Ubiquilin-mediated Small Molecule Inhibition of Mammalian Target of Rapamycin Complex 1 (mTORC1) Signaling

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Mammalian target of rapamycin complex 1 (mTORC1) is a master regulator of cellular metabolism, growth, and proliferation. mTORC1 has been implicated in many diseases such as cancer, diabetes, and neurodegeneration, and is a target to prolong lifespan. Here we report a small molecule inhibitor (Cbz-B3A) of mTORC1 signaling. Cbz-B3A inhibits the phosphorylation of eIF4E-binding protein 1 (4EBP1) and blocks 68% of translation. In contrast, rapamycin preferentially inhibits the phosphorylation of p70S6k and blocks 35% of translation. Cbz-B3A does not appear to bind directly to mTORC1, but instead binds to ubiquilins 1, 2, and 4. Knockdown of ubiquilin 2, but not ubiquilins 1 and 4, decreases the phosphorylation of 4EBP1, suggesting that ubiquilin 2 activates mTORC1. The knockdown of ubiquilins 2 and 4 decreases the effect of Cbz-B3A on 4EBP1 phosphorylation. Cbz-B3A slows cellular growth of some human leukemia cell lines, but is not cytotoxic. Thus Cbz-B3A exemplifies a novel strategy to inhibit mTORC1 signaling that might be exploited for treating many human diseases. We propose that Cbz-B3A reveals a previously unappreciated regulatory pathway coordinating cytosolic protein quality control and mTORC1 signaling.

Mammalian target of rapamycin (mTOR)3 is an evolutionarily conserved Ser/Thr kinase that serves as a master regulator of many cellular functions, including mRNA translation, autophagy, and cellular proliferation. mTOR integrates growth signals and the availability of amino acids. Deregulation of mTOR is common in cancer (1), type 2 diabetes (2), and neurodegeneration (3, 4). Inhibition of mTOR prolongs lifespan in many organisms (3, 4). Inhibition of mTOR strongly inhibits phosphorylation of p70S6k at Thr-389. However, the initial phosphorylation of 4EBP1 is rapamycin resistant, so rapamycin has comparatively little effect on mTORC1 signaling. mTORC1 and mTORC2 are targets for autophagy (10). mTORC1 inhibits autophagy by phosphorylating autophagy related protein 13 (ATG13), and UNC-51-like kinases 1 and 2 (ULK1 and ULK2). Phosphorylation of these proteins prevents phosphorylation and activation of FIP2000, an essential protein for autophagosome formation (28–30). Additionally, the lipidation of LC3 to LC3-II is required for the formation of autophagosomes (31). LC3-II migrates to a lower molecular weight than LC3 on a SDS-PAGE, and therefore is typically used to measure autophagy.

Currently, inhibitors of mTOR generally fall within two categories: rapamycin and rapamycin derivatives (rapalogs), and ATP-competitive mTOR kinase inhibitors. Rapamycin binds to mTORC1 as a complex with FKBP12, and will also inhibit mTORC2 activity after prolonged treatment (17, 32–34). Rapamycin strongly inhibits phosphorylation of p70S6k at Thr-389. However, the initial phosphorylation of 4EBP1 is rapamycin resistant, so rapamycin has comparatively little effect on 4EBP1-phosphorylated mTORC1 (10). ATP-competitive mTOR kinase inhibitors inhibit the kinase activity of both mTORC1 and mTORC2 and block the phosphorylation of all mTOR sub-
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strates (35). This class of inhibitors includes Torin 1, Torin 2, OSI-027, AZD-8055, AZD-2014, and INK128 (39).

Our laboratory recently reported that Boc₃Arg (B3A) is a small molecule degradogen that, when coupled to an appropriate recognition ligand, can induce the degradation of a target protein (Fig. 1) (40). B3A-linked trimethoprim and ethacrynic acid increase the degradation of *Escherichia coli* dihydrofolate reductase (eDHFR) and glutathione transferase (GST), respectively, in both cycloheximide-treated cells and cell lysates. B3A-induced degradation is proteasome dependent but does not appear to require ubiquitination of the target protein. We believe that B3A resembles an unfolded peptide and may usurp the cytosolic protein quality control processes.

While further investigating the mechanism of B3A-induced degradation, we unexpectedly discovered that B3A ligands inhibit translation. Here we show that Cbz-B3A blocks translation by inhibiting the mTORC1 pathway in a process that is dependent on the presence of ubiquilins 2 and 4. Unlike rapamycin, Cbz-B3A has a larger effect on the phosphorylation of 4EBP1 than p70S6k.

**Experimental Procedures**

**Materials**—Rapamycin was obtained from Gold Biotechnologies. Cycloheximide, tunicamycin, and thapsigargin were obtained from Sigma. Bortezomib was obtained from Fisher Scientific. Rabbit monoclonal anti-p70S6k (49D7, 2708), anti-p70S6k Thr-381 (108D2, 9234), anti-mTOR (7C10, 2983), anti-mTOR Ser-2448 (D9C2, 5536), anti-mTOR Ser-2481 (2974), anti-eli2α Ser-51 (D9G6, 3398), anti-LC3A/B (4108), anti-AKT Ser-473 (D9E, 4060), anti-AKT Thr-308 (D25E6, 13038), anti-Rheb (E1G1R, 13879), anti-TSC2 (D9F12, 4308), and rabbit polyclonal anti-Rheb2 (4952), and anti-p70S6k Ser-371 (9208) were obtained from Cell Signaling. Rabbit polyclonal anti-HA (ab9110), anti-raptor (ab40758), and anti-ubiquilin 4 (ab106443), mouse monoclonal anti-ubiquilin 2 (ab57150), and anti-eli2α (ab5369) were obtained from Abcam. Mouse anti-actin was obtained from Sigma. IRDye800CW donkey anti-mouse and IRDye680CW donkey anti-rabbit were obtained from Li-Cor. TMP-B3A was synthesized as previously described (40). Cbz-B3A, Ac-B3A, and amine-B3A were synthesized with a final dilution of 1:500,000 and 100 μg/ml of cycloheximide or 6 μM bortezomib. For the recombinant luciferase assay, Quantulum Recombinant Luciferase (Promega) was diluted 1:250,000 in reporter lysis buffer with 1 mg/ml of BSA, then mixed 1:1 with 200 μM compound in the same buffer for a final dilution of 1:500,000 and 100 μM compound in 20 μl. This was incubated for 10 min at room temperature, then measured with a Luciferase Assay System with Reporter Lysis Buffer from Promega according to the manufacturer’s protocol.

For the luciferase half-life experiment, stably transduced luciferase HEK-293T cells were plated in a 96-well plate with a clear bottom at 5,000 cells/well 24 h before the experiment. Cells were incubated for 4 h with the indicated concentrations of Cbz-B3A, then the media was replaced with phenol red-free DMEM supplemented with 10% heat-inactivated FBS (Sigma), then 5 mM compound in the same buffer for a final dilution of 1:500,000 and 100 μM compound in 20 μl. This was incubated for 10 min at room temperature, then measured with a Luciferase Assay System with Reporter Lysis Buffer from Promega according to the manufacturer’s protocol.

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**Real-time PCR**—Real-time PCR was conducted on a CorbettRotor-Gene 6000. RNA was reverse transcribed and cDNA was amplified with iTaq Universal SYBR Green One-Step kit (Bio-Rad) according to the manufacturer’s protocol. Firefly luciferase was amplified using primers 5’-CTCACTGAGCTACATCAGC-3’ and 5’-TCCAGATCCCAAACCTTCCGC-3’. *Renilla* luciferase was amplified using primers 5’-GGAATTAT- AATGCTTATCTACGTGC-3’ and 5’-CTTGCAAAATTGGAAGACCTTTTAC-3’. Actin was amplified using primers 5’- GCCATGGGTCAGAAGGATT-3’ and 5’-GGGGTTGTGGAGGTTCAAA-3’.

**Luciferase Assay—**Stably transduced luciferase HEK-293T cells were plated in 96-well plates 24 h prior to treatment with 100 μM Cbz-B3A, amine-B3A, acetyl-B3A, and Cbz-acetyl. After 4 h, cells were harvested and measured with Luciferase Assay System with Reporter Lysis Buffer from Promega according to the manufacturer’s protocol. Before the addition of luciferin, the protein concentration was measured by removing 2 μl of lysate from each well and adding it to diluted Bio-Rad Protein Assay Dye Reagent Concentrate, and the absorbance was measured at A₅₉₅. The protein concentration was used to normalize the luciferase signal. For translation assays, cells were pretreated for 15 min with 100 μM cycloheximide or 6 μM bortezomib. For the recombinant luciferase assay, Quantulum Recombinant Luciferase (Promega) was diluted 1:250,000 in reporter lysis buffer with 1 mg/ml of BSA, then mixed 1:1 with 200 μM compound in the same buffer for a final dilution of 1:500,000 and 100 μM compound in 20 μl. This was incubated for 10 min at room temperature, then measured with a Luciferase Assay System with Reporter Lysis Buffer from Promega according to the manufacturer’s protocol.

**Retroviral Transduction**—Firefly luciferase and firefly luciferase-eDHFR were cloned into pBABE-puro vector, which was a gift from Hartmut Land, Jay Morgenstern, and Bob Weinberg (Addgene plasmid number 1764). pBABE-puro-luciferase was co-transfected with VSV-G into GP2–293 cells (Retro-X Universal Packaging System, Clonetech, Mountain View, CA) with TransIT-2020 (MirusBio) according to the TransIT-2020 protocol. After 48 h, the viral supernatant was harvested, spun down, filtered with a 45-μm syringe filter, and added to HEK-293T supplemented with 8 μg/ml of Polybrene. After 4 h of infection, the media was replaced with fresh media. 48 h later, media was replaced with media containing 3 μg/ml of puromycin. Media was replaced with fresh media containing puromycin every 3–4 days. After 2 weeks of puromycin selection, the cells were grown out from single cell colonies and screened for luciferase expression.

**Immunoblotting—**Quantification of Western blots was performed with ImageJ (41). Cells were lysed in RIPA buffer (1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS, 10 mM TrisCl, 10 mM β-glycerophosphate, 10 mM sodium pyrophosphate, 50 mM NaF, 200 μM sodium vanadate, 1 mM EDTA, 500 μM EGTA, 1% Benzonase, and supplemented with Complete (Roche Diagnostics) protease inhibitors.)

**Cell Culture and Experiments—**HEK-293T cells and GP2–293 were cultured in DMEM supplemented with 10% heat-inactivated FBS (Sigma), 1× GlutaMax (Gibco), 100 units/ml of penicillin, and 100 μg/ml of streptomycin (Gibco) at 37°C and 5% CO₂. K562 and BaF3/p210 cells were cultured in RPMI supplemented with 10% heat-inactivated FBS (Sigma), 1× GlutaMax, 100 units/ml of penicillin, and 100 μg/ml of streptomycin at 37°C and 5% CO₂.

**Retroviral Transduction—**Firefly luciferase and firefly luciferase-eDHFR were cloned into pBABE-puro vector, which was
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Trypsinized 48 h after transfection and plated into a 96-well plate at 10,000 cells/well. The media was removed 24 h later and replaced by media containing 100 μM Cbz-B3A or DMSO and the cells were treated for 4 h. After treatment, cells were lysed with reporter lysis buffer (Promega), then substrate was added and Renilla luciferase was measured on a luminometer. For the substrate, 10 μl of 10 mM coelenterazine (Gold Biotechnology) dissolved in acidified ethanol was added to 1 ml of Renilla buffer (25 mM Na4PPi, 10 mM NaOAc, 15 mM EDTA, 0.5 mM Na2SO4, 1.0 mM NaCl, pH 5.0).

For [35S]Methionine/Cysteine Incorporation Translation Assay— 80,000 cells/well of HEK-293T cells were plated into 24-well plates 24 h before the assay. Cells were treated with the indicated concentration of compound for 4 h. After 4 h, the media was removed and replaced with DMEM without methionine and cysteine (Life Technologies) supplemented with 0.2 mCi/ml of EXPRESS [35S] protein labeling mix (PerkinElmer Life Sciences), 10% diazylated FBS (Pierce), 1× GlutaMax (Gibco), 100 units/ml of penicillin, 100 μg/ml of streptomycin (Gibco), 1 mM sodium pyruvate (Life Technologies), and the indicated concentration of compound. After 1 h, this media was removed, cells were washed with ice-cold PBS, and lysed with low deoxycholate RIPA buffer (10 mM Tris-Cl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 140 mM NaCl, 1 mM EDTA, 500 μM EGTA, 1× Roche Complete protease inhibitors, 1:100 Benzonase, pH 8.0). Lysate was centrifuged at maximum speed for 20 min at 4 °C. The concentration was measured by a Bradford assay. 10 μg of lysate was added to 100 μl of ice-cold 1 mg/ml of BSA with 0.02% sodium azide. 1 ml of ice-cold 10% TCA was added, the solutions were mixed and incubated on ice for 0.5 h. These mixtures were vacuum filtered onto GF/C Whatman glass microfiber filters. The filters were washed with 2× 5 ml of 10% TCA, then 2× 3 ml of ethanol. The filters were then dried for one-half h. The filters were placed into scintillation vials with 5 ml of scintillation fluid and read on a scintillation counter.

SILAC Lysate—HEK-293T cells were grown in DMEM minus l-lysine and l-arginine (Thermo Scientific Pierce) supplemented with 10% dialyzed FBS for SILAC (Pierce), 1× GlutaMax (Gibco), 100 units/ml of penicillin, 100 μg/ml of streptomycin (Gibco) and either 84 μg/ml of [13C/15N]l-arginine and 146 μg/ml of [13C/15N]l-lysine (Cambridge Isotope Laboratories) or 84 μg/ml of l-arginine and 146 μg/ml of l-lysine (Sigma) at 37 °C and 5% CO2 for a minimum of 6 passages. Cells were lysed by 3 quick freeze/thaws in Dulbecco’s phosphate-buffered saline supplemented with Roche Complete protease inhibitor mixture and 10 mM β-glycerophosphate, 10 mM sodium pyrophosphate, 50 mM NaF, and 200 μM sodium vanadate and used for pulldowns. Mass spectrometry was performed on a Thermo Orbitrap Elite and Thermo XL-ETD Orbitrap microcapillary LC-MS/MS. The false discovery rate was set to 0.01 (1%) for peptide and protein identifications. To determine SILAC ratios, a minimum of two ratio counts between SILAC peptide pairs was required.

Pulldowns—NHS-activated Sepharose 4 fast flow beads (GE Healthcare) were added to screw cap spin columns (Pierce) washed with phosphate buffer. Amine-acetyl or amine-B3A was dissolved in ethanol at 5 mM then diluted 5-fold into 100 mM NaPO4, 150 mM NaCl, pH 7.2, to reach a concentration of 1 mM compound in buffer. 1 mM amine-acetyl or amine-B3A was added to beads and rotated overnight at room temperature. Afterward, the beads were blocked with 100 mM ethanolamine for 3 h. The manufacturer’s protocol was followed thereafter. Briefly, HEK-293T cells were lysed by 3 quick freeze/thaws in Dulbecco’s phosphate-buffered saline supplemented with Roche Complete protease inhibitor mixture and 10 mM β-glycerophosphate, 10 mM sodium pyrophosphate, 50 mM NaF, and 200 μM sodium vanadate. Lysate was added to the beads at 3 μg/μl and rotated for 30 min at room temperature, then washed 3 times with Dulbecco’s phosphate-buffered saline and inhibitors and eluted with 1× Laemmli loading buffer at 65 °C for 20 min.

Cellular Thermal Shift Assay (CETSA)—CETSA was performed as previously described (42). Briefly, HEK-293T cells were lysed in Dulbecco’s phosphate-buffered saline with Roche Complete protease inhibitor by 3 quick freeze thaws, centrifuged at 20,000 × g for 20 min at 4 °C, and the supernatant was adjusted to 4 mg/ml. Lysate was incubated with compound for 30 min and heated to different temperatures in a thermal cycler for 3 min, then cooled to room temperature for 3 min. All samples were then centrifuged at 20,000 × g for 20 min at 4 °C and a Western blot was run on the supernatant.

RNAi Knockdown—ON-TARGETplus SMARTpool siRNA for ubiquitin 1 (catalogue number L012942-00-0005), ubiquitin 2 (L013566-00-0005), and ubiquitin 4 (L021178-01-0005) were obtained from Dharmacon and transfected into HEK-293T cells with DharmaFECT 1 (Dharmacon, Lafayette, CO) according to the manufacturer’s protocol. Cells were harvested 72 h post-transfection and analyzed by Western blot.

Cellular Growth and Cytotoxicity Assays—To determine cellular growth, a CellTiter-Glo luminescent cell viability assay (Promega) was used per the manufacturer’s instructions. To determine cytotoxicity, LDH release was measured with the LDH Cytotoxicity Assay Kit (Pierce) according to the manufacturer’s protocol. For 4-h assays, 96-well plates were seeded at 75,000 cells/ml for BaF3/p210 or K562 cells. For HEK-293T cells, 10,000 cells/well were plated 24 h before treatment. Cells were treated for 4 h at 37 °C and 50 μl was removed from every well and used in the LDH cytotoxicity assay per the manufacturer’s protocol. For 48 h assays, 7,500 cells/ml of BaF3/p210 or K562 cells were incubated with compound at 37 °C for 48 h (200 μl/well). For HEK-293T cells, 1,000 cells/well were plated 24 h prior to compound treatment. After treatment, 50 μl was removed from each well for the LDH assay for all cell lines and 100 μl of mixed cells were removed and used with the CellTiter-Glo kit for K562 and BaF3/p210 cells. For HEK-293T cells, wells were treated as directed with the CellTiter-Glo kit. Cytotoxicity or viability was measured by luminosity and absorbance on a microplate reader.

NCI-60 DTP Human Tumor Cell Line Screen—Screen was conducted as previously described (43), performed at Developmental Therapeutics Program NCI/NIH.

Statistics—All p values were determined by a standard independent 2 sample t test calculated by scipy.stats.ttest_ind within Python 3.4 (programming language).
Results

Cbz-B3A Decreases Luciferase Protein—We chose to investigate the mechanism of B3A-induced degradation in the human embryonic kidney cell line HEK-293T because these cells are easily manipulated and have been widely used to elucidate protein degradation and signaling pathways. We constructed HEK-293T cells that stably expressed firefly luciferase fused to eDHFR (luciferase-eDHFR), as well as firefly luciferase alone (wild type) as a control. Surprisingly, TMP-B3A (Fig. 1) reduced luciferase signal to similar levels in both luciferase-eDHFR and wild-type luciferase cells (Fig. 2a). Firefly luciferase is a promiscuous small molecule binder (44), suggesting that TMP-B3A might bind to luciferase and induce proteasomal degradation as previously reported for the B3A-induced degradation of eDHFR and GST (40). Therefore we removed the TMP recognition ligand to leave the free amino group at the end of the linker (amine-B3A; Fig. 1). Amine-B3A treatment did not cause a significant change in the luciferase signal. However, the positive charge of the free amine is likely to hinder cellular uptake. Therefore we also replaced TMP with the commonly used amine protecting groups carboxybenzyl and acetyl to make Cbz-B3A and acetyl-B3A, respectively (Fig. 1). Both Cbz-B3A and acetyl-B3A decreased the wild type luciferase signal as observed with TMP-B3A (Fig. 2b). In contrast, the luciferase signal was not reduced when cells were treated with Cbz-acetyl, which lacks the B3A tag. These observations indicate the B3A tag is responsible for the decrease in firefly luciferase signal, although the process is distinct from the B3A-induced proteasomal degradation observed previously.

We next wanted to determine whether the reduction in signal was due to inhibition of luciferase activity or reduced levels of luciferase protein. When recombinant firefly luciferase was
incubated with Cbz-B3A, there was no reduction of signal, indicating that Cbz-B3A does not inhibit the enzymatic activity of firefly luciferase (Fig. 2c). Moreover, when cells were treated with Cbz-B3A, the amount of luciferase protein decreased by ~40%, in good agreement with the decrease in luciferase signal (compare Fig. 2, b, d, and e). We also investigated the effect of Cbz-B3A on the levels of luciferase mRNA by quantitative RT-PCR (Fig. 2f). No change was observed in the presence of Cbz-B3A, indicating that the decrease in luciferase protein must arise from either a decrease in synthesis or an increase in degradation.

To determine whether this phenomenon was unique to our firefly luciferase construct, we repeated the assay with HEK-293T cells transfected with Renilla luciferase regulated by the RSV promoter. The Renilla luciferase signal also decreased with Cbz-B3A treatment (Fig. 2g). Thus Cbz-B3A decreased the levels of both luciferase proteins. As observed with firefly luciferase expression, the amount of Renilla luciferase mRNA was not affected by Cbz-B3A treatment. These observations indicate the Cbz-B3A modulates the biosynthesis or degradation of at least two luciferase proteins.

The Cbz-B3A-mediated Decrease in Luciferase Protein Is Not Due to Increased Degradation—To further investigate why luciferase protein levels decrease in the presence of Cbz-B3A, we treated cells with bortezomib to inhibit proteasomal degradation. Surprisingly, bortezomib alone decreased luciferase levels (Fig. 3a). The proteasome inhibitor-induced degradation of luciferase has been reported by others (45). Importantly, bortezomib did not protect firefly luciferase signal from Cbz-B3A treatment. These observations indicate that the Cbz-B3A-induced decrease firefly luciferase does not involve proteasomal degradation (Fig. 3a), further demonstrating that this process must be distinct from the B3A-dependent degradation previously reported (40).

To observe luciferase turnover, cells were treated with cycloheximide to block protein biosynthesis. Luciferase levels decreased in the presence of cycloheximide as expected (Fig. 3a). No further decrease was observed in the presence of Cbz-B3A, which suggested that Cbz-B3A does not increase luciferase turnover. We also measured the half-life of luciferase turnover in the presence and absence of Cbz-B3A (Fig. 3b). Cbz-B3A had no effect on the half-life of luciferase at concen-
trations up to 100 μM. Under these conditions, luciferase signal was reduced in a dose-dependent manner with an EC50 of 8 ± 2 μM (Fig. 3c). These observations indicate that the decrease in luciferase protein is not the result of degradation, and must instead be due to a decrease in protein synthesis.

**Cbz-B3A Inhibits Translation**—We measured the incorporation of [35S]methionine/cysteine into proteins to directly determine the effects of Cbz-B3A on global translation. Intriguingly, Cbz-B3A decreased the incorporation of [35S]methionine/cysteine into protein in a dose-dependent manner (Fig. 3d), with maximal inhibition of 68% observed at 10 μM, and an EC50 of ~3 μM. Thus Cbz-B3A is a strong inhibitor of translation.

**Cbz-B3A Does Not Inactivate elf2α—B3A resembles an unfolded peptide, which suggests that it might trigger the unfolded protein response. Translation is blocked by phosphorylation of the translation initiation factor elf2α during the unfolded protein response, as well as during ER stress, and in response to amino acid starvation (46, 47). Therefore we investigated whether B3A induced the phosphorylation of elf2α. As expected, serum starvation, tunicamycin (induces ER stress and unfolded protein response), and thapsigargin (induces ER stress and unfolded protein response) increased phosphorylation of elf2α (Fig. 3, e and f) (48). However, no increase in elf2α phosphorylation was observed after treatment with Cbz-B3A. Thus Cbz-B3A does not block translation by inducing the phosphorylation of elf2α.

**Cbz-B3A Inhibits the Phosphorylation of 4EBP1**—We next investigated the effect of Cbz-B3A on the translation repressor 4EBP1. The phosphorylation of 4EBP1 by mTORC1 inactivates 4EBP1, allowing translation to initiate. 4EBP1 has multiple phosphorylation sites, the differentiation of which is visible on a Western blot of total 4EBP1 protein (Fig. 4a). Only the hypophosphorylated form (the bottom band) binds to elf2a and blocks translation (37, 49). When cells were treated with 10 μM Cbz-B3A, a clear shift from hyperphosphorylation to hypophosphorylation was observed (Fig. 4a and b). No shift was observed with Cbz-acetyl, which indicates that the B3A tag is required for inhibition of 4EBP1 phosphorylation. The dose-response of 4EBP1 phosphorylation inhibition reached a maximum at 10 μM with an EC50 of ~2 μM, in good agreement with the dose dependence of translation inhibition (compare Fig. 4c and Fig. 3b). Moreover, the accumulation of hypophosphorylation was greater than that observed with saturating rapamycin (Fig. 4, a and b), as expected given that the Thr-37/46 site is rapamycin resistant (35, 37, 38). These observations indicate that Cbz-B3A blocks translation by inhibiting the phosphorylation of 4EBP1.

**Cbz-B3A Inhibits mTORC1 Signaling**—We measured the phosphorylation of p70S6k to further investigate the effects of Cbz-B3A on mTORC1-regulated translation. Cbz-B3A inhibits phosphorylation of p70S6k at Thr-389 (Fig. 5, a and b). The dose response is similar to that observed for the phosphorylation of 4EBP1 (Fig. 5a). Interestingly, Cbz-B3A-treated cells retained ~30% phosphorylation at Thr-389, whereas rapamycin completely blocked phosphorylation of this site. Unlike rapamycin, Cbz-B3A did not inhibit phosphorylation of p70S6k at Ser-371 (Fig. 5, b and c). We also examined the p70S6k catalyzed phosphorylation of mTOR at Ser-2448 (50). Cbz-B3A had only a small effect on the phosphorylation of mTOR at Ser-2448 (Fig. 6, a and b), indicating that the Cbz-B3A-mediated decrease in the phosphorylation of Thr-389 does not inactivate p70S6k. Importantly, the levels of mTOR did not change after treatment with Cbz-B3A (Fig. 6c), nor did Raptor levels (Fig. 6d).

Recent literature suggests that phosphorylation of p70S6k is more important than the phosphorylation of p70S6k in regulating the rate of protein biosynthesis (51). Therefore, Cbz-B3A should be a more effective translation inhibitor than rapamycin. Indeed, although saturating concentrations of Cbz-B3A inhibited translation by 68%, we found that saturating rapamycin inhibited translation by only 35% (Fig. 6, e and f).

The inhibition of mTORC1 activates autophagy, and the amount of autophagy correlates with the amount of LC3 A/B II (52). Cbz-B3A increased LC3 A/B II to equivalent levels as rapamycin (Fig. 6, g and h), indicating that both compounds increase autophagy. This observation is further evidence that Cbz-B3A inhibits mTORC1 signaling.

**FIGURE 4. Cbz-B3A inhibits the phosphorylation of 4EBP1. a** Cbz-B3A reduces 4EBP1 phosphorylation. Anti-4EBP1 Western blot of lysate from HEK-293T cells treated with DMSO, Cbz-Acetyl (10 μM), Cbz-B3A (10 μM), or rapamycin (20 nM) for 4 h. b, quantification of a with hyper representing the top band, mid the middle band, and hypo the bottom band. c, dose response for inhibition of 4EBP1 phosphorylation. Cbz-B3A inhibits 4EBP1 phosphorylation with an EC50 of ~2 μM. The graph represents the quantification of 4EBP1 found in the top band (hyperphosphorylation) and bottom band (hypophosphorylation) in a dilution curve of HEK-293T cells treated with Cbz-B3A for 4 h. Significance was determined in comparison to DMSO; n.s., not significant, p > 0.05; *, p < 0.05; **, p < 0.01; ***, p < 0.001; ****, p < 0.0001.
Cbz-B3A Does Not Inhibit mTORC2—Previously reported inhibitors of mTORC1 also inhibit mTORC2, so we examined the effects of Cbz-B3A on the mTORC2-catalyzed phosphorylation of Akt at Ser-473 (17). No decrease in Akt phosphorylation was observed when cells were treated with Cbz-B3A, indicating that Cbz-B3A only inhibits the mTORC1 complex (Fig. 6, i and j). Akt is also upstream of mTORC1, so this observation also indicates Akt does not mediate the inhibition of mTORC1 by Cbz-B3A.

The phosphorylation of mTOR at Ser-2481 is a marker of the mTORC2 complex (53). There was no change in Ser-2481 phosphorylation of mTOR after treatment with Cbz-B3A, indicating that there was no shift of mTOR from mTORC1 to mTORC2 (Fig. 6a). Thus Cbz-B3A does not decrease the levels of mTOR and Raptor, nor does it change the ratio of mTORC1 and mTORC2.

Cbz-B3A Binds Ubiquilins—We designed a SILAC bead pull-down assay to identify proteins that bind the B3A tag. HEK-293T cells were incubated with either media supplemented with [13C/15N]-l-arginine and [13C/15N]-l-lysine (R6K6) or unlabelled l-arginine and l-lysine (R0K0). B3A beads were incubated with R6K6 lysate and acetyl beads were incubated with R0K0 lysate for a negative control. Proteins were eluted with 1x Laemmli buffer and the eluant was analyzed by mass spectrometry. mTOR and the other components of mTORC1 (Raptor, mLST8, PRAS40, and DEPTOR) were not found in this sample, indicating that mTORC1 does not bind Cbz-B3A directly. 146 proteins were identified with a B3A to acetyl ratio of greater than 2:1, including 47 proteins that were only found in the B3A sample (supplemental Table S1). Ubiquilin 2 was at the top of this list, with seven peptides accounting for 19.9% sequence coverage, including 3 unique peptides. Two homologs of ubiquilin 2, ubiquilin 1 and ubiquilin 4, also bind selectively to the B3A resin, with B3A to acetyl ratios of 7.3:1 and 2.7:1, respectively. Intriguingly, ubiquilin 1 has been reported to bind to mTOR, although the effects of this interaction are unknown (54).

We verified that ubiquilins bind to B3A with immunoblotting. B3A beads bound ubiquilin 1 and ubiquilin 2 sufficiently to deplete the lysate (Fig. 7a). Ubiquilin 4 bound and eluted from B3A beads but did not appear to bind as strongly as ubiquilin 1 and 2. In contrast, no ubiquilin eluted from acetyl beads. mTOR was present in both B3A and acetyl eluants, demonstrating that this interaction was not specific and further indicating that B3A does not bind directly to mTOR.

To further confirm Cbz-B3A interacts with ubiquilins, we performed CETSA (42). In CETSA, the thermal stability of a protein is monitored by incubating lysate at different temperatures. When the protein of interest denatures, it precipitates and is depleted from the soluble fraction. Ligands usually stabilize a protein and are expected to shift the melting curve to higher temperatures, although shifts to lower melting points

![Figure 5. Cbz-B3A inhibits mTOR. a, Cbz-B3A inhibits p70S6k Thr-389 phosphorylation. The graph represents the average and standard deviation of the quantification of anti-Thr-389 phosphorylation Western blots done in three independent replicates. b, Cbz-B3A inhibits Thr-389 but not Ser-371 phosphorylation of p70S6k. p70S6k Western blot of HEK-293T cells treated with DMSO, Cbz-Acetyl (10 μM), Cbz-B3A (10 μM), or rapamycin (20 nM) for 4 h. Total anti-p70S6k and anti-phosphoantibodies against Thr-389 and Ser-371 were used for blotting. c, quantification of Fig. 5b. Bars represent the average and standard deviation of the Western blots shown. No change was observed in total p70S6k protein, although a reduction of phosphorylation explains the compactness of the band. n.s., not significant. **, p < 0.01; ***, p < 0.001.](image-url)
have also been observed (55). When lysate was incubated with Cbz-B3A, there was a clear shift to a lower melting temperature for ubiquilin 4 (Fig. 7b), indicating Cbz-B3A destabilizes this protein. No shift was seen in actin, demonstrating that this was a specific effect. This observation suggests that Cbz-B3A binds to ubiquilin 4 and induces a conformational change. Unfortu-
nately, neither ubiquilin 1 nor ubiquilin 2 melted within the accessible temperature range.

**Cbz-B3A Inhibits mTOR through Ubiquilins**—To address whether the association of Cbz-B3A with ubiquilins mediates the inhibition of mTORC1, we performed RNAi knockdowns of ubiquilin 1, 2, and 4. Ubiquilin 1, 2, and 4 were knocked down 60, 65, and 52%, respectively (Fig. 8, a and b). No off-target or compensating effects were observed on other ubiquilins. Ubiquilin 2 RNAi increased the hypophosphorylated form of 4EBP1 (Fig. 8, c and d), suggesting that ubiquilin 2 activates mTORC1. No change in 4EBP1 phosphorylation was observed with either ubiquilin 1 or ubiquilin 4 knockdown.

We also observed the effect of ubiquilin knockdown in the context of Cbz-B3A treatment. Cells were treated with 3 μM Cbz-B3A so that either an increase or a decrease in 4EBP1 phosphorylation could be detected. The knockdown of both ubiquilin 2 and ubiquilin 4 decreased the ability of Cbz-B3A to block the phosphorylation of 4EBP1, whereas the knockdown of ubiquilin 1 had no effect on Cbz-B3A (Fig. 8, c–e). Thus ubiquilins 2 and 4 mediate the inhibition of mTORC1 by Cbz-B3A.
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Ubiquilins 2 and 4 Do Not Interact with mTOR—We hypothesized that ubiquilin 2 and/or 4 might interact with mTORC1. Because neither mTOR nor any components of mTORC1 were found in SILAC pulldowns, either ubiquilins do not form a stable complex with mTORC1 or B3A disrupts this complex. However, we were unable to detect any ubiquilins in mTOR pulldowns in the absence or presence of Cbz-B3A under a variety of lysis/wash buffers and different lysis methods. We were able to verify that ubiquilin 1 binds the first 670 amino acids of mTOR in a yeast 2-hybrid (54), but only when ubiquilin 1 was the prey. We did not observe a similar interaction with mTOR(1–670) and ubiquilin 4 (data not shown).

We also performed FRET assays to determine whether ubiquilin binds to mTOR within the context of living cells. HEK-293T cells were transfected with ubiquilins 1, 2, or 4 fused to Cerulean as the donor and mTOR fused with Venus as the acceptor. Both the N-terminal and C-terminal fusion proteins were tested. FRET efficiency was measured by FRET after acceptor photobleaching. No evidence of FRET was observed with any of the combinations of ubiquilin and mTOR in the absence or presence of Cbz-B3A (data not shown).

Cbz-B3A Does Not Change Levels of TSC2 or Rheb—Ubiquilins modulate protein degradation, increasing the degradation of some proteins and protecting others (56). However, no change in the levels of mTOR, 4EBP1, p70S6k, or Raptor was observed with Cbz-B3A treatment (see Figs. 4a, 5b, and 6a and d). Two proteins that modulate mTORC1 activity were also enriched in the B3A eluant, Ras homolog enriched in brain (Rheb) and its GTPase activating protein TSC2 (supplemental Table S1) (15). Rheb is a direct upstream activator of mTORC1, whereas TSC2 inhibits Rheb action (14, 15). Rheb was ranked 109 with a B3A:acetyl ratio of 2:5:1. TSC2 ranked 22nd and was only found in the B3A eluant. No effect was observed on the quantity of either of these proteins after cells were treated with Cbz-B3A (Fig. 9a, f and g).

Cbz-B3A Slows Cellular Growth—Because inhibition of mTORC1 slows cellular proliferation (57), we investigated the proliferation of several different cell lines after treatment with Cbz-B3A. The proliferation of HEK-293T, K562, and BaF3/p210 cells, measured by CellTiter Glo, was significantly slowed by treatment with as little as 1 μM Cbz-B3A for 48 h. Maximum growth inhibition was seen at 10 μM Cbz-B3A treatment. Proliferation of HEK-293T, K562, and BaF3/p210 was inhibited by 64, 52, and 68%, respectively (Fig. 9a). Importantly, Cbz-B3A was not cytotoxic to the cells as measured by the release of lactate dehydrogenase (Fig. 9b).

Cbz-B3A was also evaluated in the NCI-60 DTP Human Tumor Cell Line Screen (43). The average decrease in proliferation was 29% for all cell lines after 48 h treatment with 10 μM Cbz-B3A. The inhibition of proliferation for K562 cells was 39%, consistent with CellTiter Glo findings (Fig. 10). The most sensitive cell lines were MOLT-4 and SR with a decrease in proliferation of 80 and 83%, respectively (Fig. 9c). MOLT-4 and SR cell lines were also more sensitive to 10 μM Cbz-B3A than 10 μM rapamycin. This is consistent with other reports that show MOLT-4 may be dependent on mTORC1 for cellular growth (58). Both MOLT-4 and SR cell lines derive from leukemia patients and Cbz-B3A has the largest effect within this category of cancer (Fig. 10).

Discussion

Taken together, our experiments show that Cbz-B3A inhibits its translation by blocking the phosphorylation of 4EBP1. Although 4EBP1 appears to be a substrate for several protein kinases, the majority of 4EBP1 phosphorylation occurs via the action of mTORC1. Cbz-B3A also inhibits two mTORC1-regulated events, the phosphorylation of p70S6k and autophagy. These observations strongly suggest that mTORC1 signaling is the target. However, Cbz-B3A does not appear to interact directly with mTORC1. Instead, inhibition is mediated by ubiquilins 2 and 4, although the exact mechanism remains to be determined. Unlike other mTOR inhibitors, Cbz-B3A efficiently blocks 4EBP1 phosphorylation but only partially inhibi-
the phosphorylation of p70S6k. In contrast, rapamycin, preferentially inhibits the phosphorylation of p70S6k by mTORC1, whereas Torin inhibits the phosphorylation of all mTOR substrates in the contexts of both mTORC1 and mTORC2. Cbz-B3A does not inhibit mTORC2 under the conditions of our experiments, although it is possible that mTORC2 inhibition might occur with extensive incubation, as observed with rapamycin. Both our data and previous literature suggest that the phosphorylation of 4EBP1 is more important than p70S6k phosphorylation for translation regulation (51). We find that Cbz-B3A inhibits a larger fraction of translation than rapamycin even though Cbz-B3A does not inactivate p70S6k. Torin 1 has little effect on translation in 4EBP1 double knock-out cells (51). Thus it follows that 4EBP1 is the critical downstream effector of mTORC1 for translation control.

Ubiquilins have a well recognized role in protein homeostasis, and our results suggest that ubiquilins may provide a previously unappreciated link between mTOR signaling and cytosolic protein quality. All ubiquilins contain a ubiquitin-like (Ubl) domain at the N terminus and a ubiquitin association (Uba) domain at the C terminus. There is strong evidence that both ubiquilin 1 and ubiquilin 2 interact with the proteasome through the Ubl domain (59) and that ubiquilin 1 binds polyubiquitinated chains through the Uba domain (60, 61). Furthermore, these proteins play a role in diseases caused by protein aggregation in neurodegeneration (62–65), which suggests that ubiquilins are involved in protein homeostasis within the cytoplasm. Additionally, ubiquilins are involved in autophagy mediated protein degradation (66, 67). Ubiquilin 4 associates with LC3 and facilitates the localization of ubiquilin 1 to autophagosomes (68). Ubiquilin 1 also binds to mTOR(1–670), although overexpression of ubiquilin 1 has no apparent effect on mTOR activity (54). We show that the knockdown of ubiquilin 2 decreases 4EBP1 phosphorylation, indicating that ubiquilin 2 activates mTOR. Both ubiquilin 2 and ubiquilin 4 mediate the inhibition of mTORC1 by Cbz-B3A. However, we have not observed an interaction between mTOR and ubiquilin 2 or ubiquilin 4 in co-immunoprecipitates, FRET assays, or yeast 2-hybrid experiments. Both the mTORC1 activator Rheb, and Rheb inhibitor TSC2 were found in the B3A eluant, indicating that it is possible that ubiquilin is mediating mTORC1 inhibition through these proteins, although the mechanism is still unclear. It is also possible that the decrease in 4EBP1 phosphorylation results from activation of a phosphatase rather than inhibition of the mTORC1 kinase. How Cbz-B3A and ubiquilins interact to inhibit mTORC1 signaling remains to be elucidated.

mTOR is a target for the treatment of many diseases, including cancer, diabetes, neurodegeneration, as well as aging. Rapamycin is used as an immunosuppressive and in cancer combination therapy, and torins have entered cancer clinical trials. Rapamycin is also beneficial in animal models of Parkinson, Huntington, and Alzheimer diseases (69), and prolongs lifespan in yeast, worms, fruit flies, and mice (5–8). Many of these effects have been attributed to the inhibition of translation (7, 70). The inhibition of 4EBP1 phosphorylation in partic-
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ular is believed to mediate the effects of rapamycin on cancer (71) and Parkinson disease (72). Cbz-B3A provides a novel strategy to block translation via 4E BP1 phosphorylation that may also be an effective treatment for these devastating diseases.

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