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Research Article

Small molecule H89 renders the phosphorylation of S6K1 and AKT resistant to mTOR inhibitors

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The mammalian target of rapamycin (mTOR) is an evolutionarily conserved Ser/Thr kinase that comprises two complexes, termed mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). mTORC1 phosphorylates S6K1 at Thr 389, whereas mTORC2 phosphorylates AKT at Ser 473 to promote cell growth. As the mTOR name implies it is the target of natural product called rapamycin, a clinically approved drug used to treat human disease. Short-term rapamycin treatment inhibits the kinase activity of mTORC1 but not mTORC2. However, the ATP-competitive catalytic mTOR inhibitor Torin1 was identified to inhibit the kinase activity of both mTORC1 and mTORC2. Here, we report that H89 (*N*-(2-(4-bromocinnamylamino) ethyl)-5-isoquinolinesulfonamide), a well-characterized ATP-mimetic kinase inhibitor, renders the phosphorylation of S6K1 and AKT resistant to mTOR inhibitors across multiple cell lines. Moreover, H89 prevented the dephosphorylation of AKT and S6K1 under nutrient depleted conditions. PKA and other known H89-targeted kinases do not alter the phosphorylation status of S6K1 and AKT. Pharmacological inhibition of some phosphatases also enhanced S6K1 and AKT phosphorylation. These findings suggest a new target for H89 by which it sustains the phosphorylation status of S6K1 and AKT, resulting in mTOR signaling.

Introduction

Rapamycin was isolated from the bacterium *Streptomyces hygroscopicus* in 1972, and was characterized to have antifungal and antibacterial activity [1]. Subsequent studies revealed that rapamycin possessed immunosuppressive and anti-tumor properties [2,3]. Approximately 20 years after the isolation of rapamycin, scientists discovered the target of rapamycin in yeast (TOR) [4]. Shortly after, in 1994 the mammalian target of rapamycin (mTOR, also referred to as mechanistic target of rapamycin) was identified [5–7]. mTOR is a conserved Ser/Thr kinase that belongs to the phosphatidylinositol 3-kinase (PI3K)-related kinase family (reviewed, [8,9]). Both mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) contain the catalytic subunit mTOR. mTORC1 consists of: mTOR; regulatory-associated protein of mTOR (Raptor), involved in mTOR substrate recognition; the positive mTOR regulator mammalian lethal with SEC13 protein 8 (mLST8; also known as GβL); the negative mTOR regulators 40 kDa Pro-rich AKT substrate (PRAS40; also referred to as AKT1S1) and DEP domain-containing mTOR-interacting protein (DEPTOR) [10–16]. mTORC1 is regulated by nutrients, stress and energy status (reviewed here [8]). Downstream processes such as cell growth, metabolism and autophagy are regulated by mTORC1. mTORC1 phosphorylates ribosomal S6 kinase 1 (S6K1) at Thr 389 and eIF4E binding protein (4EBP1, also known as PHAS-1) at multiple sites (Thr 37/46 and Ser 65) to promote protein synthesis (reviewed here [17]). Moreover, mTORC1 can inhibit autophagy through the direct phosphorylation of UNC-51 like autophagy activating kinase 1 (ULK1) at Ser 758 [18]. Rapamycin and analogs of rapamycin (rapalogs) allosterically inhibit mTORC1 kinase activity by binding to 12 kDa FK506 binding protein 12 (FKBP12), an immunophilin and peptidyl-prolyl cis-trans isomerase [19–21]. The rapamycin-FKBP12 complex binds to the FKB-rapamycin binding (FRB) domain on mTOR narrowing the catalytic space and blocking some

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substrates from the active site [22]. Phosphorylation of S6K1 at Thr 389, as well as phosphorylation of 4EBP1 at Ser 65 is potently inhibited by rapamycin. However, phosphorylation of 4EBP1 at Thr 37/46 and ULK1 at Ser 758 is largely insensitive to rapamycin treatment [23]. In addition to mTOR, glycogen synthase kinase-3β (GSK3β) and leucine-rich repeat kinase 2 (LRRK2) can phosphorylate 4EBP1 at Thr 37/46, whereas extracellular receptor kinase (ERK) and proviral integration sites of Moloney 2 (PIM2) can phosphorylate 4EBP1 at Ser 65 [24–27].

mTORC2 contains: mTOR; rapamycin-insensitive companion of mTOR (Rictor); the positive mTOR regulator mLST8 (GβL); the negative mTOR regulator DEPTOR; the regulatory subunit mammalian stress-activated Mitogen-activated protein kinase (MAPK)-interacting protein 1 (mSin1), which contains a pleckstrin homology (PH) domain that binds to Phosphoinositide 3-kinase (PI3K)-generated Phosphatidylinositol (3,4,5)-triphosphate (PIP_3) at the plasma membrane; and protein observed with Rictor 1 and 2 (Protor1/2) [19,21,28–31]. mTORC2 promotes ion transport, cell survival, and cytoskeletal remodeling. Several members of the AGC kinase group, like Protein Kinase C (PKC: PKCα, PKCδ, PKCζ, PKCγ, and PKCε) and serum and glucocorticoid-regulated kinase 1 (SGK1) are phosphorylated by mTORC2 [19,32–36]. mTORC2 also regulates growth factor signaling and mTORC1 activity by phosphorylating RAC-alpha Ser/Thr protein kinase (AKT also known as protein kinase B) at Ser 473, which is required for AKT activation [19]. AKT phosphorylates downstream substrates such as forkhead box O1/3a (Foxo1/3a), GSK3α/β and tuberous sclerosis complex 2 (TSC2) [28,37]. mTORC2 is insensitive to short-term rapamycin treatment, but prolonged rapamycin treatment can sequester mTOR and inhibit mTORC2 assembly and activity [38,39]. The ATP-mimetic Torin1 was recently identified in a biochemical screen to inhibit both the activity of mTORC1 and mTORC2 [40]. Torin1 is only suitable for research purposes due to its highly hydrophobic nature, and the issue of poor drug solubility remains a current issue in the clinic. Although, rapamycin and rapalogs are approved in the clinic for human disease, there are many limitations. For example, rapamycin and rapalogs are cytostatic instead of cytotoxic, they fail to inhibit all of mTORC1 mediated processes, and mTORC1 inhibition relieves multiple negative feedback loops preventing other growth factor signaling cascades [41]. Therefore, understanding the precise molecular mechanisms involved in mTOR signaling may lead to the identification of better therapeutic targets.

In this study, we report that pretreatment of cells with protein kinase inhibitor H89 (*N*-(2-(4-bromocinnamyl amino) ethyl)-5-isoquinolinesulfonamide) renders S6K1 and AKT phosphorylation resistant to mTOR inhibitors (rapamycin and Torin1) in multiple cell lines. We show that resistance of S6K1 and AKT phosphorylation to mTOR inhibitors is specific to H89, as other kinase inhibitors have no effect. H89 does not alter the kinase activity of mTOR or mTOR binding components. Moreover, H89 targeted kinases are not involved in promoting S6K1 and AKT phosphorylation. The pharmacological inhibition of some phosphatases also render S6K1 and AKT phosphorylation resistant to mTOR inhibition. Thus, H89 maintains the phosphorylation status of S6K1 and AKT to promote mTOR signaling.

Experimental

Antibodies

The following antibodies were purchased from Cell Signaling and used at the indicated dilution for Western blot analysis: S6K1 (#9202, 1 : 1000), phospho-S6K1 (#9234, 1 : 1000), AKT (#9272S, 1 : 1000), phospho-AKT S437 (#4058S, 1 : 1000), 4EBP1 (#9452, 1 : 1500), phospho-4EBP1 (#9451, 1 : 1000) ULK (8054S, 1 : 1000), phospho-ULK S757 (#6888, 1 : 1000), FoxO3a (12829S, 1 : 1000), phospho-FoxO1(T24)/FoxO3a(T32) (9464T, 1 : 1000), GSK3β (12456S, 1 : 1000), phospho-GSK3α/β S21/9 (12456S, 1 : 1000), TSC2 (3635S, 1 : 1000), phospho-TSC2 (3617S, 1 : 1000), mTOR (#2983, 1 : 1000), Raptor (#2280, 1 : 1000), mlST8 (#3274, 1 : 1000), DEPTOR (#11816, 1 : 1000), PRAS40 (#2691, 1 : 1000), Sin1 (#12860S, 1 : 1000), phospho-CREB (#9198S, 1 : 1000), CREB (#9197S, 1 : 1000), phospho-Thr (#9381S, 1 : 1000), phospho-β-catenin (#9561, 1 : 1000), PKA Cat α (#4782, 1 : 1000), phospho-glycogen synthase (#3891, 1 : 1000), and actin (#3700, 1 : 1000). Flag (#F3165, 1 : 10 000) was obtained from Sigma. HA (#sc-7392 or #sc-805, 1 : 500) and Myc (#sc-40, 1 : 1000), FKBP12 (#sc-133067, 1 : 1000) were obtained from Santa Cruz. EGFP (#632380, 1 : 2000) was from Clontech. Horseradish peroxidase (HRP) linked secondary antibodies (#NXA931V anti-mouse, 1 : 8000 or #NA934V anti-rabbit, 1 : 4000) were from GE Healthcare.

Chemicals

Rapamycin was from Calbiochem (#53123-88-9). Torin1 (#S2827), BI-D1870 (#S2843), CKI-7 (#S2248), GSK-429286A (#S1474), GSK-650394 (#S7209) were from SelleckChem. Forskolin (#1099),

3-isobutyl-1-methylxanthine (IBMX, #2845) and H89 (#2910) were from Tocris. Insulin (#I1507) and calyculin A (#101932-71-2) were from Sigma. Okadaic acid (#O-5857) was from LC Laboratories.

Cell lines and tissue culture

Human embryonic kidney 293A (HEK293A), Mouse embryonic fibroblasts (MEFs), human bone osteosarcoma epithelial cells (U20S), hepatocellular carcinoma cell line (HepG2), invasive ductal carcinoma (MDA-MB-231a), human pancreatic carcinoma (MIA PaCa-2), and HeLa cells were maintained at 37°C with 5% CO_2, cultured in high-glucose DMEM (#D5796 from Sigma) supplemented with 10% Fetal Bovine Serum (FBS) (#F2442 from Sigma) and penicillin/streptomycin (#P0781 from Sigma, 100 units penicillin and 100 μg streptomycin/ml). Human bronchial epithelial cells (HBEC) were maintained at 37°C with 5% CO_2, cultured in keratinocyte serum-free medium (KSFM) (Thermo Fisher #17005042) containing 50 μg/ml bovine pituitary extract (BPE) (Thermo Fisher #13028014), 5 ng/ml of human EGF (Thermo Fisher #PHG0311L) and penicillin/streptomycin (#P0781 from Sigma, 100 units penicillin and 100 μg streptomycin/ml).

cDNA transfection

Cells were transfected with plasmid DNA using PolyJet™ DNA *In Vitro* Transfection Reagent (#SL100688 from SignaGen Laboratories) according to manufacturer's instructions. For transfection experiments, HEK293A cells were plated in 10-cm culture dishes ∼24 h prior to transfection. For mTORC1 and mTORC2 co-immunoprecipitation experiments, cells were transfected with 2 μg of Myc-tagged mTOR and 2 μg HA-tagged Raptor, HA-tagged Rictor or empty vector in HEK293A cells. Fresh medium was added 6 h after the transfection. 24 h post transfection, cells were treated accordingly and samples were collected for immunoprecipitation.

Cell lysis and immunoprecipitation

Cells were rinsed with ice-cold PBS and lysed in ice-cold CHAPS lysis buffer [40 mM HEPES pH 7.5, 120 mM NaCl, 1 mM EDTA, 10 mM pyrophosphate, 10 mM glycerophosphate, 50 mM NaF, 0.5 mM Orthovanadate, 0.3% CHAPS, and one tablet of EDTA-free protease inhibitors (#11873580001 from Roche) per 25 ml, for the co-immunoprecipitation experiment]. The soluble fractions from cell lysates were isolated by centrifugation at 13 000 rpm for 10 min in a microfuge at 4°C. An amount of 50 μl of cell lysate was taken as an input control. For immunoprecipitations, ∼30 μl of anti-HA (#PI88836 from Thermo Fisher Scientific), beads were added to each sample and incubated with rotation for 2 h at 4°C. Immunoprecipitates were washed three times with lysis buffer. Immunoprecipitated proteins were denatured by adding 50 μl of sample buffer and boiling for 5 min, resolved by 10%–15% SDS–PAGE, and analyzed via Western blot analysis.

mTORC1 kinase assay

S6K1 immunoprecipitation

HEK293A cells were transfected with Myc-tagged S6K1 24 h before the experiment. Cells were rinsed with ice-cold PBS and collected in sonication buffer [20 mM Tris HCl pH 7.5, 20 mM NaCl, 20mM beta-glycerol phosphate, 20 mM NaF, 4 mM Orthovanadate, 1 mM DTT, and protease inhibitors]. Cells were sonicated in a water bath. The soluble fractions from cell lysates were isolated by centrifugation at 13 000 rpm for 10 min in a microfuge at 4°C. For immunoprecipitations, anti-Myc (ThermoScientific, #20168) beads were added sonication buffer [20 mM Tris HCl pH 7.5, 20 mM NaCl, 20 mM beta-glycerol phosphate, 20 mM NaF, 4 mM Orthovanadate, 1 mM DTT, and protease inhibitors], once with mTORC1 kinase buffer [25 mM HEPES-KOH pH 7.4, 20 mM KCl]. Beads were incubated with Myc peptides (Sigma, #M2435-1MG) to elute Myc-S6K1.

mTORC1 kinase assay

HEK293A cells were transfected with HA-tagged Raptor and Myc-tagged mTOR 24 h before the experiment. Cells were rinsed with ice-cold PBS and collected in sonication buffer [20 mM Tris HCl pH 7.5, 20 mM NaCl, 20 mM beta-glycerol phosphate, 20 mM NaF, 4 mM Orthovanadate, 1 mM DTT, and protease inhibitors]. Cells were sonicated. The soluble fractions from cell lysates were isolated by centrifugation at 13,000 rpm for 10 min in a microfuge at 4°C. For immunoprecipitations, anti-HA (#PI88836 from Thermo Fisher Scientific) beads were added to each sample and incubated with rotation for 2 h at 4°C. Immunoprecipitates were washed one time with high salt buffer [25 mM HEPES-KOH pH 7.4, 20 mM KCl, 500 mM NaCl], twice with kinase washing buffer [25 mM HEPES-KOH pH 7.4, 20 mM KCl]. Beads were incubated in mTORC1 kinase buffer

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[25 mM HEPES-KOH pH 7.4, 50 mM KCl, 10 mM $MgCl_2$] with eluted Myc-tagged S6K1 and 500 μM ATP in a 50 μl reaction for 30 min at 30°C. Reactions were terminated by adding 4× loading buffer and boiled at 95°C for 10 min.

mTORC2 kinase assay

HEK293A cells were transfected with HA-tagged Rictor and Myc-tagged mTOR 24 h before the experiment. Cells were rinsed with ice-cold PBS and lysed in ice-cold CHAPS lysis buffer [40 mM HEPES pH 7.5, 120 mM NaCl, 1 mM EDTA, 10 mM pyrophosphate, 10 mM glycerophosphate, 50 mM NaF, 0.5 mM orthovanadate, 0.3% CHAPS, and one tablet of EDTA-free protease inhibitors (#11873580001 from Roche) per 25 ml, for the co-immunoprecipitation experiment]. The soluble fractions from cell lysates were isolated by centrifugation at 13 000 rpm for 10 min in a microfuge at 4°C. anti-HA (#PI88836 from Thermo Fisher Scientific) beads were added to each sample and incubated with rotation for 2 h at 4°C. Beads were washed twice with CHAPS lysis buffer, and once with mTORC2 kinase assay buffer [25 mM HEPES-KOH pH 7.4, 100 mM potassium acetate, 1 mM $MgCl_2$]. Beads were incubated in kinase buffer in 15 μl reaction, supplemented with 500 μM ATP and 250 ng recombinant His-AKT (Upstate Biotechnology). Kinase reactions were incubated at 37°C for 30 min. Reactions were terminated by adding 4× loading buffer and boiled at 95°C for 10 min.

Western blot

Cells were rinsed with PBS and lysed with Laemmli sample buffer (50 mM Tris pH 6.8, 2% SDS, 0.025% Bromophenol Blue, 10% glycerol, 5% BME) and boiled for 10 min before separation by 10%–15% SDS–PAGE and transfer to polyvinylidene difluoride membranes (#162-0177 from Bio-Rad). Blots were then blocked in 5% milk for 1 h, probed with primary antibodies and horseradish peroxidase (HRP) conjugated secondary antibodies, and developed with SuperSignal™ West Dura Substrate (#34075 from Thermo Fisher Scientific).

Generation of PKA Cat α/β knockout cells using CRISPR/Cas9 genome editing

The 20 nucleotide guide sequences targeting human PRKACA and PRKACB were designed using the CRISPR design tool at http://www.genome-engineering.org/crispr/ [42] and cloned into the expression vector SpCas9-2A-Puro V2.0 (pX459) V2.0 (Addgene #62988). The guide sequence targeting Exon 1 of human PRKACA and Exon 9 of human PRKACB are shown below.

PRKACA

3′-AGAACCGCCGCCGCCGCAAC-5′

PRKACB

5′-UAAAAUCGGUCAGUUUCAUC-3′

The single guide RNAs (sgRNAs) in the pX459 vector (500 ng) were transfected into HEK293A cells (6-well) using PolyJet™ DNA *In Vitro* Tranfection Reagent according to manufacturer's instructions. 24 h after transfection the medium was again changed and puromycin (#ant-pr-1 from Invitrogen) added to a final concentration of 5 μg/ml. Under these conditions, non-infected cells died within 24–48 h. For the surviving the cells, the medium was changed to medium not containing puromycin, and the cells were grown to ∼80% confluence. The cells were trypsinized, washed with PBS, and re-suspended in fluorescence-activated cell sorting (FACs) buffer (PBS, 5 mM EDTA, 2% FBS and Pen/Strep). Cells were single cell sorted by FACs (UCSD; Human Embryonic Stem Cell Core, BDInflux) into 96-well plate format into DMEM containing 30% FBS and 50 μg/ml penicillin/streptomycin. Single clones were expanded, and screened for PKA Cat α/β by protein immunoblotting.

RNA interference

siRNA

Cells were plated and allowed to reach a confluency of ∼60%. ON-TARGET plus SMART pool siRNA) against PPP1CA (Dharmacon #L-008927-00-0005), PPP1CB (Dharmacon #L-008685-00-005), PPP1CC (Dharmacon #L-006827-00-0005) were used at 50nM and transfected using DharmaFECT transfection reagent (Dharmacon, #T-2001-03) for 24 h.

shRNA

The short-hairpin RNAs (shRNAs) against human PPP1CA/PPP1CB/PPP1CC were mixed with the packaging plasmids psPAX2 and pMD2.G and co-transfected into HEK293A cells using PolyJet™ DNA *In Vitro* Transfection reagent (SignaGen Laboratories, #SL100688) according to manufacturer's instructions. After 48 h, virus was collected and applied with Polybrene (Millipore, #TR1003G) to new HEK293A cells. After 24 h, virus was removed and replaced with complete media for 24 h. After 24 h cells were selected using puromycin (5 μg/ml; InvivoGen #ant-pr-1). The shRNA sequences are shown below.

PPP1CA

sh-1: CCGGTGAGTGCAAGAGACGCTACAACTCGAGTTGTAGCGTCTCTTGCACTCATTTTT
sh-2: CCGGACTACGACCTTCTGCGACTATCTCGAGATAGTCGCAGAAGGTCGTAGTTTTTT

PPP1CB

sh-1: CCGGGATGAATGCAAACGAAGATTTCTCGAGAAATCTTCGTTTGCATTCATCTTTTTG
sh-2: CCGGCGACAGTTGGTAACCTTATTTCTCGAGAAATAAGGTTACCAACTGTCGTTTTTG

PPP1CC

sh-1: CCGGGCGAATTATGCGACCAACTGACTCGAGTCAGTTGGTCGCATAATTCGCTTTTT
sh-2: CCGGACATTTGGTGCAGAAGTGGTTCTCGAGAACCACTTCTGCACCAAATGTTTTTT

Results

H89 is a well-characterized PKA inhibitor ($IC_{50} \sim 50$ nM) [43] and has been shown to potently inhibit ribosomal protein S6 kinase beta-1(S6K1, $IC_{50} \sim 80$ nM), mitogen and stress activated protein kinase 1 (MSK1, $IC_{50} \sim 120$ nM), rho-associated coiled-coil containing protein kinase (ROCK-II, $IC_{50} \sim 270$ nM), protein kinase B alpha (PKBα, $IC_{50} \sim 2.6$ μM) and MAPK activated protein kinase 1b (MAPKAP-K1b, $IC_{50} \sim 2.8$ μM) [44]. Treatment of human embryonic kidney 239A (HEK293A) cells with H89 under normal culturing conditions increased the phosphorylation of S6K1 at Thr 389 and AKT at Ser 473 (Figure 1A). As expected, treatment of cells with rapamycin or Torin1 inhibited S6K1 phosphorylation (Figure 1B, lanes 2 and 3). Moreover, Torin1 also inhibited AKT phosphorylation (Figure 1B, lane 3). Interestingly, pretreatment of H89 prior to rapamycin or Torin1 treatment, at least to some extent prevented the decrease in S6K1 and AKT phosphorylation in response to the mTOR inhibitors (Figure 1B, lanes 5 and 6). However, if cells were first pretreated with rapamycin or Torin1 prior to the addition of H89, rapamycin or Torin1 could still inhibit S6K1 and AKT phosphorylation (Figure 1B, lanes 7 and 8). We assessed other cell lines where we pretreated with H89, then added rapamycin and Torin1, and observed the phosphorylation status of S6K1 and AKT (Figure 1C). Mouse embryonic fibroblast (MEF), human bronchial epithelial (hBE), human bone osteosarcoma epithelial (U20S), hepatocellular carcinoma (HepG2), human pancreatic carcinoma (MIA PaCa-2), and mouse myoblast (C2C12) cells were also resistant to rapamycin and Torin1 treatment when pretreated with H89. Taken together, H89 enhances S6K1 and AKT phosphorylation. Moreover, H89 can prevent rapamycin and Torin1 from inhibiting S6K1 and AKT phosphorylation.

mTORC1 regulates downstream cellular processes through multiple substrates [45]. It has previously been demonstrated that cells treated with rapamycin does not inhibit all mTORC1 phosphorylation sites [46], while Torin1 has been shown to be more effective [40]. In order to determine if H89 can prevent rapamycin and Torin1 inhibition of mTORC1 substrates other than S6K1, we analyzed the phosphorylation status of Unc-51 like autophagy activating kinase 1 (ULK1) at Ser 758 [47] and eukaryotic translation initiation factor 4E-binding protein 1 (4EBP1) at Ser 65 [17] (Supplementary Figure S1). As expected Torin1, but not rapamycin significantly decreased ULK1 and 4EBP1 phosphorylation (Supplementary Figure S1, lanes 2 and 4). In contrast with S6K1 and AKT phosphorylation (Figure 1A), HEK293A cells pretreated with H89 prior to rapamycin or Torin1 treatment did not alter ULK1 or 4EBP1 phosphorylation (Supplementary Figure S1, lanes 5 and 6). Moreover, it did not appear that H89 enhanced ULK1 or 4EBP1 phosphorylation under normal culturing conditions (Supplementary Figure S1, lane 4). Thus, Torin1 inhibits ULK1 and 4EBP1 phosphorylation, regardless of H89 treatment.

Next, we examined other inhibitors that targeted a range of different kinases (Figure 2A,B): BI-D1870 (RSK1/2/3/4 inhibitor) [48], CKI-7 (CSK1, SGK, S6K1, MSK1 inhibitor) [49], GSK429286A (ROCK1 and

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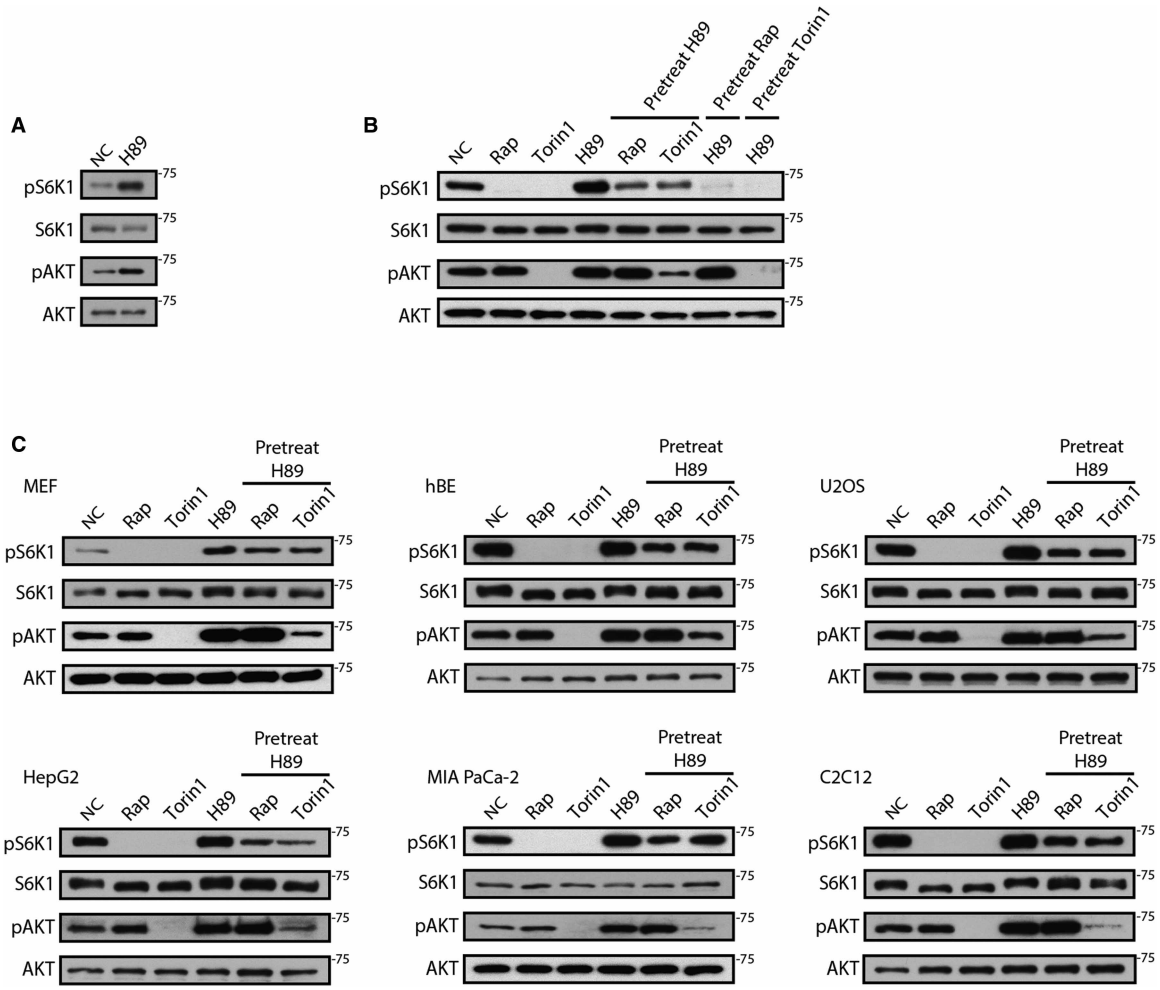

Figure 1. H89 renders S6K1 and AKT phosphorylation resistant to rapamycin and Torin1.

(**A**) Human embryonic kidney 293A (HEK293A) cells were treated DMSO or with or without 10 μM H89 for 45 min. Cell lysates were immunoblotted for the phosphorylation status of S6K1 (pS6K1) at Thr 389 and phosphorylation status of AKT (pAKT) at Ser 473. S6K1 and AKT were used as loading control. (**B**) HEK293A cells were treated with DMSO or either 20 nM rapamycin (2nd lane) or 150 nM Torin1 (3rd lane) for 30 min, 10 μM H89 for 45 min (4th lane), pretreated with 10 μM H89 for 45 min and then treated with 20 nM rapamycin (5th lane) or 150 nM Torin1 (6th lane) for 30 min, or first pretreated with 20 nM rapamycin (7th lane) or 150 nM Torin1 (8th lane) for 30 min and then treated with 10 μM H89 as indicated. (**C**) Mouse embryonic fibroblasts (MEFs), Human bronchial epithelial (hBE), human bone osteosarcoma epithelial cells (U2OS), hepatocellular carcinoma cell line (HepG2), human pancreatic carcinoma (MIA PaCa-2), and mouse C3H muscle myoblast (C2C12) cells were treated as described in (B). NC denotes normal conditions treated with DMSO. The experiments were performed at least 3 times. Rap = rapamycin.

ROCK2 inhibitor) [50,51] and GSK650394 (SGK1 and SGK2 inhibitor) [52]. HEK293A cells were pretreated with either H89, BI-D1870, CKI-7, GSK429286A or GSK650394 prior to rapamycin treatment (Figure 2C). As expected, the kinase inhibitor BI-D1870 slightly decreased mTORC1 activity. RSK phosphorylates tuberous sclerosis complex 2 (TSC2) at Ser 1798 and inactivates its activity, resulting in an increase in mTORC1 activity and S6K1 phosphorylation [53]. Only H89, and not the other kinase inhibitors, could block the effect of rapamycin treatment on S6K1 and AKT phosphorylation.

Because mTORC1 phosphorylates S6K1 at Thr 389 and mTORC2 phosphorylates AKT at Ser 473, we investigated whether H89 could directly alter mTOR kinase activity or the binding of known components found in both mTORC1 and mTORC2. HA-tagged Raptor and Myc-tagged mTOR were expressed in HEK293A cells, and HA-tagged Raptor was immunoprecipitated followed by *in vitro* kinase reactions using exogenously expressed Flag-tagged S6K1 as a substrate (Figure 3A). mTORC1 phosphorylated S6K1 at Thr 389 under

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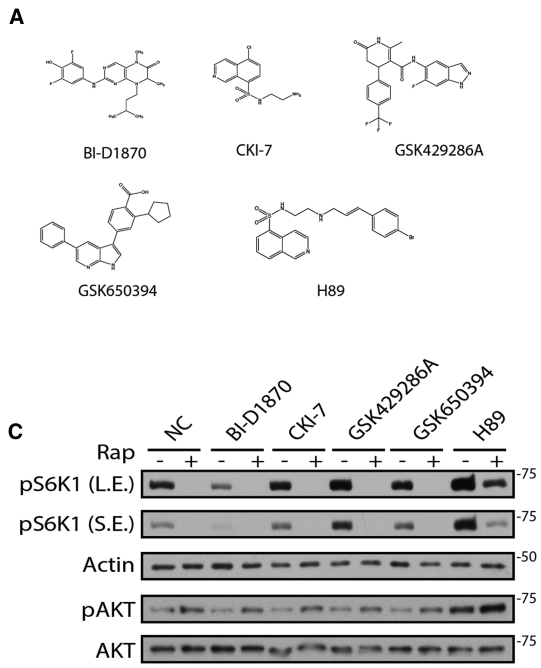

B

Kinase Inhibitor	Kinase	IC_{50}	Reference
BI-D1870	RSK1/2/3/4	31nm, 24nM, 18nM, 15nM	Sapkota et al. Biochem J. 2007
CKI-7	CSK1 SGK S6K1 MSK1	6μM	Chijiwa et al. JBC 1989
GSK429286A	ROCK1 ROCK2	14nM 63nM	Goodman et al. J Med Chem 2007 Nichols et al. Biochem J. 2009
GSK650394	SGK1 SGK2	62nM 103nM	Sherk et al. Cancer Res. 2008
H89	S6K1 MSK1 PKA ROCK2 PKBα MAPKAP-K1b	80nM 120nM 135nM 270nM 2600nM 2800nM	Davies et al. Biochem J. 2000

Figure 2. Similar kinase inhibitors to H89 do not alter S6K1 and AKT phosphorylation.

(**A**) The chemical structures of the kinase inhibitors BI-D1870, CKI-7, GSK429286A, GSK650394 and H89 are depicted. (**B**) A table summarizing the targets of the kinase inhibitors described in (**A**), the targets, the kinase inhibitor IC_{50} and reference. (**C**) Human embryonic kidney 293A (HEK293A) cells were treated with DMSO or pretreated with 10 μM of indicated kinase inhibitors for 45 min, followed by 20 nM rapamycin treatment for 30 min. The phosphorylation of S6K1 (pS6K1) at Thr 389 and AKT (pAKT) at Ser 473 were analyzed. Actin and AKT were used as loading control. NC denotes normal conditions treated with DMSO. L.E. denotes long exposure and S.E. denotes short exposure. The experiments were performed at least 1 time. Rap = rapamycin.

normal culturing conditions (Figure 3A, lane 3), and Torin1 treatment prevented mTORC1 from phosphorylating S6K1 (Figure 3A, lane 4). Phosphorylation of S6K1 by mTORC1 occurred regardless of H89 treatment in cell culture (Figure 3A, lane 5) or adding H89 *in vitro* directly to the kinase reaction (Figure 3A, lane 6). Moreover, we investigated if the rapamycin-FKBP12 association with mTORC1 changed with H89 treatment (Figure 3B). Similar to previous reports, treatment of cells with rapamycin increased FKBP12 binding to mTORC1 (Figure 3B, lane 3), and H89 had no effect on mTORC1-FKBP12 binding in response to rapamycin treatment (Figure 3B, lane 5). Next, we looked at mTORC2 activity and expressed HA-tagged Rictor and Myc-tagged mTOR in HEK293A cells (Figure 3C). HA-tagged Rictor was immunoprecipitated followed by *in vitro* kinase reactions using recombinant AKT as a substrate [54]. mTORC2 could phosphorylate AKT at Ser 473 under normal culturing conditions (Figure 3C, lane 3), and as expected Torin1 treatment prevented mTORC2 from phosphorylating AKT (Figure 3C, lane 4). Phosphorylation of AKT by mTORC2 occurred despite H89 treatment in cell culture (Figure 3C, lane 5) or adding H89 *in vitro* to the kinase reaction (Figure 3C, lane 6). To look at the binding of components found in both mTORC1 and mTORC2, we expressed HA-tagged Raptor and Myc-tagged mTOR in cells (Figure 4A). Cells were treated with or without H89 and HA-tagged Raptor was immunoprecipitated. H89 did not affect the binding between HA-tagged Raptor, Myc-tagged mTOR, mLST8, and DEPTOR. Similarly, H89 treatment did not alter the binding among HA-tagged Rictor, Myc-tagged mTOR, mLST8 or DEPTOR (Figure 4B,C). Taken together, H89 does not alter the kinase activity of mTORC1 or mTORC2, or rapamycin-FKBP12 association with mTORC1. Moreover, H89 does not change the binding of components in mTORC1 or mTORC2.

Protein kinase A (PKA) is usually advertised as the main target of H89 [44]. To test if inhibition of PKA rendered the phosphorylation of S6K1 and AKT resistant to rapamycin and Torin1 treatment, we used a biochemical approach to inhibit PKA. PKA catalytic subunits form a complex with PKA regulatory subunits under low cyclic adenosine 3′5′ monophosphate (cAMP) levels resulting in PKA inhibition [55]. Elevated

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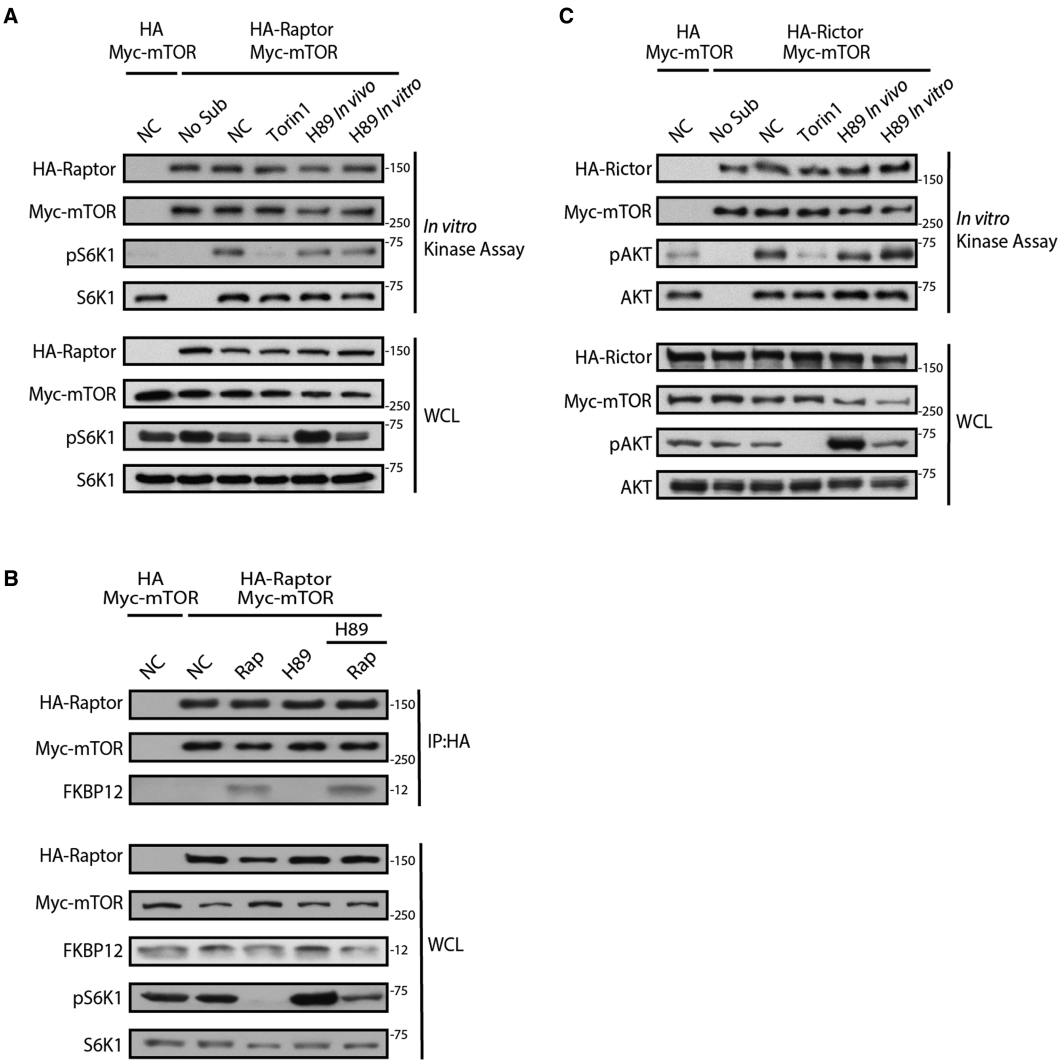

Figure 3. H89 does not alter mTOR kinase activity or FKBP12 binding to mTORC1.

(**A**) Human embryonic kidney 293A (HEK293A) cells were co-transfected with HA-tagged Raptor and Myc-tagged mTOR for 24 h. Cells were treated with DMSO, 1 μM Torin1, or 10 μM H89 for 45 min. Co-immunoprecipitation experiments were performed by pulling down HA-tagged Raptor, followed by *in vitro* kinase assay reactions. Cell lysates were immunoblotted for Myc, HA, and phosphorylation of S6K1 (pS6K1) at Thr 389. S6K1 was used as loading control. (**B**) HEK293A cells were transfected with Myc-tagged mTOR and HA-tagged Raptor. After 48 h cells were treated with DMSO, 10 μM H89, 20 nM rapamycin or pretreated with 10 μM H89, followed by 20 nM rapamycin as indicated. mTORC1 was isolated by the immunoprecipitation of HA-tagged Raptor. Cell lysates and HA immunoprecipitates were analyzed for HA-tagged Raptor, Myc-tagged mTOR, FKBP12, pS6K1 and S6K1. (**C**) HEK293A cells were co-transfected with HA-tagged Rictor and Myc-tagged mTOR for 24 h. Cells were treated with either DMSO, 1 μM Torin1, or 10 μM H89 for 45 min. Co-immunoprecipitation experiments were performed by pulling down HA-tagged Rictor, followed by *in vitro* kinase assay reactions. Cell lysates were immunoblotted against Myc, HA, and phosphorylation of AKT (pAKT) at Ser 473. AKT was used as loading control. IP denotes immunoprecipitation and WCL denotes whole cell lysate. NC denotes normal conditions treated with DMSO. The experiments were performed at least 1 time. Rap = rapamycin.

cAMP bind to the regulatory subunits of PKA releasing the active PKA catalytic subunits. Mutations in PKA regulatory subunits, such as PKA regulatory subunit Iα, are unresponsive to increased cAMP levels and cannot release the PKA catalytic subunits [56]. Overexpression of the mutant PKA Iα regulatory subunit in cells sequesters all of the endogenous PKA catalytic subunits and inhibits the kinase activity of PKA [57,58].

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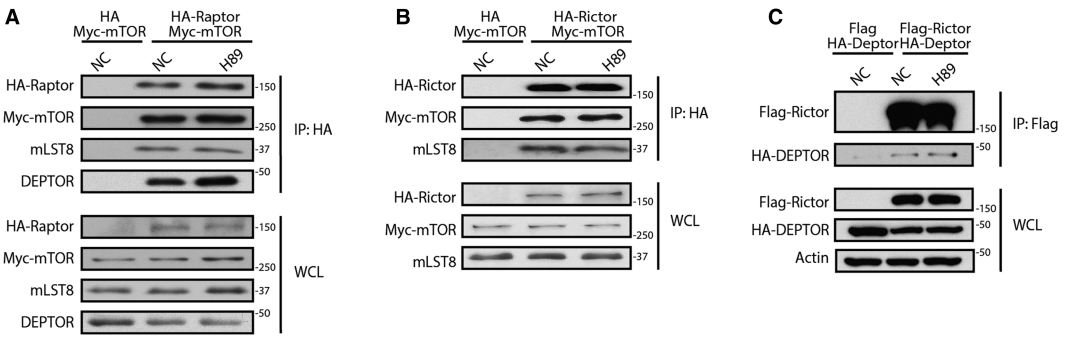

Figure 4. H89 treatment does not alter mTOR complex binding.

(**A**) Human embryonic kidney 293A (HEK293A) cells were co-transfected with Myc-tagged mTOR and HA-tagged Raptor. After 48 h, cells were treated with DMSO or 10 μM H89 as indicated. mTORC1 was isolated by the immunoprecipitation of HA-tagged Raptor. HA-tagged Raptor, Myc-tagged mTOR, mLST8 and DEPTOR were immunobloted. (**B**) HEK293A cells were co-transfected with Myc-tagged mTOR and HA-tagged Rictor. After 48 h, cells were treated with DMSO or 10 μM H89 as indicated. mTORC2 was isolated by the immunoprecipitation of HA-tagged Rictor. HA-tagged Rictor, Myc-tagged mTOR, mlST8. (**C**) HEK293A cells were co-transfected with empty vector and HA-tagged DEPTOR or Flag-tagged Rictor and HA-tagged DEPTOR. Cells were treated with DMSO or with or without 10 μM H89 and immunoprecipitated for Flag. Flag-tagged Rictor, HA-tagged DEPTOR and Actin were immunoblotted. IP denotes immunoprecipitation and WCL denotes whole cell lysate. NC denotes normal conditions treated with DMSO. The experiments were performed at least 3 times.

EGFP-tagged mutant PKA regulatory subunit Iα was expressed in HEK293A cells and then cells were treated with either rapamycin or Torin1 (Figure 5A). As expected, rapamycin and Torin1 did not affect S6K1 and AKT phosphorylation with pretreatment of H89 (Figure 5A, lanes 4 and 5). However, rapamycin or Torin1 decreased S6K1 and AKT phosphorylation even with the overexpression of EGFP-tagged mutant PKA regulatory subunit Iα (Figure 5A, lanes 6 and 7). As a control, to ensure PKA inhibition by the over expression of EGFP-tagged mutant PKA regulatory subunit Iα, we looked at the phosphorylation of cAMP response element-binding protein (CREB) at Ser 133 (Figure 5B). CREB is a well-characterized substrate of PKA [59]. Cells were treated with forskolin and IBMX to increase intracellular cAMP levels to activate PKA. Forskolin increases cAMP through the activation of adenylyl cyclase; whereas, isobutyl-1-methylxanthine (IBMX) is a phosphodiesterase inhibitor leading to increased cAMP levels [60]. The overexpression of EGFP-tagged mutant PKA regulatory subunit Iα prevented the activation of PKA by increased intracellular cAMP levels, as judged by the phosphorylation of CREB. Moreover, PKA catalytic α/β HEK293A KO cells were pretreated with H89 then with rapamycin or Torin1, and S6K1 and AKT phosphorylation was evaluated (Figure 5C). In the absence of PKA catalytic α/β, H89 still rendered S6K1 and AKT phosphorylation resistant to rapamycin and Torin1 treatment. Consistently, H89 blocked rapamycin or Torin1 effect on S6K1 and AKT phosphorylation even with the overexpression of Flag-tagged PKA catalytic α (Figure 5D). Collectively, the effects of H89 on S6K1 and AKT phosphorylation are not through PKA.

In addition to PKA, H89 can inhibit other AGC kinases like S6K1, MSK1, ROCK-II, PKBα and MAPKAP-K1b [44]. The ROCK-II inhibitor GSK429286A did not alter S6K1 or AKT phosphorylation (Figure 2), so we determined ROCK-II was probably not involved. In a dose-dependent manner we overexpressed Flag-tagged RSK1, MAPKAP-K1b, MSK1, S6K1, PKBα, PKBβ, GSKα and GSKβ (Figure 6A). Overexpression of these kinases were unable to relieve H89 from blocking the decrease in S6K1 phosphorylation in response to rapamycin or Torin1 treatment. We saw a slight decrease in the phosphorylation of S6K1 when we overexpressed GSKβ at high levels even in the presence of H89. However, S6K1 phosphorylation decreased from rapamycin after pretreatment of cells with a GSKβ inhibitor called CHIR99021 (Figure 6B, left). Moreover, GSKβ activity was maintained in cells treated with H89, as judged by the phosphorylation of the GSKβ substrates β-catenin at Ser 33/37 and Thr 41, and glycogen synthase at Ser 641 (Figure 6B, right) [61]. Because H89 has been reported to inhibit multiple AGC kinases [44], we overexpressed numerous AGC kinases and assessed S6K1 phosphorylation with rapamycin treatment (Figure 6C). Flag-tagged GRK4, GRK7, PRKG1, PDPK1, STK32A, STK32B, MAST1, MAST2, PRKCH, PRKCE, PRKC1, PRKCZ, PKN1, MSK2, RSK3,

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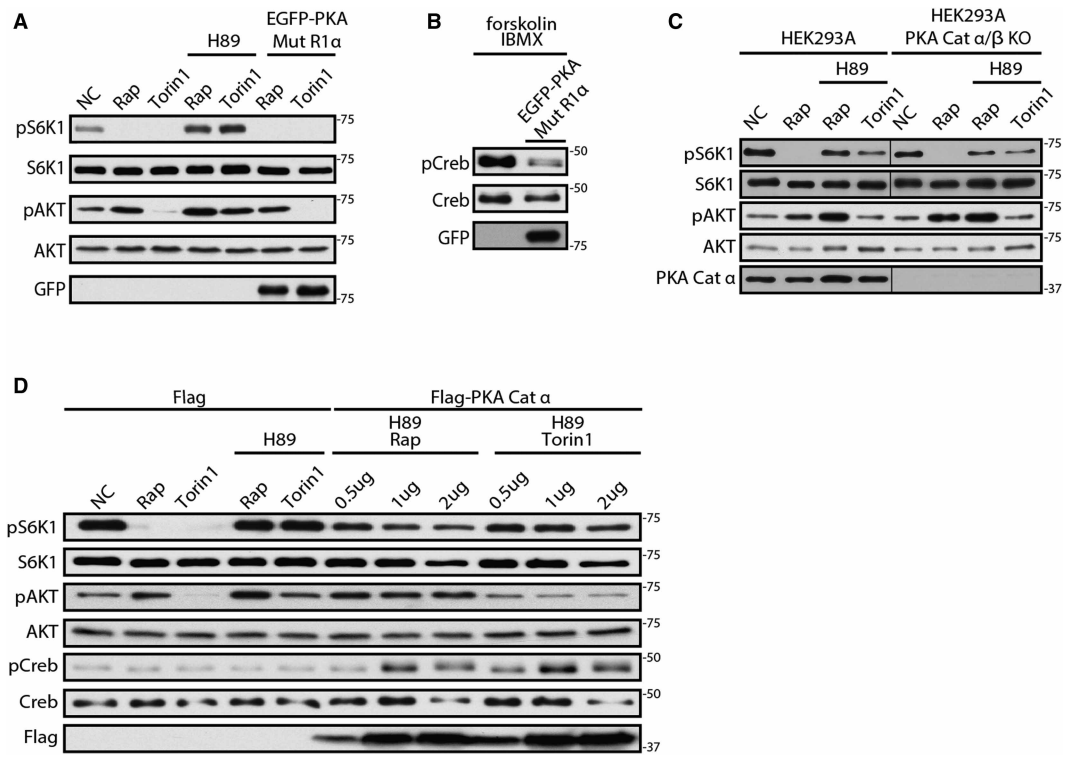

Figure 5. H89 does not target PKA to increase S6K1 and AKT phosphorylation.

(**A**) Human embryonic kidney 293A (HEK293A) cells were treated with DMSO or 20 nM rapamycin or 150 nM Torin1 for 30 min (2nd and 3rd lane), pretreated with 10 μM H89 for 45 min then treated with 20 nM rapamycin or 150 nM Torin1 for 30 min (4th and 5th lane), or transfected with EGFP-tagged PKA regulatory subunit mutant RIα (EGFP PKA mutant RIα) plasmid 48 h and then treated with 20 nM rapamycin or 150 nM Torin1 as indicated. Cell lysates were immunoblotted for the phosphorylation status of S6K1 (pS6K1) at Thr 389 and the phosphorylation status of AKT (pAKT) at Ser 473. S6K1 and AKT were used as loading control. Cell lysates were also immunoblotted for EGFP-tagged PKA mutant RIα to assess expression level. (**B**) To confirm that EGFP-tagged PKA mutant RIα inhibits PKA, HEK293A cells were transfected with EGFP-tagged PKA mutant RIα for 48 h. Cells were treated with 20 μM Forskolin and 20 μM IBMX for 45 min and PKA activity was analyzed by immunoblotting for the phosphorylation of CREB (pCREB) at Ser 133. CREB was used as loading control. EGFP-tagged PKA mutant RIα was also probed. (**C**) HEK293A and PKA knockout (KO) HEK293A cells were treated with DMSO or with 20 nM rapamycin for 30 min (2nd and 6th lane), pretreated 10 μM H89 for 45 min then treated with 20 nM rapamycin or 150 nM Torin1 for 30 min (3rd–4th lane and 7th–8th lane). Cell lysates were immunoblotted for the phosphorylation status of S6K1 (pS6K1) at Thr 389 and the phosphorylation status of AKT (pAKT) at Ser 473. S6K1 and AKT were used as loading control. Cell lysates were also immunoblotted for PKA Cat α to assess expression level. (**D**) HEK293A cells were transfected with increasing amount of Flag-tagged PKA (0.5 μg, 1 μg, 2 μg) for 48 h before being treated with 10 μM H89 for 45 min, followed by 20 nM rapamycin or 150 nM Torin1 treatment for 30 min. Flag was used to confirm Flag-tagged PKA expression level. S6K1 and AKT was used as loading control. The experiments were performed at least 1 time. Rap = rapamycin.

GRK3, GRK2, DMPK, STK38, AKT3, SGK1, SGK2, SGK3, PRKCA, PRKCB and PRKCG were overexpressed in cells. Cells were pretreated with H89 prior to the addition of rapamycin and S6K1 phosphorylation was assessed. Similar to the other AGC kinases (Figure 6A,B), overexpression of these kinases could not relieve the effect of H89 on S6K1 phosphorylation in response to rapamycin treatment. In addition to AGC kinases, inhibition of MAPKAP-K2 [62] with PF3644022 [63] showed no change in the phosphorylation S6K1 or AKT (Supplementary Figure S2). Taken together, these AGC kinases or MAPKAP-K2 do not appear to be the target of H89 in respect to the enhancement of S6K1 and AKT phosphorylation.

mTORC1 is activated in response to upstream stimuli including amino acids and growth factors [45]. Therefore, we asked if H89 had an effect on the phosphorylation of S6K1 and AKT if we inhibited the PI3K-AKT pathway

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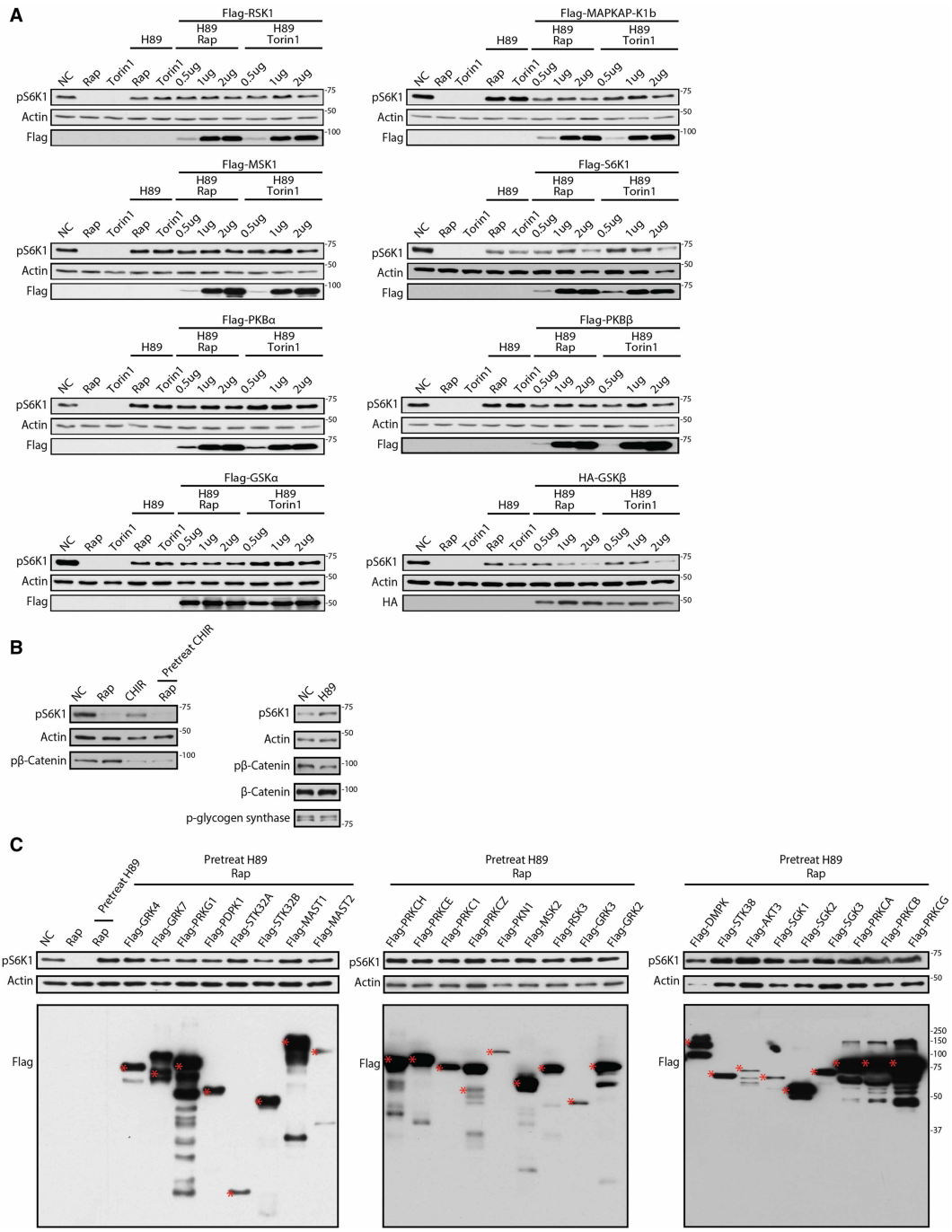

Figure 6. Other known H89 targeted kinases do not alter S6K1 and AKT phosphorylation. Part 1 of 2

(**A**) Human embryonic kidney 293A (HEK293A) cells were transfected with increasing amount of Flag-tagged AGC kinase 48 h before being pretreated with 10 μM H89 for 45 min, followed by 20 nM rapamycin or 150 nM Torin1 treatment for 30 min. Cell lysates were immunoblotted for the phosphorylation of S6K1 (pS6K1) at Thr 389. Flag was immunoblotted to confirm expression level of each kinase. Actin was used as loading control. (**B**) HEK293A cells were treated with 20 nM rapamycin for 30 min (2nd lane), 3 μM CHIR for 30 min (3rd lane) or 3 μM CHIR pretreatment for 30 min followed by 20 nM rapamycin treatment for 30 min (4th lane). Cell lysates were immunoblotted against phosphorylation status of S6K1 (pS6K1) at Thr 389 and β-catenin at Ser 33/37 Thr 41. Actin was used as loading control (left). HEK293A cells were treated with or without 10 μM H89 for 45 min. Cell lysates were immunoblotted as in left panel, and the phosphorylation of glycogen synthase (p-glycogen synthase) at Ser 641. NC denotes normal conditions treated with DMSO (right). (**C**) HEK293A cells were transfected with Flag

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Figure 6. Other known H89 targeted kinases do not alter S6K1 and AKT phosphorylation. Part 2 of 2

empty vector (EV) or specified Flag-tagged proteins for 24 h. Cells were treated with 20 nM rapamycin for 30 min, or pretreated with 10 μM H89 for 45 min followed by 20 nM rapamycin treatment for 30 min. The phosphorylation of S6K1 (pS6K1) at Thr 389 was analyzed. Actin was used as loading control. Flag was used to confirm exogenous protein expression. Red asterisk indicates expected molecular mass. The experiments were performed at least 1 time. Rap = rapamycin.

with the PI3K inhibitor (GDC-0941) [64] (Figure 7A). As expected, cells treated with GDC-0941 decreased the phosphorylation of S6K1 and AKT (Figure 7A, lane 3), even with the addition of H89 after GDC-0941 treatment (Figure 7A, lane 4). However, pretreatment of H89 prior to GDC-0941 appeared to maintain the S6K1 and AKT phosphorylation (Figure 7A, lane 5). Next, we starved cells of FBS with or without H89 in the media (Figure 7B). The phosphorylation of AKT was decreased in the absence of FBS, but was somewhat retained when H89 was added to the media. We also starved HEK293A cells of amino acids with or without H89 (Figure 7C). Treatment with H89 before and during amino acid starvation maintained the phosphorylation of S6K1 (Figure 7C, lane 3). Overall, it appears that H89 can maintain AKT and S6K1 phosphorylation even under growth factor and amino acid depletion experiments, suggesting that H89 may work through a phosphatase.

The removal of Ser or Thr phosphorylation on proteins is catalyzed by Ser/Thr phosphatases [65,66]. The protein Ser/Thr phosphatases are categorized into three main families: the phosphoprotein phosphatases (PPPs), metal-dependent protein phosphatases (PPMs) and the aspartate-based phosphatases [65]. PPP is the family with the largest number of known members, containing phosphatases such as PP1 and PP2A. Because H89 can maintain S6K1 phosphorylation under starvation conditions, we tested whether phosphatase inhibitors could rescue rapamycin and Torin1 treatment (Figure 7D,E). We used the phosphatase inhibitors calyculin A and okadaic acid [67]. Calyculin A has been reported to inhibit both PP1 and PP2A, whereas okadaic acid appears more specific to PP2A. Human bronchial epithelial (hBE) cells were pretreated with or without calyculin A prior to rapamycin or Torin1 treatment and the S6K1 and AKT phosphorylation was assessed (Figure 7D). Like H89, calyculin A inhibited rapamycin and Torin1 from decreasing S6K1 and AKT phosphorylation (Figure 7D, lanes 8 and 9). Interestingly, pretreatment of okadaic acid maintained S6K1 and AKT phosphorylation in response to rapamycin and Torin1 treatment (Figure 7E, lanes 8 and 9). These results suggest that PP2A may play a role in H89 enhancing S6K1 and AKT phosphorylation. However, after H89 treatment, S6K1 or AKT phosphorylation was unaffected by the overexpression of 29 phosphatases (Flag-tagged PPP1CA, PPP1CB, PPP1CC, PPP2CA, PPP2CB, PPP3CA, PPP3CB, PPP3CC, PPP4C, PPP5C, PPP6C, PPEF1, PPEF2, PPM1A, PPM1B, PPM1D, PPM1E, PPM1F,PPM1G, PPM1H, PP1J, PPM1K, PPM1L, PPM1M PDP1, PDP2, PHLPP1, PHLPP2 and ILKAP) including the PP2A family (PPP2CA and PPP2CB) (Supplementary Figure S3). Consistently, S6K1 or AKT phosphorylation was sustained after the depletion of the PP1 family (PPP1CA, PPP1CB, PPP1C) either with small interfering RNA (siRNA) (Supplementary Figure S4A) or short hairpin RNA (shRNA) (Supplementary Figure S4B) with H89 treatment. Taken together, results from calyculin A and okadaic acid appear similar in the prevention of rapamycin and Torin1 from decreasing S6K1 and AKT phosphorylation. However, overexpression of PP1 and PP2, as well as many other phosphatases, did not appear to change the phosphorylation status of S6K1 and AKT.

Discussion

In this study, we show that the kinase inhibitor H89 enhances the phosphorylation of S6K1 and AKT. We made this finding when dissecting the cross-talk between the mTORC1 and PKA pathway [58]. Importantly, pretreatment of cells with H89 renders S6K1 and AKT phosphorylation resistant to mTOR inhibitors (rapamycin and Torin1) and nutrient starvation. Increased S6K1 and AKT phosphorylation is specific to H89, and other similar kinase inhibitors had no effect on the phosphorylation of S6K1 and AKT. S6K1 phosphorylation at Thr 389 and AKT phosphorylation at Ser 473 promote cell growth and tumorigenesis. Therefore, understanding the molecular mechanism for how H89 enhances S6K1 and AKT phosphorylation is important. Moreover, the target of H89 could perhaps be therapeutically beneficial for patients who develop rapamycin resistance. For example, for patients with mutations in the FKBP12-rapamycin binding (FRB) domain of mTOR which disrupts the ability of rapamycin to inhibit mTORC1 [68]. Although we did not identify the precise target of H89 involved in the enhancement of S6K1 and AKT, we generated a working model (Figure 7E). Under nutrient rich conditions, the mTOR phosphorylates downstream substrates such as S6K1 and AKT. In contrast, a limitation in available nutrients results in the dephosphorylation of S6K1 and AKT.

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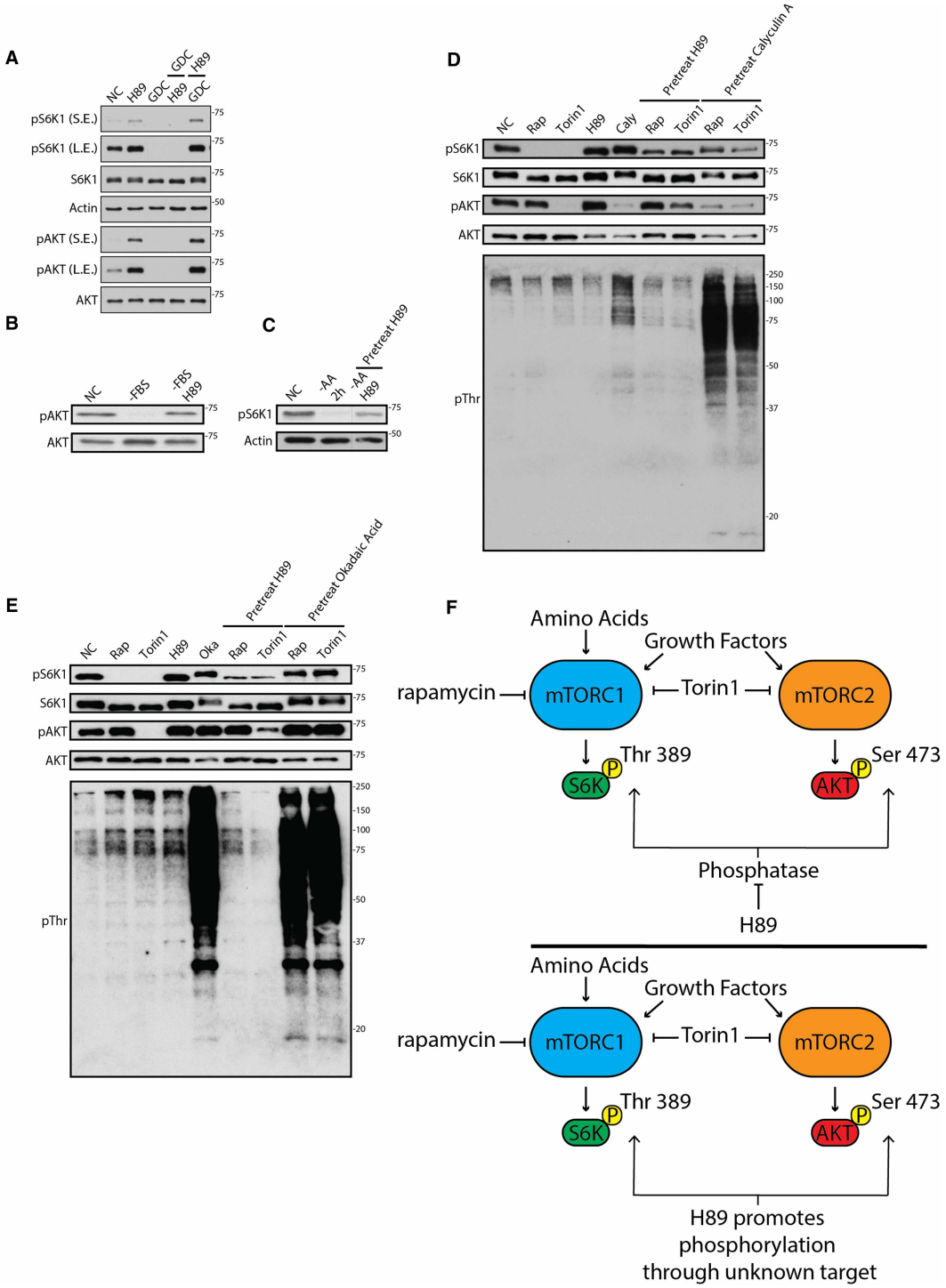

Figure 7. H89 maintains S6K1 and AKT phosphorylation under nutrient depletion conditions. Part 1 of 2

(**A**) Human embryonic kidney 293A (HEK293A) cells were treated with DMSO, 10 μM H89 for 45 min (2nd lane), 1 μM GDC-0941 for 1 h (3rd lane), pretreated with 1 μM GDC-0941 for 1 h then 10 μM H89 for 45 min (4th lane), or pretreated with 10 μM H89 for 45 min and then treated with 1 μM GDC-0941 for 1 h (5th lane). The phosphorylation of S6K1 (pS6K1) at Thr 389 and AKT (pAKT) at Ser 473 were analyzed. Actin was used as loading control. NC denotes normal conditions treated with DMSO. L.E. denotes long exposure and S.E. denotes short exposure. (**B**) HEK293A cells were starved with serum for 16 h without or with 10 μM H89 in the serum free media (2nd and 3rd lane). The phosphorylation of AKT (pAKT) at Ser 473 was analyzed. AKT were used as loading control. (**C**) HEK293A cells were starved of all amino acids for 2 h (2nd lane) or pretreated

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Figure 7. H89 maintains S6K1 and AKT phosphorylation under nutrient depletion conditions. Part 2 of 2

with 10 μM H89 for 45 min in normal conditions before being starved with 10 μM H89 for 2 h (3rd lane). The phosphorylation of S6K1 (pS6K1) at Thr 389 was analyzed. Actin was used as loading control. (**D**) Human bronchial epithelial (hBE) cells were treated with DMSO, 20 nM rapamycin (2nd lane), 150 nM Torin1 (3rd lane), 10 μM H89 (4th lane), 5 nM calyculin A (5th lane) pretreated with 10 μM H89 then 20 nM rapamycin (6th lane), pretreated with 10 μM H89 then 150 nM Torin1 (7th lane), pretreated with 5 nM calyculin A then 20 nM rapamycin (8th lane), or pretreated with 5 nM calyculin A then 150 nM Torin1 (9th lane). The phosphorylation of S6K1 (pS6K1) at Thr 389 and AKT (pAKT) at Ser 473 were analyzed. The phosphorylation of threonine residues was analyzed to indicate phosphatase inhibition. Actin was used as loading control. (**E**) Human bronchial epithelial (hBE) cells were treated with DMSO, 20 nM rapamycin (2nd lane), 150 nM Torin1 (3rd lane), 10 μM H89 (4th lane), 1 μM okadaic acid (5th lane) pretreated with 10 μM H89 then 20 nM rapamycin (6th lane), pretreated with 10 μM H89 then 150 nM Torin1 (7th lane), pretreated with 1 μM okadaic acid then 20 nM rapamycin (8th lane), or pretreated with 1 μM okadaic acid then 150 nM Torin1 (9th lane). The phosphorylation of S6K1 (pS6K1) at Thr 389 and AKT (pAKT) at Ser 473 were analyzed. The phosphorylation of threonine residues was analyzed to indicate phosphatase inhibition. Actin was used as loading control. NC denotes normal conditions treated with DMSO. (**F**) Schematic of how H89 promotes the phosphorylation of S6K1 and AKT. H89 targets a phosphatase that dephosphorylates S6K1 and AKT (*Top panel*). H89, through an unknown target, promotes phosphorylation of S6K1 and AKT (*Bottom panel*). The experiments were performed at least 2 times. FBS = fetal bovine serum, AA = amino acid, Rap = rapamycin, Caly = calyculin A, Oka = okadaic acid.

Moreover, rapamycin inhibits mTORC1, and Torin1 inhibits the activity of both mTORC1 and mTORC2. We propose that H89 could inhibit a phosphatase leading to the increase in S6K1 and AKT phosphorylation (Figure 7E, *Top panel*). Alternatively, H89 could promote the phosphorylation of S6K1 and AKT through an unknown target, such as a kinase (Figure 7E, *Bottom panel*).

H89 has historically been used an inhibitor of PKA [43]; however, AGC kinases can also be inhibited by H89 [62]. AGC kinases like S6K1 and AKT are vital for the downstream signaling cascade of mTORC1 and mTORC2. The overexpression of a multitude of AGC kinases (Figure 6) were unable to effect H89 pretreatment on S6K1. However, future studies using other specific kinase inhibitors might provide more insight into the specific mechanism of H89 [69,70]. H89 may work through an alternative pathway, with one possibility being phosphatase. PP2A phosphatases consist of a core dimer scaffold, a catalytic subunit and a regulatory subunit [71]. PP2A has been shown to regulate major signaling pathways [72]. For example, PP2A has previously been demonstrated to associate with S6K1 [73] and AKT [74], resulting in a negative regulation of the mTOR pathway. Interestingly, inhibition of PP2A occurs under growth factor stimulating conditions [75]. H89 promotes the phosphorylation of mTOR substrates S6K1 and AKT, even under nutrient depleted conditions. Therefore, it is plausible that H89 acts on an unknown target (such as phosphatase) that regulates growth signaling pathways. Alternatively, H89 may act on S6K1 and AKT directly. Future work should investigate if H89 can alter S6K1 and AKT conformation preventing dephosphorylation. Identification of the target of H89 that mediates mTOR signaling will provide insight into mTOR biology and perhaps be a potential therapeutic target.

Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

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Author Contributions

C.H.M. and J.L.J. designed the experiments. C.H.M. conducted the experiments. C.H.M. and J.L.J. wrote the manuscript.

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Abbreviations

DEPTOR, DEP domain-containing mTOR-interacting protein; FACs, fluorescence-activated cell sorting; FKBP12, FK506 binding protein 12; FRB, FKB-rapamycin binding; HEK293A, human embryonic kidney 293A; SGK1, serum and glucocorticoid-regulated kinase 1; TSC2, tuberous sclerosis complex 2; ULK1, Unc-51 like autophagy activating kinase.

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