# Supporting Information

## Selective ATP-competitive inhibitors of TOR suppress rapamycin insensitive function of TORC2 in S. *cerevisiae*

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### Materials and Methods

Drugs QL-IX-55, QL-VIII-86, and Torin1 were prepared in lab following previously reported procedures.(*1*,*2*). AZD8055, KU63796, PP242, WYE354 were purchased from Haoyuan Chemexpress (Shanghai, China). GFP-4EBP1 was purchased from Invitrogen. Antibody of pYpk1 was generated in Ted powers' Lab in UCSD, USA.

### Strains

Yeast *Saccharomyces cerevisiae* RDY98 (*MATa, erg6 Δ TRP1$^{cg}$, pdr1 Δ KAN, pdr3 Δ HIS5+, ade2, trp1, his3, leu2, ura3, can1*) and RDY84 (*MATa, pdr1 Δ KAN, pdr3 Δ*

*HIS5+, ade2, trp1, his3, leu2, ura3, can1)* were obtained from Russell Dorer (Harvard University). Both strains are in W303 background. Yeast strain SH121 and SH121 harboring pRS314[$^{HA}$*TOR*2] plasmid were obtained from Yoshinori Ohsumi (National Institute for Basic Biology, Japan).

**Drug induced mutagenesis**

RDY84 cells were grown in 10 ml of YPD liquid medium at 30°C to mid-log phase ($A_{600}$=1.0) and then diluted to $A_{600}$=0.05 using fresh medium containing 200 nM of QL-IX-55 for 2 days incubation. A couple of cycles later, 200 μl of culture was plated onto a YPD agar plate containing 300 nM of XL-IX-55 for 3 days until colonies appeared. 72 Colonies were picked and streaked on a fresh YPD agar plate containing 300 nM of QL-IX-55. Single colonies were picked and resuspended in 50~100 μl of 0.2% SDS, boiled for 5 min and pelleted by centrifugation at 14,000rpm for 1 min. One μl of supernatant was used as a template for PCR. The ATP pocket region in TOR1 was amplified with primers 5'- GACAAAAAGCGGCTCTTTCA-3' and 5'- TGCCCCCTTCCTTAA TAATTC; the ATP pocket region in TOR2 was amplified with primers 5'-CAACATGTGTCGCCAAAACT-3' and 5'-TCCACTCACTTCCATTGCAT-3'. Sequencing results indicated that (clone?) No.16 harbored the double mutations I2290S/K2293I.

**Generation of yeast mutants**

Homologous recombination was used for generation of yeast mutants.(*3*) A fragment of TOR2 was amplified using total DNA of yeast mutant bearing I2290S/K2293I in TOR2

as template by the following oligonucleotides: TOR2-6259 (5'-GGGCTGCAGGGTGGAAAACCAATTGTTAAAATATC-3') and TOR2-ds (5'-GGGAATTCGCATTTTTATACAACACTTTTACAGGC-3') and then inserted into the PstI-EcorI sites of YIplac211, which we designated plasmid YIplac211-TOR2d. The YIplac211-TOR2d construct was used to transform *Escherichia coli* DH5α and plasmid DNA was isolated by mini-prep. The YIplac211-TOR2d DNA was digested by KpnI and used to transform RDY98 using a LiAc method and transformants were selected on an SCD minus Ura solid agar plate. The colonies were picked and restreaked on 5-fluoroorotic acid (5-FOA) plate. The TOR2 DNA fragment was and sequenced to confirm the presence of the yeast TOR2 mutations. In addition, site-directed mutagenesis was used to construct plasmids YIplac211-TOR2/I2290s, YIplac211-TOR2/K2293I, YIplac211-TOR2/Y2166F, and YIplac211-TOR2/Y2166F/I2290 using Genscript.

**Yeast growth inhibition assay**

One ml of yeast overnight culture was inoculated into a flask containing 100 ml of fresh YPD medium and then incubated with shaking at 250 rpm at 30°C to mid-log phase. The cells were harvested by centrifugation and diluted to $A_{600}$=0.05, and then 30 μl of the culture was seeded in 384-well plates per well using a Matrix WellMate. 300 nl of compounds or DMSO (carrier) were transferred by robotics to the assay plates using stainless steel pin arrays. The assay plates were incubated at 30°C for 16~20 hours and $OD_{600}$ was measured using an EnVision plate reader

**mTORC1 in Vitro Kinase Assay**

mTORC1 was obtained from HEK-293T cell lines that stably express N-terminally FLAG-tagged Raptor and was purified as previously reported.[4] Kinase assay was performed using Lanthascreen assay kit (Invitrogen). Serially diluted compounds were transferred using a pin-transfer system to mTORC1 in kinase buffer (25 mM HEPES, pH 7.4, 6 mM $MnCl_2$, and 8 mM $MgCl_2$) in a 384-well low-volume assay plate (Corning). After 30 min incubation, an equal volume of substrate mixture (0.6 μM GFP-4EBP1, 20 μM ATP, and 8 mM DTT) in the kinase buffer was added and incubated for 80 min at room temperature. Reactions were stopped by the addition of the same volume of stop buffer containing Tb-labeled p-4EBP1 (T37) antibody (3 nM) and 30 mM EDTA. After 30 min incubation, FRET signal between GFP and Tb was detected using Envision plate reader (PerkinElmer) and the IC50's were calculated using Prism4 software (GraphPad).

**TORC1 and TORC2 Kinase Assay**

TORC1 and TORC2 were purified from exponentially growing *KOG1-HA* (RL54-1b: TB50a *MATa leu2 ura3 trp1 his3*Δ *rme1 GAL+ HMLa KOG1-3HA::KanMX6*) and *AVO3-HA* (RL69-1c: TB50 *MATa leu2 ura3 trp1 his3*Δ *rme1 GAL+ HMLa AVO1-3HA::KanMX4*) cells, respectively. Cells were grown at 30°C in YPD to mid-log phase. Cells were harvested by centrifugation, washed with $H_2O$, and resuspended in lysis buffer (1× PBS pH 7, 10% glycerol, 0.5% Tween 20, protease inhibitor cocktails (Roche), 1 mM PMSF and phosphatase inhibitors). Equal volume of glass beads was added, and the cells were disrupted in a Fast Prep machine at 4°C (FastPrep-120: 7× 30 s at max. speed). Crude lysates were cleared at 1000 × g for 5 min, and the extracts were precleared over

Protein A Sepharose. Protein concentrations were measured by Bradford method. Equal amounts of proteins were incubated with Protein A sepharose crosslinked with mouse monoclonal HA antibody for 2 hr at 4 ºC. Beads were washed 6 times with lysis buffer (without protease inhibitors). Inhibitors were tested using 8-fold serial dilutions from 100 µM to 10 nM. Kinase reactions were performed in kinase buffer (1× PBS, 20% glycerol, 0.5% Tween 20, 4 mM $MnCl_2$, 10 mM DTT, protease inhibitor cocktails [−EDTA] (Roche), phosphatase inhibitors) in a final volume of 30 µl containing 20 µg of PHAS-I (4EBP1). Kinase reactions were started with the addition of 80 µM ATP and 5 µCi [γ-$^{32}$P] ATP, shaken 15 min at 30°C, and terminated by cooling on ice. 23 µL of the supernatants were transfer to Filter P18 (Whatman). The filters were washed 6 times with 0.75% phosphoric acid, and incorporation of $^{32}$P was measured by Liquid-Scintillation Analyzer. $IC_{50}$ values were determined by fitting the data to a sigmoid dose-response-curve using the BioDataFit 1.02 software package.

**ATP competition assay**

The HA-TOR2 protein expression and isolation was performed by a modified version of the procedure described by Kamada et al.(*5*) SH121cell harboring pRS314[$^{HA}$*TOR2*] plasmid and pRS314[$^{HA}$*TOR2 mutant*] grown in SCD minus Ura medium were collected, washed once with distilled water, and suspended in ice-cold TOR lysis buffer (10 µl/optical density at 600 nm cell) (1x phosphate-buffered saline [PBS] [pH 7.4], 10% glycerol, 4 mM $Na_3VO_4$, 50 mM KF, 15 mM Na-PPi [pH 7.5], 15 mM *p*-nitrophenylphosphate [*p*NPP], 1X PhsoSTOP (Roche), and protease inhibitor cocktail (Sigma)). An equal volume of glass beads (425 to 600 µm) was added to this suspension, and cells were broken by vigorous vortexing using Bullet Blender® Homogenizerfor for

10 min at 4°C. A half volume of lysis buffer containing 2% Tween 20 was added, and the mixture was further incubated for 10 min with mild rotation. The beads and cell debris were removed by centrifugation for 10 min at 10,000 x *g* at 4°C, and the supernatant was further clarified by an additional 10-min centrifugation. For immunoprecipitation of $^{HA}$Tor2 protein, 2 mg of cell lysate was brought up to 1 ml with immunoprecipitation buffer (TOR lysis buffer containing 0.5% Tween 20 and 0.25% gelatin) and incubated with 10 µl of Monoclonal Anti-HA-Agarose (Sigma) for 2 h at 4°C with gentle rotation. Immunocomplexes were transferred into fresh microcentrifuge tubes and washed four times with 1 ml of immunoprecipitation buffer (without gelatin) with gentle rotation and twice with 1 ml of 25 mM HEPES-KOH (pH 7.5). The resultant immunocomplexes were resuspended in 27 µl of TOR kinase assay buffer (25 mM HEPES-KOH [pH 7.5], 50 mM NaCl, 10 mM $MnCl_2$, 15 mM *p*NPP) containing 4 µg of substrates (4E-BP1-GFP [Invitrogen]) and 100 nM of QL-IX-55. This mixture was preincubated for 30 min at 30°C before the reaction was initiated by adding 3 µl of different amount of ATP (ATP final concentration is 0, 10, 100, 500, 1000, 5000, and 10000 respectively). After incubation for 60 min at 30°C, the reaction was terminated by addition of 10 µl of 4x sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer and incubation for 5 min at 95°C. Samples were subjected to 7.5% SDS-PAGE gels and analyzed by immunoblotting using Phospho-Ser-65 4E-Bp1 (Cell Signaling Technology). The membrane was then stripped and re-immunoblotted using 4E-Bp1 antibody (Cell Signaling Technology).

**Colonogenic assay and Halo assay**

Colonigenic Assay. Drug resistance profiles of RDY98 (TOR2-WT) and *TOR2* mutant cells determined by a spot assay. For this assay, 10 µl cells were spotted in a fivefold dilution series from left to right (starting $A_{600}$ =0.8) on YEPD plates in the presence of QL-XI-55. Growth differences were recorded following incubation of the plates for 48 h at 30°C.

Halo assay. Yeast was grown in YPD medium overnight. ~10 µl of the culture (equal cell number) was added to 2 ml of 2× YPD, and then 2 ml of 1% agar (cooled to 50°C) was added and the cell suspension was poured onto the surface of a YPD plate. Dilutions of the drug (or carrier) were made into DMSO and 12.5 µl of each dilution of the drug was pipetted onto the center of a sterile 6-mm filter disk (Whatman). The disk was then placed on the top agar and the plates were incubated at 30 °C for 2–3 days until halos were clearly visible.

**Northern Blot Analysis**

Procedures were conducted essentially as described.(*6*) Briefly, 10-ml cultures were grown to mid log phase ($A_{600}$ = 0.5) and harvested by centrifugation, and total RNA was collected and run on a 1.5% agarose/6.9% formaldehyde gel. The RNA was transferred to a Duralon-UV membrane, and probed overnight at 65°C with DNA probes that target specified RNAs. After washing, membranes were exposed to a phosphorimaging screen, and analyzed using a Storm 860 imaging system (GE Healthcare).

**Western Blot Analysis**

Protein extracts were prepared using a NaOH cell lysis method (*7*). Briefly, cells were lysed in 0.255 M NaOH, 1% 2-mercaptoethanol, and proteins were precipitated by treatment with trichloroacetic acid at final concentration of 6.1%. The protein pellet was then washed with 1 M Tris-HCl, pH 6.8, and resuspended in SDS-PAGE sample buffer (65 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 100 mM dithiothreitol, 0.002% bromophenol blue). Samples were loaded onto SDS-PAGE gels and transferred to nitrocellulose membrane. Membranes were probed with primary antibodies α-HA (12CA5, 1:5000; Covance), α-phospho-Ypk1 that recognizes the phosphorylated hydrophobic motif (HM) position T662 in Ypk1 (1:20,000), and α-G6PDH (1:100,000; Sigma-Aldrich). Membranes were then probed with appropriate secondary antibodies conjugated to IRDye (1:5000; LI-COR Biosciences) and visualized using an Odyssey Infrared Imaging System (LI-COR Biosciences). Images were quantified using ImageQuant software (GE Healthcare).

**Actin-staining and Fluorescence Microscopy**

Actin-staining and detection in yeast cells was performed as described.(*8*) Briefly, cells were treated with the appropriate inhibitor for the time indicated, fixed and stained with rhodamine-phalloidin. Fluorescence microscopy was performed using a Nikon E600 fluorescent microscope and an Orca ER charge-coupled device camera (Hamamatsu) controlled by SimplePCI software. All images are Z series projections.

**Protein kinase assay of TOR2 protein**

This assay was performed by a modified version of the procedure described by Kamada et al. (*5*)

The HA-TOR2 protein expression and isolation method was showed in " ATP competition assay". After immunoprecipatation, the isolated complexes were transferred into fresh microcentrifuge tubes and washed four times with 1 ml of immunoprecipitation buffer (without gelatin) with gentle rotation and twice with 1 ml of 25 mM HEPES-KOH (pH 7.5). The resultant immunocomplexes were resuspended in 26.5 µl of TOR kinase assay buffer (25 mM HEPES-KOH [pH 7.5], 50 mM NaCl, 10 mM $MnCl_2$, 15 mM *p*NPP) containing 4 µg of substrates (4E-BP1-GFP [Invitrogen]). 1 µl of drug was added. This mixture was preincubated for 30 min at 30°C before the reaction was initiated by adding 3 µl of 1 mM of ATP and 0.5 µl of 10 µCi/ul [$\Upsilon$-$^{32}$P] ATP (PerkinElmer). After incubation for 60 min at 30°C, the reaction was terminated by addition of 10 µl of 4x SDS-PAGE sample buffer and incubation for 5 min at 95°C. Samples were subjected to 7.5% SDS-PAGE gels. After electrophoresis, gels were immersed in 12.5% trichloroacetic acid for 30 min, followed by several washes in distilled water. Proteins were detected by Coomassie staining (GelCode Blue; Pierce). Phosphorylated proteins were detected by autoradiography.

**Reference:**


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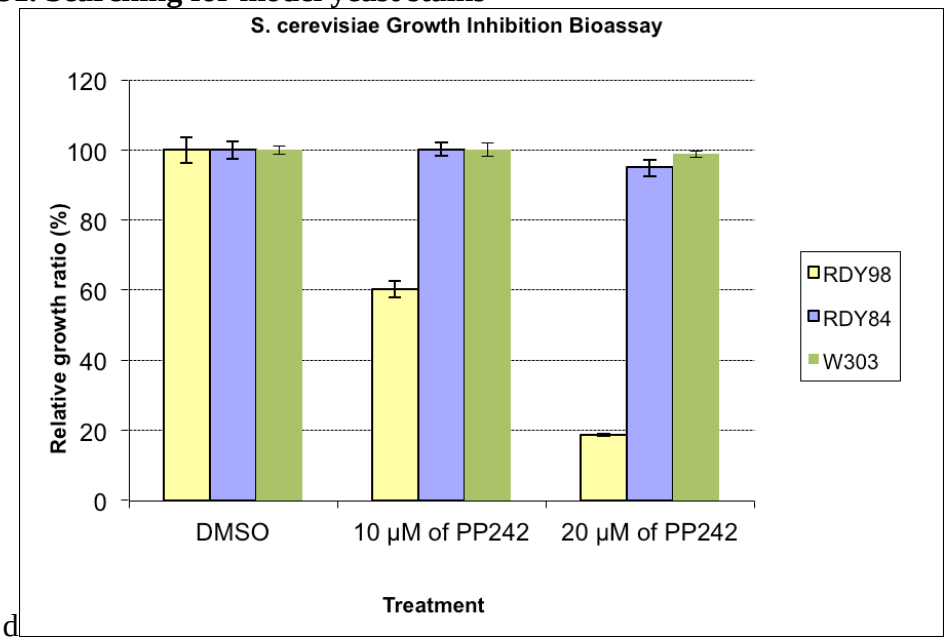
## Figures and Legends

**Figure S1. Searching for model yeast stains**

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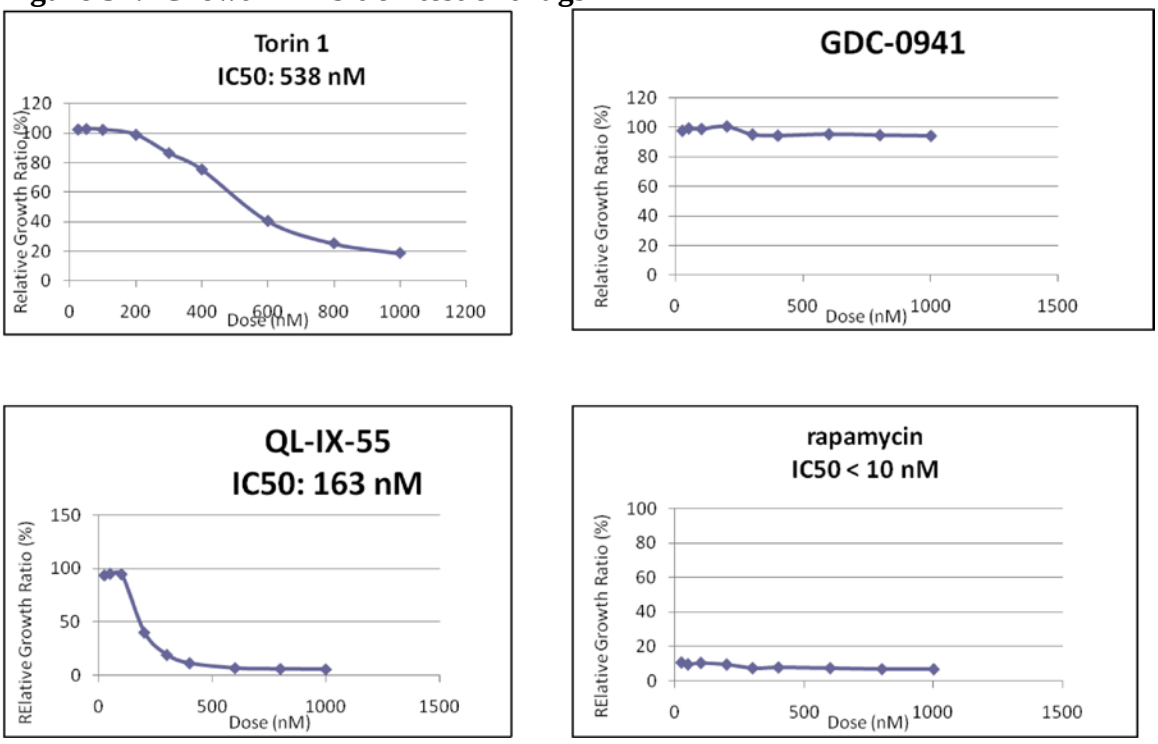
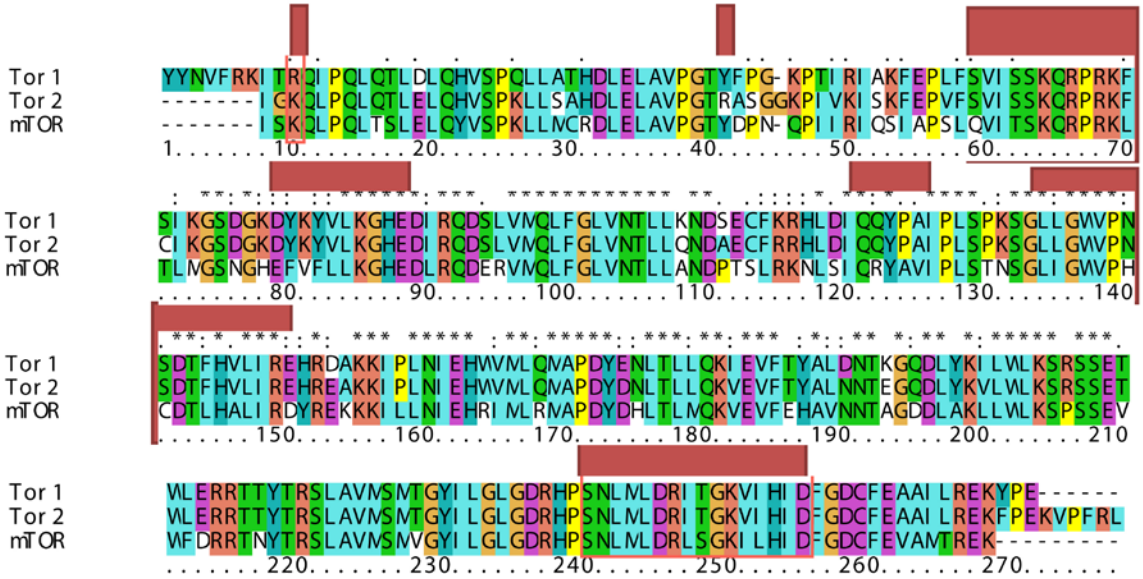
Yeast growth inhibition assay was performed in 384 well plates. $OD_{600}$ was measured at 18 hours after addition of different concentration of small molecules.

**Figure S2: Growth inhibition test of drugs**




Overnight cultures of RDY98 was transferred into fresh medium for 6 hours incubation until OD600 reached 0.8. Cells were seeded into 384 well plates (starting $A_{600}$=0.1) and small molecules were transferred into wells in plates in different final concentration as shown in the figures. Relative growth ration was calculated using DMSO treated cells as positive control.

**Figure S3. Sequence alignment of TOR1/2 and mTOR**




Sequence alignment was done by Clustalx software(V2) and the different colors is used to hightlight the sequece similarity. Red color bars above each row highlited the amino acids residues involved in forming ATP binding pocket based on the molecule modeling.

**Table S1. Growth inhibition assay of mTOR inhibitors**

| Drugs | 75 μM (% growth) | 25 μM (% growth | 5 μM (% growth | 1 μM (% growth |
|---|---|---|---|---|
| Torin1 | 6.8 | 6.5 | 5.5 | 5 |
| PP242 | 2.3 | 3.7 | 95 | 98 |
| AZD8055 | 18.4 | 77.8 | 97.6 | 100 |
| KU63794 | 16.7 | 82.1 | 100 | 100 |
| WYE354 | 11.5 | 70.1 | 100 | 100 |

**Table S3: Tor2 mutations resistant to QL-IX-55 treatment.**

| Yeast | RDY98(WT) | I2290S/K2293I | Y1266F | I2290S | Y2166F/I2290S |
|---|---|---|---|---|---|
| $GI_{50}$(nM) | 180 | 773 | 656 | 864 | 1602 |
| $GI_{50}$ ratio | 1 | 4.29 | 3.64 | 4.8 | 8.9 |