

Chapter 2

Biochemical and Pharmacological Inhibition of mTOR by Rapamycin and an ATP-Competitive mTOR Inhibitor

Ker Yu and Lourdes Toral-Barza

Abstract

The mammalian target of rapamycin (mTOR) is the catalytic subunit of two multiprotein complexes, mTOR complex-1 (mTORC1) and mTOR complex-2 (mTORC2). Clinically used rapamycin and rapalogs are FKBP12-dependent allosteric inhibitors of mTORC1. The recently discovered WYE-125132 and related drugs represent a new generation of ATP competitive and highly specific inhibitors targeting mTOR globally. As mTORC1 and mTORC2 mediate diverse sets of both redundant and distinctive cellular pathways of growth, nutrient and energy homeostasis, rapamycin and WYE-125132 elicit both overlapping and distinctive pharmacological properties with important implications in treating cancer, metabolic, and age-related degenerative diseases. Detailed methods are described for the determination of mTOR inhibition by rapamycin and WYE-125132 in assays of recombinant mTOR enzyme, immunoprecipitated native mTOR complexes, growth factor- and amino acid-induced cellular phosphorylation cascades as well as the PI3K/AKT/mTOR hyperactive breast tumor model *in vitro* and *in vivo*. The methods have been particularly useful in discovery and biochemical characterization of mTOR inhibitors, cellular and *in vivo* mTOR substrate phosphorylation analysis, and in deciphering novel biomarkers of mTORC1 and mTORC2 signaling pathways.

Key words: mTOR, Rapamycin, WYE-125132, mTOR inhibitor, DELFIA

1. Introduction

Mammalian target of rapamycin (mTOR) is the founding member of a family of unconventional phosphoinositide-3-kinase (PI3K)-related kinases (PIKKs) that are uniquely large molecular weight (>250 kDa) serine/threonine kinases with catalytic sites homologous to those of PI3K but different from those of the conventional protein kinases (1, 2). mTOR resides in at least two functional multiprotein complexes, mTOR complex-1 (mTORC1) and mTOR complex-2 (mTORC2), mediating diverse signals from

growth factors, nutrients, and energy supply (3, 4). mTORC1 is well known for its critical roles in protein synthesis and growth, which involves phosphorylation of the ribosomal protein S6 kinase 1 (S6K1) and the eukaryotic translation initiation factor eIF4E-binding protein 1 (4EBP1). mTORC2 phosphorylates and activates AKT, a key regulator of cell growth, metabolism, and survival. For more than a decade we have relied largely on the use of rapamycin to study mTOR function but it now appears that rapamycin is a partial inhibitor of mTOR through allosteric inhibition of mTORC1 but not mTORC2. Recent development of highly potent and specific active-site mTOR inhibitors, such as WYE-125132 (5), not only offers new therapeutic potentials but also provides invaluable tools for deciphering novel insights into the signaling network of mTOR complexes (6, 7).

Traditionally, biochemical assessment of mTOR kinase activity has been challenging due to its exceedingly large molecular weight and intricate multiprotein complexes that are often sensitive to buffer conditions. Employing a truncated and catalytically active form of mTOR expressed in and affinity-purified from HEK293 cells, we illustrate a high-capacity Dissociation-Enhanced Lanthanide Fluorescence Immunoassay (DELFI) for the determination of inhibition kinetic mechanism of rapamycin and WYE-125132. Native mTORC1 and mTORC2 are immunoprecipitated from cytoplasmic extracts and it is shown that mTORC1-specific substrate phosphorylation of S6K1 and mTORC2-specific phosphorylation of AKT are differentially inhibited by rapamycin and WYE-125132. Likewise in cellular models, signaling biomarkers of mTORCs induced by insulin-like growth factor 1 (IGF-1), amino acids, or by PI3K hyperactivation in breast cancer cells are only partially inhibited by rapamycin but are globally targeted by WYE-125132.

2. Materials

2.1. Assay of Recombinant mTOR via Dissociation- Enhanced Lanthanide Fluorescence Immunoassay

1. The FLAG-tagged recombinant mTOR enzyme and the kinase assay substrate His6-S6K1 are obtained as described (8) and stored in -80°C freezer.
2. Assay buffer: 10 mM HEPES (pH 7.4), 50 mM NaCl, 50 mM β -glycerophosphate, 10 mM MnCl_2 , 0.5 mM dithiothreitol (DTT), 0.25 μM Microcystin LR, and 50 $\mu\text{g}/\text{mL}$ bovine serum albumin (BSA) (see Note 1).
3. Adenosine-5'-triphosphate (ATP): 20 mM stock in distilled water and store in -20°C in small single use aliquots.
4. Inhibitor chemicals: Rapamycin and WYE-125132 (Wyeth Research) dissolved in dimethyl sulfoxide (DMSO) as 20 mM

stock solutions. His6-FKBP12 is obtained as described (8) or equivalent.

5. Reaction stop buffer: 20 mM HEPES (pH 7.4), 20 mM ethylenediaminetetraacetic acid (EDTA), and 20 mM ethylene glycol tetraacetic acid (EGTA).
6. Inhibitor dilution plate: Polypropylene 96-well round bottom plate (Costar #3359).
7. Assay plate (for carrying kinase reaction): Nontreated 96-well round bottom polystyrene plate (Costar #3795), plate adhesive film (VWR), and plate shaker (Lab-Line Instruments).
8. DELFIA detection plate: MaxiSorp plate 96-well (Nunc #44-2404-21).
9. Dulbecco's phosphate-buffered saline without calcium and magnesium (DPBS, Mediatech).
10. DELFIA assay buffer (PerkinElmer).
11. Eu-P(T389)-S6K1 antibody: P(T389)-S6K1 polyclonal antibody labeled with europium-N1-ITC (Eu) (Cell Signaling, PerkinElmer). Dilute Eu-P(T389)-S6K1 antibody to 40 ng/mL with DELFIA assay buffer.
12. DELFIA wash buffer: DPBS with 0.05% Tween-20 (Bio-Rad).
13. DELFIA enhancement solution (PerkinElmer).
14. VICTOR Model Plate reader (PerkinElmer).

2.2. Immune-Complex Kinase Assay of mTOR complexes

1. HEK293 cells from American Type Culture Collection (ATCC).
2. Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 µg/mL penicillin, and 50 µg/mL streptomycin (Gibco/Invitrogen).
3. Cytosolic fraction extraction buffer: 5 mM HEPES (pH 7.4), 5 mM β-glycerophosphate, 100 µg/mL digitonin, 1 mM DTT, 1 mM MgCl₂, 50 nM Microcystin LR, 1× protease inhibitor cocktail (Roche).
4. Immunoprecipitation (IP) antibody: Anti-FRAP (N19) goat antibody and control goat IgG (Santa Cruz Biotechnology).
5. Agarose beads: Protein A/G PLUS-Agarose (Santa Cruz Biotechnology).
6. Wash buffer: extraction buffer supplemented with 50 mM NaCl and 0.5% NP40 (Roche).
7. Assay buffer (see Subheading 2.1, item 2).
8. Inhibitor chemicals: WYE-125132 and CCI-779 (Wyeth Research) dissolved in DMSO as 20 mM stock solutions, His6-FKBP12 (8) or equivalent.

9. Substrate mix: dilute the His6-S6K1 to 0.6 μ M and the inactive His6-AKT1 to 0.34 μ M in assay buffer.
10. ATP mix: dilute ATP to 200 μ M in assay buffer.
11. NuPAGE LDS sample buffer (4 \times) (Invitrogen) to be used as kinase reaction stop buffer.
12. SDS-PAGE and Western blotting system: All materials for SDS-PAGE and Western blotting are purchased from Invitrogen unless otherwise specified (see Note 2).
13. NuPAGE Sample Reducing Agent.
14. Precast gels in a 26-well midi format, 4–12% NuPAGE Bis–Tris and 3–8% NuPAGE Tris–acetate.
15. Running buffer: NuPAGE MOPS SDS (20 \times) and NuPAGE Tris–acetate SDS (20 \times). Dilute to 1 \times with distilled water.
16. Molecular weight markers: dilute 100 μ L Sharp Pre-Stained Protein Standard 90 μ L 1 \times sample buffer and add 10 μ L MagicMark XP Western Standard.
17. Transfer buffer: 2 \times NuPAGE transfer buffer, 0.2% NuPAGE Antioxidant, and 20% methanol.
18. Transfer system: iBlot Dry Blotting System and iBlot Gel Transfer Stacks in nitrocellulose or comparable manual transferring unit.
19. Membrane wash buffer: TBS-T (Tris-buffered saline). Dilute 100 mL of 10 \times TBS (Bio-Rad) with 900 mL distilled water plus 1 mL Tween-20.
20. Blocking buffer: 5% nonfat dry milk (Bio-Rad) in TBS-T.
21. Primary antibodies: mTOR, Raptor, P-S6K1(T389), AKT, P-AKT (S473) (Cell Signaling), Rictor (Novus Biologicals), Anti-polyhistidine (clone-1) (Sigma).
22. Dilution buffer for primary antibodies: 3% BSA in TBS-T supplemented with 0.05% sodium azide.
23. Secondary antibodies: goat antirabbit IgG-HRP and goat antimouse IgG-HRP (Santa Cruz Biotechnology).
24. Detection: Enhanced chemiluminescent (ECL) reagents and Amersham Hyperfilm for ECL (GE Healthcare).

2.3. Analysis of mTOR Activity in Cultured Cells

1. Rat1 and HEK293 cells (ATCC) grown in DMEM growth medium (see Subheading 2.2, item 2).
2. MDA361 cells (ATCC), grown in Minimum Essential Medium (MEM) supplemented with 10% fetal bovine serum, 100 μ g/mL penicillin and 50 μ g/mL streptomycin, 1 mM Na pyruvate (Gibco/Invitrogen).
3. Low serum DMEM, replace FBS concentration to 0.1%.

4. Inhibitor chemicals WYE-125132, rapamycin, 17-hydroxy-wortmannin (Wyeth Research) prepared as 1,000× concentrate in DMSO.
5. Recombinant human IGF-1 (R&D Systems), reconstituted according to the manufacturers' recommendation. Store in aliquots at -80°C .
6. Amino acid (AA)-free medium: Earle's balanced salt solution (EBSS) 10×, MEM vitamins (100×), MEM nonessential amino acids (NEAA) (100×), 7.5% sodium bicarbonate, 45% D(+)-glucose solution, dialyzed FBS. To make 100 mL of AA-free medium: 10 mL EBSS, 1 mL vitamins, 1 mL NEAA, 2.7 mL sodium bicarbonate, 1 mL glucose, 10 mL FBS and bring to 100 mL with distilled water. Filter sterilized.
7. Amino acid solution (50×) (Sigma #M5550).
8. Cell lysis buffer: NuPAGE LDS sample buffer (4×) diluted to 1.1× with distilled water and stored at room temperature.
9. Protein concentration determination: Bio-Rad RC/DC protein Assay.
10. SDS-PAGE (see Subheading 2.2, items 13–16).
11. Total protein staining Ponceau S Solution (Sigma).
12. Western blotting (see Subheading 2.2, items 17–24).
13. Additional primary antibody: P-AKT (T308), AKT, FKHL1, P-FKHL1 (T32), GSK3, P-GSK3 (S21/9) (Cell Signaling).

**2.4. mTOR inhibition
and antitumor activity
by WYE-125132
against Tumor
Xenografts in Nude
Mice**

1. MDA361 cells (ATCC).
2. MDA361 xenograft tumors in female nude mice (5).
3. Formulation vehicle: 5% ethanol, 2% Tween-80, 5% polyethylene glycol (PEG)-400.
4. WYE-125132 (Wyeth Research).
5. Tumor lysis buffer: 25 mM HEPES, pH 7.5, 100 mM NaCl, 20 mM β -glycerophosphate, 1.5 mM MgCl_2 , 0.5 mM EGTA, 0.25 mM EDTA 1% NP-40, 10 mM Na_3VO_4 , 10 $\mu\text{g}/\text{mL}$ aprotinin, 10 $\mu\text{g}/\text{mL}$ leupeptin, 1 mM PMSF, 1 μM microcystin LR, and 0.1% 2-mercaptoethanol.
6. Probe homogenizer (Ultra-Turrax T8, IKA Labortechnik Staufen).
7. SDS-PAGE (see Subheading 2.2, items 13–16).
8. Western blotting (see Subheading 2.2, items 17–24).
9. Additional primary antibody: Cleave PARP (Cell Signaling) and β -actin (Chemicon International).

3. Methods

3.1. In Vitro Biochemical Assays of mTOR

3.1.1. Assay of Recombinant Flag-mTOR via DELFIA

For full details on the production of recombinant mTOR enzyme and substrate His6-S6K1, readers are referred to Toral-Barza et al. (8). The protocol assumes assays in 96-well plate format with reagent volumes intended for each assay well. The kinase assay and DELFIA detection protocols are carried out at room temperature.

1. Prepare inhibitor serial dilution in DMSO in the inhibitor dilution plate according to the desired dose range to be tested. In general, prepare inhibitors as 50× concentrate so that they will be diluted 50× in the final assay (see Note 3). Rapamycin serial dilution was prepared in DMSO, while FKBP12 was prepared in the assay buffer (this is to keep the concentration of DMSO in the assay at 2%).
2. Prepare enzyme mix: dilute FLAG-mTOR enzyme to 12 nM in assay buffer.
3. Prepare substrate-ATP mix: dilute the His6-S6K1 to 2.5 μM and ATP to 200 μM in assay buffer.
4. Dispense 12 μL enzyme mix to assay plate (see Note 4). The assay buffer alone is used as background control.
5. Add 0.5 μL of inhibitor or DMSO to assay plate (see Note 5). Seal the plate with adhesive film and incubate for 10 min on a plate shaker (500 rpm) to thoroughly mix enzyme and inhibitor.
6. Initiate kinase reaction by adding 12.5 μL of substrate-ATP mix. The reaction is continued for 2 h with shaking.
7. Stop kinase reaction by adding 25 μL of reaction stop buffer and return the plate to plate shaker for 10 min.
8. Dispense 60 μL DPBS to DELFIA detection plate. Add 40 μL of the stopped reaction mixture and let the peptide substrate to bind to the plate for 2 h on plate shaker.
9. Aspirate the binding mixture from the DELFIA plate and wash the plate (see Note 6) once with DPBS.
10. Dilute Eu-P(T389)-S6K1 antibody to 40 ng/mL with DELFIA assay buffer. Add 100 μL of Eu-P(T389)-S6K1 antibody. Incubate for 1 h on plate shaker.
11. Aspirate and wash the plate 4× with DELFIA wash buffer. Add 100 μL DELFIA enhancement solution and incubate for 30 min without shaking.
12. Measure DELFIA TF fluorescence in VICTOR Model Plate reader.
13. Calculate the inhibitor activity as percent of DMSO control with background (without enzyme) value subtracted.

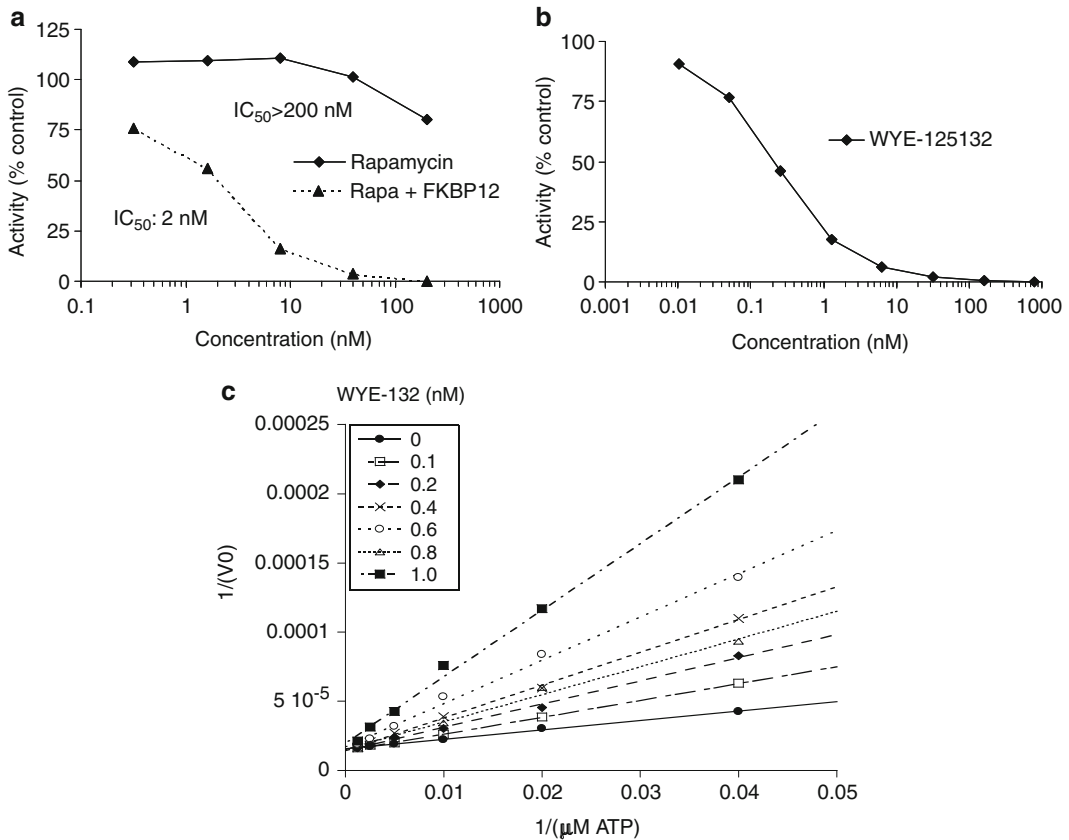


Fig. 1. *In vitro* kinase assay of recombinant mTOR by DELFIA. Dose-response curves of rapamycin alone, rapamycin plus FKBP12 (**a**), or an ATP-competitive inhibitor WYE-125132 (**b**). Note that rapamycin-FKBP12 complex but not rapamycin alone potently inhibits mTOR, whereas WYE-125132 alone inhibits mTOR. (**c**) WYE-125132 versus ATP matrix competition assay. The initial enzyme rates (V_0) against 0, 0.1, 0.2, 0.4, 0.6, 0.8, and 1 nM WYE-125132 at various concentrations of ATP were measured to generate the double-reciprocal plot (**c** reproduced from ref. 5 with permission from the American Cancer Society).

14. Determine the half maximal inhibitory concentration (IC_{50}) of the inhibitor by constructing inhibitor dose-response curves. Examples of the results produced are shown in Fig. 1a, b.

3.1.2. Inhibitor Versus ATP Matrix Assay

Follow methods in Subheading 3.1.1, steps 1–12 with modifications as follows.

1. Prepare inhibitors as 50× concentration of 0.5, 1, 2, 3, 4, and 5 times the inhibitor's IC_{50} value. For example, for an inhibitor with IC_{50} value of 2 nM, prepare 50× concentration of 1, 2, 4, 6, 8, and 10 nM.
2. Prepare enzyme mix: dilute FLAG-mTOR enzyme to 20 nM in assay buffer.

3. Prepare substrate–ATP mix: dilute His6-S6K1 to 2.5 μM with varying concentrations of ATP (50, 100, 200, 400, 800, and 1,600 μM) in assay buffer.
4. Reduce kinase reaction time to 30 min.
5. Transfer 15 μL of the stopped reaction mix to DELFIA detection plate with predispensed 85 μL DPBS.
6. Generate a Lineweaver–Burk analysis by plotting the reciprocal of the initial enzyme rate (V_0) versus the reciprocal of ATP. An example of results produced is shown in Fig. 1c.

3.2. Immune-Complex Kinase Assay of mTORC1 and mTORC2

1. HEK293 cells were grown in 150 mm culture dish until 80% confluence. Cells are freshly fed 1 day before experiment.
2. Prepare cell extraction buffer and keep on ice.
3. Aspirate growth medium from plate and wash 2 \times with cold DPBS and place plate on ice.
4. Add 3 mL cell extraction buffer to the plate and incubate for 15 min.
5. Collect the supernatant (cytosolic extract) and centrifuge it for 5 min at 1,000 rpm to remove cell debris.
6. Carefully transfer the extracted protein into a new tube and determine the protein concentration by the Bradford method (Bio-Rad).
7. To immunoprecipitate (IP) mTOR complexes, prepare a 1 mg/mL protein extract in a microcentrifuge tube and supplement with NaCl to final 50 mM. Add 2 μg anti-FRAP/mTOR (N19) or control IgG and incubate for 1.5 h while rocking at 4°C. Add 20 μL of a 50% slurry of agarose beads and incubate for 2 additional hours at 4°C.
8. Spin down the beads at 3,000 rpm for 5 min. Wash the beads 3 \times with 0.5 mL wash buffer and once with 0.5 mL assay buffer. Remove as much of the assay buffer without disturbing the beads.
9. Resuspend the beads in 24 μL substrate mix and add 1 μL of 50 \times inhibitor. Preincubate for 10 min at 25°C with shaking in a microcentrifuge mixer (see Note 7).
10. Initiate kinase reaction by adding 25 μL ATP mix. Incubate the reaction for 1 h at 25°C in a microcentrifuge mixer.
11. Stop the reaction by adding 17 μL 4 \times LDS sample buffer and proceed with SDS-PAGE (see Note 8).
12. Add 7 μL 10 \times reducing agent and denature samples at 70°C for 10 min.
13. Spin the sample for 5 s at 14,000 rpm and transfer the supernatant to a new microcentrifuge tube.

14. Run 5 μL of molecular weight markers and samples in a 26-well 3–8% Tris–acetate gels using Tris–acetate SDS running buffer to resolve mTOR, Rictor, and Raptor. His6-AKT and His6-S6K1 are separated in a 26-well 4–12% gels using MOPS SDS running buffer.
15. Remove gel from the cassette and soak in the transfer buffer for 5 min on an orbital shaker.
16. Place the gel on top of the iBlot nitrocellulose anode stack. Soak an iBlot filter paper in the transfer buffer and place it on top of the gel. Remove any trapped air bubbles by using a roller. Then place the iBlot cathode stack on top and remove air bubbles.
17. Transfer using the iBlot dry blotting system (see Note 9).
18. Incubate the membrane in 50 mL blocking buffer for 1 h in a rocking platform at room temperature.
19. Discard the blocking buffer and rinse membrane 3 \times for 2 min each with TBS-T. Incubate the membrane with various primary antibodies of mTOR, Rictor, Raptor, P-S6K1 (T389), P-AKT (S473), and His6 (1:1,000 dilution) overnight on a rocking platform at 4°C.
20. Remove the primary antibody and wash the membrane 3 \times for 5 min each with TBS-T. Add the secondary antibody diluted to 1:5,000 in blocking buffer and incubate for 1 h at room temperature on a rocking platform.
21. Discard the secondary antibody and wash 3 \times for 5 min each with TBS-T and 2 \times with TBS only.
22. Prepare the ECL reagent in just enough volume to completely cover the membrane. Discard the final wash and add the ECL reagent and incubate for 1 min.
23. Place the membrane between sheets of Saran wrap and remove excess ECL reagent with a roller and Kim-Wipes. Tape the corners of the wrapped membrane in a film cassette.
24. Expose to ECL film from 1 s to several minutes to obtain desired exposure for each antigen. An example of the results produced is shown in Fig. 2.

3.3. Pharmacological Inhibition of mTOR in Cultured Cells

3.3.1. Inhibition of IGF-1-Induced mTOR Activation

1. Plate Rat1 cells in six-well culture dish in growth medium for 1–2 days.
2. Cells (60% confluence) are washed 1 \times with serum-free DMEM and incubated in 3 mL low serum DMEM for 24 h.
3. Serum-starved Rat1 cells are pretreated with 3 μL inhibitor (1,000 \times dilution from the prepared stocks) for 1 h followed by IGF-1 stimulation (100 ng/mL) for 30 min.
4. Cells are washed 1 \times with DPBS and lysed in 200 μL 1.1 \times NuPAGE LDS sample buffer followed by 1 min of water-bath

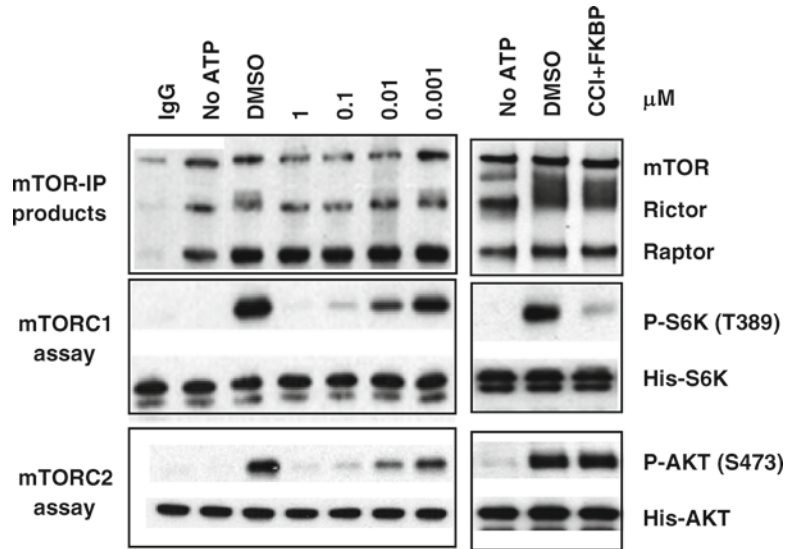


Fig. 2. Immune-complex kinase assay of mTORC1 and mTORC2. Native mTOR complexes immunoprecipitated from HEK293 cytosolic lysates were assayed for substrate phosphorylation of S6K (for mTORC1 activity) or AKT (for mTORC2 activity) without or with inhibitors. The assay products were immunoblotted. Note that CCI-779 (a rapamycin analog) in complex with FKBP12 only inhibits mTORC1, whereas WYE-125132 inhibits both mTORC1 and mTORC2 (the *left* and *right panels* reproduced from refs. 5 and 10, respectively, with permission from the American Cancer Society).

sonication in a cold room. Keep samples on ice or store at -80°C for later use.

5. Perform Western blotting (Subheading 3.2, steps 14–24) for P-S6K1(T389), P-AKT (S473), and P-AKT (T308). An example of the results produced is shown in Fig. 3a, left panel.

3.3.2. Inhibition of Amino Acid-Induced mTOR Activation

1. Plate HEK293 cells in six-well culture dish in growth medium and let cells to reach 70–80% confluence.
2. Very carefully wash the cells once with amino acid-free medium and incubate in 3 mL amino acid-free medium for 2 h.
3. Amino acid-starved cells are pretreated with 3 μL inhibitors (1,000 \times dilution from the prepared stocks) for 2 h. Cells are then stimulated with amino acids (add 60 μL of the 50 \times solution) for 1 h.
4. Prepare total cell lysate as described for the IGF-1 experiment.
5. Perform Western blotting (Subheading 3.2, steps 14–24) for P-S6K1(T389), P-AKT (S473), and P-AKT (T308). An example of the results produced is shown in Fig. 3a, right panel.

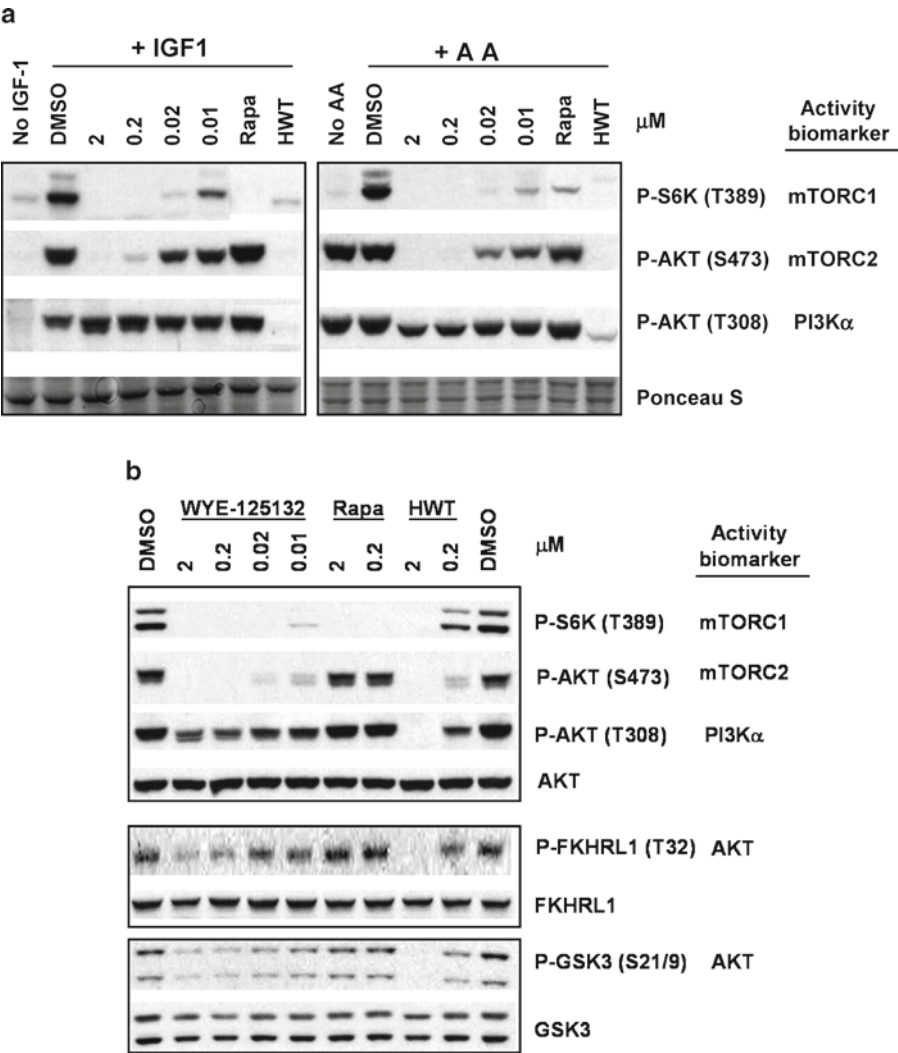


Fig. 3. Differential inhibition of mTORC1 and mTORC2 by rapamycin and WYE-125132 in cultured cells. (a) Inhibition of IGF1-induced mTOR activation in Rat1 cells (*left panel*) and inhibition of amino acid (AA)-induced mTOR activation in HEK293 cells (*right panel*). (b) Inhibition of mTOR signaling function in PI3K-hyperactive MDA361 breast cancer cells. Protein lysates were immunoblotted. Note that consistent with the targeting of mTORC1 and mTORC2, WYE-125132 inhibits cellular P-S6K (T389) and P-AKT (S473) as well as AKT downstream targets P-FKHRL1 (T32) and P-GSK3 (S21/9), whereas mTORC1-selective rapamycin only inhibits P-S6K (T389). Note also that WYE-125132 does not significantly inhibit the PI3K biomarker P-AKT (T308), whereas the PI3K inhibitor 17-hydroxywortmannin (HWT) inhibits P-AKT (T308) (*a, left panel* reproduced from ref. 5 with permission from the American Cancer Society).

**3.3.3. Inhibition of mTOR
in PI3K Pathway
Hyperactive MDA361
Breast Cancer Cells**

1. Plate MDA361 cells in six-well culture dish in growth medium (3 mL) for 24 h.
2. The cells (50–60% confluence) are treated with 3 μ L inhibitor (1,000 \times dilution from the prepared stocks) for 6 h in growth medium.
3. Prepare total cell lysate as described for the IGF-1 and amino acids experiments.
4. Perform Western blotting (Subheading 3.2, steps 14–24) for P-S6K1(T389), P-AKT (S473), P-AKT (T308), P-FKHRL1 (T32), FKHRL1, P-GSK3 (S21/9), and GSK3. An example of the results produced is shown in Fig. 3b.

**3.4. Inhibition of mTOR
in MDA361 Xenograft
Tumors and Antitumor
Efficacy by WYE-
125132**

**3.4.1. Inhibition of mTOR
Signaling Biomarkers in
MDA361 Xenograft Tumors**

1. Prepare MDA361 xenograft tumors in female nude mice as described (5).
2. Stage tumors when they reach approximately 400 mm³ and randomize them to treatment groups.
3. Formulate WYE-125132 in the specified vehicle.
4. Dose tumor-bearing mice ($n=3$) by a single oral injection with vehicle or 50 mg/kg WYE-125132.
5. Harvest and dissect tumors at 0, 3, 6, 12, 24, and 36 h after oral injection.
6. Quickly mince tumor tissues and lyse them in the specified tumor lysis buffer using a probe homogenizer (Ultra-Turrax T8, IKA Labortechnik Staufen).
7. Centrifuge the tumor lysate for 10 min at 14,000 rpm at 4°C.
8. Carefully transfer the clear lysate into a new tube and determine the protein concentration by the Bradford method (Bio-Rad).
9. Perform Western blotting (Subheading 3.2, steps 14–24) for P-S6K1(T389), P-AKT (S473), P-AKT (T308), AKT, cleaved PARP and β -actin. An example of the results produced is shown in Fig. 4a.

**3.4.2. Antitumor Efficacy
of WYE-125132**

1. Stage MDA361 tumors when they reach approximately 200 mm³ and randomize them to treatment groups.
2. Formulate WYE-125132 in the specified vehicle.
3. Dose tumor-bearing mice ($n=10$) with vehicle, 5, 10, and 20 mg/kg WYE-125132 orally via cycle dosing (one cycle consists of 5 days on and 2 days off) for four cycles.
4. Measure tumor volume twice a week using sliding vernier calipers and analyze tumor growth data (9). An example of results produced is shown in Fig. 4b.

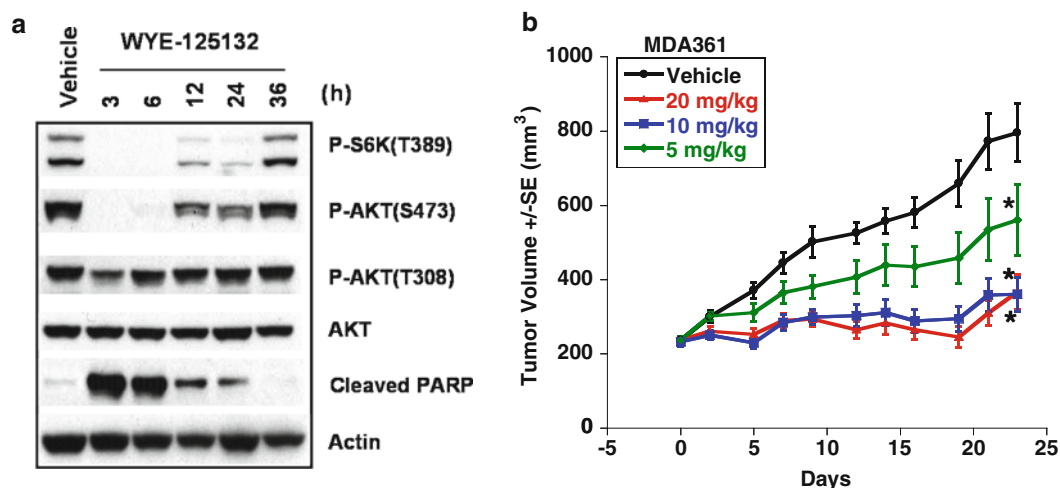


Fig. 4. WYE-125132 inhibits mTOR signaling and tumor growth in PI3K/AKT/mTOR-hyperactivated MDA361 tumors *in vivo*. (a) Mice bearing MDA361 tumors were dosed orally with vehicle or 50 mg/kg WYE-125132. Tumor lysates at the indicated time points after dosing are immunoblotted. (b) Mice bearing MDA361 tumors were dosed with vehicle, 5, 10, or 20 mg/kg WYE-125132 for cycles (one cycle consists of 5 days on and 2 days off). Tumor growth curves are shown. * $p < 0.05$, WYE-125132 versus vehicle (reproduced from ref. 5 with permission from the American Cancer Society).

4. Notes

1. 5 \times Basal assay buffer without MnCl₂, Microcystin, and BSA can be prepared and stored it in -20°C . 1 \times Assay buffer is made when needed by dilution of 5 \times basal assay buffer with molecular grade water, then supplement with MnCl₂, Microcystin, and BSA.
2. Although the SDS-PAGE and Western blotting protocols described here employ the Invitrogen reagents and equipment system, feel free to replace them with other compatible systems that you prefer.
3. Inhibitor dilutions can be prepared ahead of time and stored at -20°C . When working with sticky inhibitors, it is best to change pipette tips during serial dilutions.
4. A multichannel repeater pipette is recommended for this procedure. We use the 250 μL Matrix 12-channel pipettor for delivering the enzyme and substrate mix.
5. A 1–10- μL EDP3-Plus Rainin 12-channel electronic pipette is used in adding the inhibitors. Change pipette tips as you add row by row to avoid carry over.
6. Washing is done by using a 12-channel Nunc-Immuno washer. The wells are filled to the top with washing buffer and aspirated.

7. Shaking is important to keep the beads in suspension. We use an Eppendorf Thermomixer set to mix at 700 rpm.
8. After stopping the reaction, the samples could be stored in -80°C or processed immediately for Western blotting. Since we use Invitrogen's NuPAGE precast gels and premix running buffers and blotting system, we generally follow their instructions for running SDS-PAGE and blotting. If you do not use the Invitrogen system please feel free to replace them with other compatible systems.
9. Right after the iBlotting, you may incubate the membrane in Ponceau S solution for 1 min. Rinse several times with distilled water to wash away the background. Make a scan (or photo copy) of the Ponceau S staining to document protein loading.

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mTOR

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