassie blue staining according to the previously published protocol[22].

4.2. The stained protein bands should be very clear after destaining. Pour off the used destain solution and add fresh 1% acetic acid solution to cover the gel. Put the gel under the light to observe the bands with significant differences between groups with (sample group) or without (control group) the small molecule.

4.3. Cut the two corresponding bands from the gel with a sterile instrument and perform LC-MS/MS immediately.

NOTE: LC-MS/MS needs to be done as soon as possible because the proteins in the gel will degrade continuously.

4.4. Digest the bands, extract peptides and perform LC-MS/MS analysis[23].

4.4.1. To analyze LC-MS/MS data, first identify all the proteins in the individual band. Second, use peptide spectrum match (PSM) to represent the abundance of each protein.

NOTE: PSM provides the total number of identified peptide sequences for the protein, including those redundantly identified. In general, how often a peptide is identified/sequenced can be used as a rough estimate of how abundant the protein is in the sample. Proteins enriched in the sample group over the control group are proteins of interest.

4.5. Procure the primary antibodies of the selected proteins. Perform western blot to verify that the small molecule can bind directly to the potential target proteins[24].

4.5.1. Load equal amounts of protein into the wells of an 8% SDS-PAGE gel, along with an appropriate molecular weight marker. Run the gel for 30 min at 80 V, then adjust the voltage to 120 V and continue running for 1–2 h.

4.6. Quantify the different target protein bands using image processing and analysis software (see the **Table of Materials**).

4.7. Analyze the data and draw the curves.

4.7.1. Determination of the proteolytic curve for a target protein

4.7.1.1. The pronase:protein ratio is varied when performing proteolysis. Normalize data from image analysis by attributing the band intensity values corresponding to the undigested bands to 100%.

4.7.1.2. Use nonlinear regression analysis of the statistical analysis and drawing software to plot a curve of normalized data for relative band intensity and pronase:protein ratios.

4.7.1.3. First, open the software, select the type of table and graph as XY and allow for 3 replicate values in side-by-side subcolumns. Then enter the corresponding data in the space below x and y. Under x, input the numbers 0, 1, 2, 3, 4, 5 (as place holders corresponding pronase:protein ratios).

4.7.1.4. In the y column, enter the normalized data for relative band intensities. Perform "Nonlinear regression" and implement a "Dose-response-Stimulation" using the "Log (agonist) versus response—Variable slope (four parameters)" equation.

4.7.1.5. Convert the annotations of the X-axis in the curve to the corresponding pronase:protein ratios.

4.7.2. Determination of the dose-dependence curve for a target protein

NOTE: For dose-dependence analysis, the molarity of the small molecule is differed across samples. The stable pronase:protein ratio should be informed by analysis of proteolysis data. The pronase:protein ratio that showed maximal difference in target protein intensity during the proteolytic curve should be used for the dose-dependence experiment.

4.7.2.1. Quantify the different target protein bands using an image analysis software. As in generation of the dose-dependence curve, normalize data from image analysis by attributing the band intensity values corresponding to the least digested band to 100%.

4.7.2.2. Apply the nonlinear regression analysis within the statistical analysis and drawing software to the normalized data. First, open the software, select the type of table and graph as XY, and enter 3 replicate values in side-by-side subcolumns.

4.7.2.3. Then, enter the corresponding data in the space below x and y. For the 'x' variable, input different concentrations of the small molecule; 'y' includes the corresponding normalized data for relative band intensity.

4.7.2.4. Transform x values using X = Log(X). Finally, employ nonlinear regression, and implement a dose-response stimulation using the "Log (agonist) versus response—Variable slope (four parameters)" equation.

REPRESENTATIVE RESULTS:

The flow chart of the experiment is outlined in **Figure 1**. The result of Coomassie blue staining is shown in **Figure 2**. Incubation with the small molecule confers protection against proteolysis. Three bands that appear to be protected by incubation with rapamycin over vehicle control are found. The expected results from proteolytic curve experiment are shown in **Figure 3**. As a proof-of-principle, we examined the well-studied protein mTOR, which is the target for the drug rapamycin[25]. Western blotting illustrates the presence of mTOR protein at low pronase:protein ratios and its reduction and loss with increasing ratios (**Figure 3A**). Proteolysis of mTOR by pronase is clearly inhibited by the presence of rapamycin and the addition of rapamycin generates an obvious shift in the proteolytic curve (**Figure 3B**). To investigate effects of drug concentration, we maintained a constant pronase:protein ratio while varying concentrations of rapamycin. As ligand concentration nears target binding saturation, an increased presence of target protein is observed. Rapamycin dose-dependently enhanced the level of mTOR, suggesting the rising stability of mTOR with rapamycin treatment (**Figure 4A**). Quantification of the target protein band intensities allows representation of target stability as a function of ligand concentration as exemplified by the curve in **Figure 4B**. These results strongly suggest that mTOR is the target protein of rapamycin.

FIGURE LEGENDS:

Figure 1: Schematic of the DARTS approach for drug target semi-quantitative analysis. Cell lysate is incubated in the presence or absence of a small molecule, followed by proteolysis and protein electrophoresis. Protected protein bands are excised and subjected to mass spectroscopy. Protein targets are identified as those proteins that display increased protease resistance in the presence of the small molecule. Then western-blot is used to identify and semi-quantitatively analyze the target proteins. Data are expressed as means ± standard deviation (SD).

Figure 2: Example of Coomassie blue staining visualization of DARTS with the small molecule rapamycin. Red dots flank the protected bands.

Figure 3: Illustration of the remaining amount of mTOR accessible for detection as a function of the pronase:protein ratio used for treatment of 293T cell lysates. (**A**) Protection of mTOR from proteolysis by rapamycin was evaluated by western blot analysis. (**B**) The intensity of the mTOR

bands were quantified using the statistical analysis and drawing software. The line was fitted with a four-parameter logistic curve. Data were obtained from the three independent experiments and were expressed as mean ± SD.

Figure 4: Illustration of the amount of stabilized mTOR accessible for detection in the presence of increasing concentrations of rapamycin. 293T cell lysates were incubated with rapamycin (0, 1, 10, 100, 1000, 10000 nM) for 1 h, then the cell lysates were subjected to digestion at the pronase:protein ratio of 1:400. (**A**) The stabilization effect of rapamycin on mTOR was evaluated by western blot. (**B**) The intensity of the mTOR bands were quantified using the statistical analysis and drawing software. The line was fitted with a four-parameter logistic curve. Data were obtained from the three independent experiments and were expressed as mean ± SD.

DISCUSSION:

DARTS allows for identification of small molecule targets by exploiting the protective effect of protein binding against degradation. DARTS does not require any chemical modification or immobilization of the small molecule[26]. This allows small molecules to be used to determine their direct binding protein targets. Standard assessment criteria for the classical DARTS method include gel staining, mass spectrometry and western blotting[12,13]. The classic methodology also mentions that these data can be quantitatively analyzed, but there is no such example provided. Here, we use principles of the cellular thermal shift assay (CETSA) to semi-quantitatively analyze the data, and obtain parameters similar to those supplied by CESTA (Tm and EC50) which increases the utility of DARTS analysis[8]. The ligand–target interaction can be plotted against pronase:protein ratio to display obvious shifts in proteolytic curves. Carrying out proteolysis using different pronase:protein ratios can help in narrowing down the concentration of pronase that should be used in downstream experiments. Further, using the optimized concentration of pronase, the proteolysis carried out in the presence of different concentrations of the small molecule may provide an indirect measure of the affinity of the small molecule with its target protein. In addition, generation of a dose-dependence curve allows for approximation of effects on target proteins dependent upon ligand concentration. Inclusion of analytic capacity for dose-dependence is a powerful expansion of DARTS methodology; providing a straightforward and quick approach to probing the therapeutic mechanism of small molecules. This gel-based approach is the easiest to implement. It can be used for high-throughput screening for compounds that bind a specific protein[20,27,28]. Additionally, DARTS can be utilized for analyzing true interactions with low affinity, because washing is not included as an experimental step[12,26]. Moreover, compared with CETSA, DARTS has advantages in identifying the targets of membrane proteins as DARTS allows a better assessment of membrane proteins through use of mild, stabilizing detergents[11].

The experiment also has some limitations. First, when the cell lysate has a low abundance of target protein, the DARTS method cannot be used to easily visualize alterations in proteolysis of the target protein. Additional steps to concentrate these proteins are required in order to apply this methodology. Second, we only test rapamycin/mTOR interaction. The interaction is known to be potent and stable. However, some small molecules may bind to their targets less selectively,

or transiently, and it is not clear if such small molecules can be analyzed with this assay. Third, some target proteins may be extremely sensitive or resistant to the proteases used.

DARTS assay analysis allows for identification of potential protein interactions through assessment of proteolytic curves generated across a range of pronase:protein ratios in the presence or absence of a small molecule ligand. Excitingly, our modifications to the standard procedural outline of the DARTS assay highlight the capacity of this method to be used in generation of concentration-response curve. These outputs are of special utility in drug development, allowing identification of mechanistically relevant drug concentrations. Moreover, comparison of concentration-response curves generated using disparate ligands offers insight to the comparative binding affinities for several ligands of the same target; a capacity potentially useful in prediction of small molecule efficacy and refinement of dosing. We hope that this demonstration of expanded analytic power of DARTS will be useful in the development, implementation, and understanding of small molecule drugs, particularly for ligands and targets difficult to analyze using alternative approaches.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

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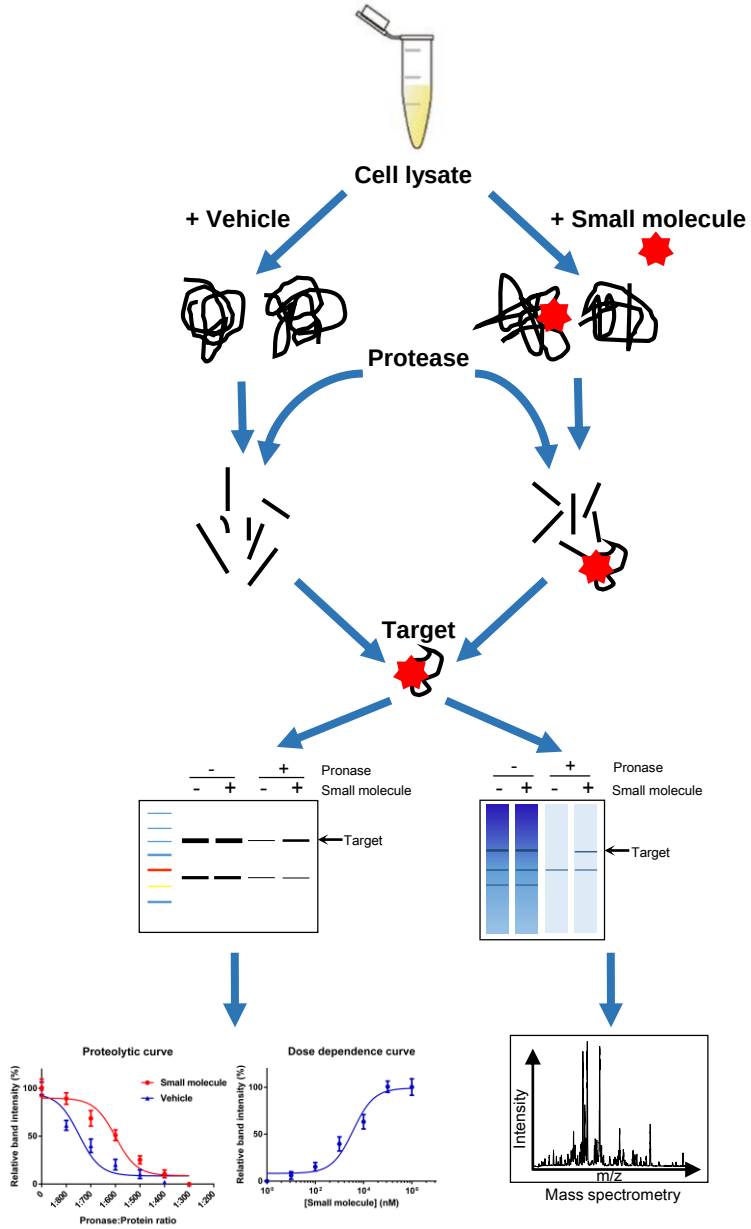


Figure 2

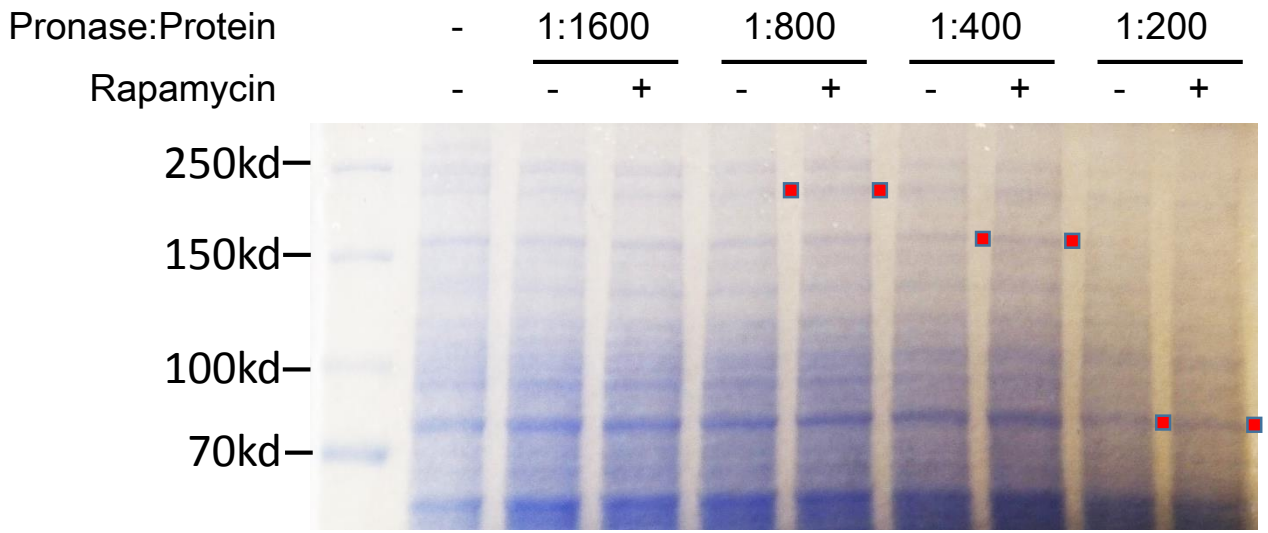
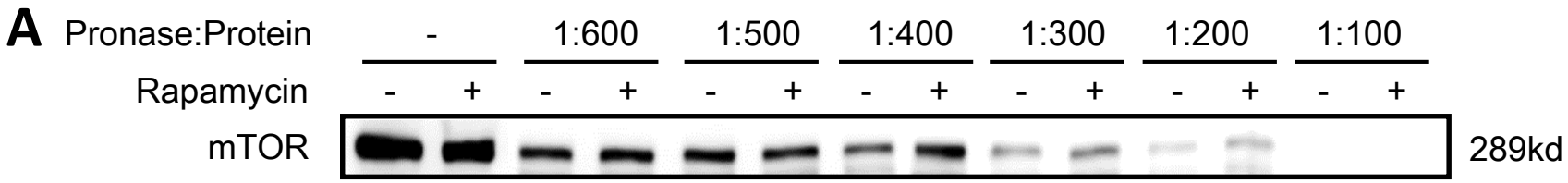


Figure 3



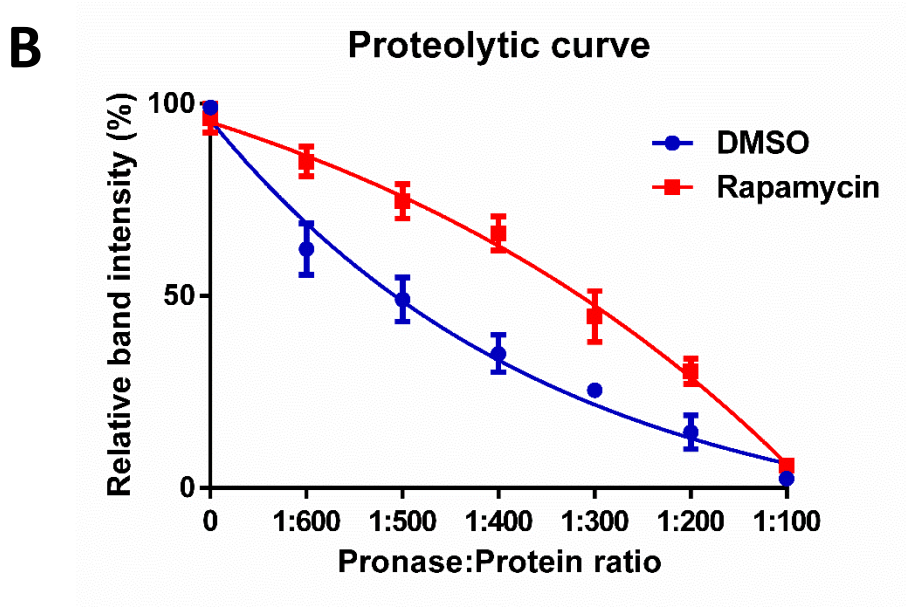
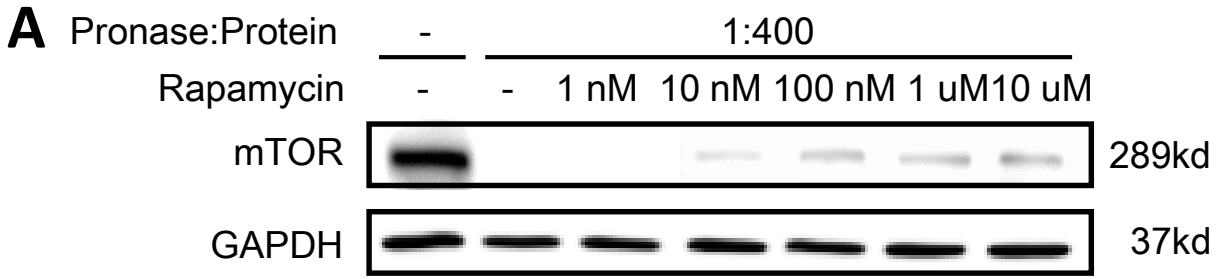
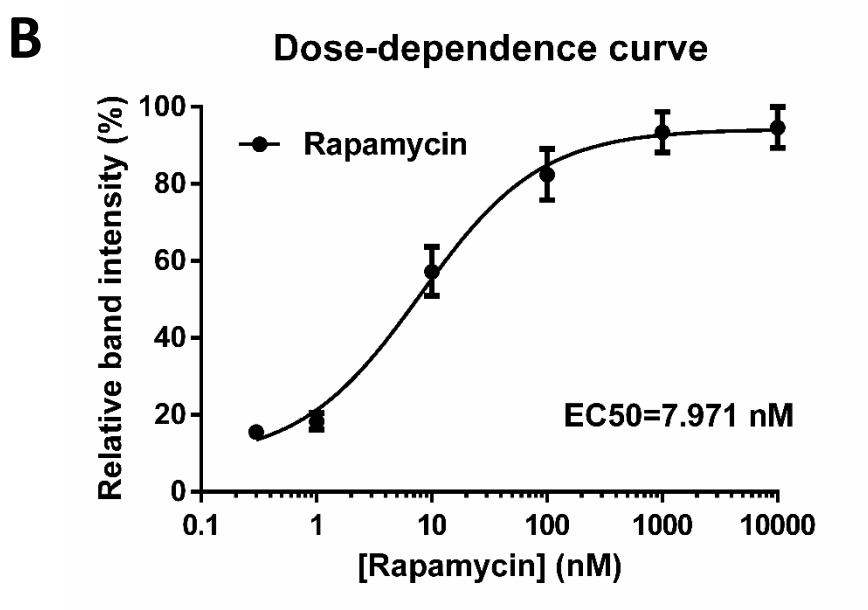


Figure 4





Name of Material/Equipment	Company	Catalog Number	Comments/Description
100X Protease inhibitor cocktail	Sigma-Aldrich	P8340	Dilute to 20X with ultrapure water
293T cell line	ATCC	CRL-3216	DMEM medium with 10% FBS
Acetic acid	Sigma-Aldrich	A6283	
BCA Protein Assay Kit	Thermo Fisher	23225	
Calcium chloride	Sigma-Aldrich	C1016	
Cell scraper	Thermo Fisher	179693	
Coomassie Brilliant Blue R-250 Staining Solution	Bio-Rad	1610436	
Dimethyl sulfoxide(DMSO)	Sigma-Aldrich	D2650	
GraphPad Prism	GraphPad Software	Version 6.0	statistical analysis and drawing software
Hydrochloric acid	Sigma-Aldrich	H1758	
ImageJ	National Institutes of Health	Version 1.52	image processing and analysis software
M-PER Cell Lysis Reagent	Thermo Fisher	78501	
Phosphate-buffered saline (PBS)	Corning	R21-040-CV	
Pronase	Roche	PRON-RO	10 mg/ml
Sodium chloride	Sigma-Aldrich	S7653	
Sodium fluoride	Sigma-Aldrich	S7920	
Sodium orthovanadate	Sigma-Aldrich	450243	
Sodium pyrophosphate	Sigma-Aldrich	221368	
Trizma base	Sigma-Aldrich	T1503	adjust to pH 8.0
β-glycerophosphate	Sigma-Aldrich	G9422	

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Signature:	
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Dear Dr. DSouza,

Again, thank you for the opportunity to further revise our manuscript. We have revised our manuscript according to reviewer's comments. Point by point responses to the reviewers' comments are described in detailed below.

Reviewer 2#

Response: We apologize for the typo, which has been corrected in the revision (Figure 4A).

Reviewer 3#

Response: We thank reviewer for the insightful point! Yes, internal control GAPDH is quite resistant to the protease used. We routinely used GAPDH as an internal control, and found that GAPDH exhibited negligible or slight reduction in response to the addition of protease. Figure 4A shows one representative experiment. Our findings were also consistent with the literature that first reported the DARTS method (doi: 10.1073/pnas.0910040106). From the DARTS experiments with various small molecules that we have tested, we observed that larger proteins tend to be more sensitive to proteolysis, which may be explained by the increased number of flexible regions across the full length of the protein and/or the increased number of peptide bonds (protease substrates). On the other hand, many small proteins (e.g. GAPDH),

especially those consisting of a single domain, are relatively resistant to proteolysis and therefore require more protease or increased digestion time. Regardless of this variability in susceptibility to proteolysis among different proteins, protection of the target protein(s) can be seen across a range of protease concentrations in which the target is partially or fully digested in the vehicle-treated control.

Reviewer 4#

Response: 1) In this paper, we highlighted the differences and advantages of this method from the classical DARTS method in detail (line 249-267). The objective of this study is to establish a semi-quantitative DARTS assay and to demonstrate the capacity of this modified DARTS approach in studying the small compounds/target proteins interactions. In this study, we present a significant improvement to the original DARTS approach by monitoring the changes in target protein stability with small molecule and characterizing the small molecule/protein binding affinity. Specifically, using the optimized concentration of pronase, the proteolysis carried out in the presence of different concentrations of the small molecule provides an indirect measure of the affinity of the small molecule with its target protein. We choose the classic rapamycin/mTOR interaction as an example as it is well characterized and results are easily reproducible.

2) The DARTS approach is aimed to identify the interaction between small molecules and proteins, not for the interactions between peptide ligands and proteins. DARTS leverages the thermodynamic stabilization of the target protein that occurs upon

small molecule binding by detecting the binding-induced increase in resistance to proteolysis.

3) Done as suggested. Corrections have been made in the revised version (line 88, 91-94). Other lysis buffers with various detergents (e.g. Triton X-100 or NP-40) can be used with DARTS as long as they are non-denaturing. Membrane proteins or nuclear proteins can be extracted by adding 0.4% of Triton X-100 or 0.4% NP-40 to the cell lysate.

4) As suggested, we have added additional information requested. Corrections have been made in the revised version (line 120-122). 2.2. Weigh enough of the small molecule to make a beginning stock concentration of 10 mM. When performing DARTS, one may begin with a higher concentration of the small molecule (5-10X the EC50 value) to ensure optimal binding.

We hope that the revised version of the manuscript is now acceptable for publication.

Yours sincerely,

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