The mammalian target of rapamycin complex 1 (mTORC1) integrates multiple signals from growth factors, nutrients, and cellular energy status to control a wide range of metabolic processes, including mRNA biogenesis; protein, nucleotide, and lipid synthesis; and autophagy. Deregulation of the mTORC1 pathway is found in cancer as well as genetic disorders such as tuberous sclerosis complex (TSC) and sporadic lymphangioleiomyomatosis. Recent studies have shown that the mTORC1 inhibitor rapamycin and its analogs generally suppress proliferation rather than induce apoptosis. Therefore, it is critical to use alternative strategies to induce death of cells with activated mTORC1. In this study, a small-molecule screen has revealed that the combination of glutaminase (GLS) and heat shock protein 90 (Hsp90) inhibitors selectively triggers death of TSC2-deficient cells. At a mechanistic level, high mTORC1-driven translation rates in TSC1/2-deficient cells, unlike wild-type cells, sensitizes these cells to endoplasmic reticulum (ER) stress. Thus, Hsp90 inhibition drives accumulation of unfolded protein and ER stress. When combining proteotoxic stress with oxidative stress by depletion of the intracellular antioxidant glutathione by GLS inhibition, acute cell death is observed in cells with activated mTORC1 signaling. This study suggests that this combination strategy may have the potential to be developed into a therapeutic use for the treatment of mTORC1-driven tumors.

Significance

The mammalian target of rapamycin complex 1 (mTORC1)-mediated signaling regulates protein translation, cell size/growth, cell survival, and metabolism. This signaling is commonly deregulated in cancer as well as genetic disorders such as tuberous sclerosis complex and sporadic lymphangioleiomyomatosis. Recent studies have shown that the mTORC1 inhibitor rapamycin and its analogs generally decrease proliferation rather than inducing cell death. In this study, we found a strategy that rapidly triggers death of cells with activated mTORC1-mediated signaling by using the combination of aminohydrolase enzyme glutaminase and chaperone protein heat shock protein 90 inhibitors. We believe this combination strategy may have potential to be developed into therapeutic use for the treatment of mTORC1-driven tumors.

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potentially be developed into clinical use for mTORC1-driven cancers with the potential for improved outcomes over cytostatic rapamycin-based therapies.

Results

A Targeted Hsp90 Inhibitor Sensitization Screen in a TSC Cell Line Model. To evaluate the possibility that inhibition of proteins involved in energy metabolism might modulate the cellular response to the Hsp90 inhibitor 17-N-allylamino-17-demethoxygeldanamycin (17AAG) and to assess this possibility in a relatively unbiased fashion, we conducted a sensitization screen using 17AAG and a small-molecule library in Tsc2−/− MEFs. The library was composed of known inhibitors of glycolysis, glutaminolysis, fatty acids synthesis/oxidation, nucleotide synthesis, and kinase activity (Table S1). 17AAG has been reported to inhibit mTORC1 signaling (14).

In agreement, we also observed decreased S6K1 phosphorylation at a high concentration of 1 μM after 72 h of treatment (Fig. 1A). To ensure that the mTORC1 was ON, we performed dose–response experiments by assessing the phosphorylation of the ER stress marker [PKR-like ER kinase (PERK) at T980] and the mTORC1 substrate (S6K1 at T389). We found increased phosphorylation of PERK along with sustained S6K1 phosphorylation at a low dose of 17AAG (Fig. 1 and Fig. S1B). Accordingly, we performed the screen using 0.3 μM 17AAG, a dose in which we effectively induced ER stress [elevated phosphorylated PERK (P-PERK)] under the condition of sustained mTORC1 activation (high P-S6K1) (Fig. 1A). At this concentration of 17AAG, we observed minimum effects on the viability of Tsc2−/− MEFs (see Fig. 3A). Cells were treated with three different concentrations of small molecules in combination with either 17AAG or the drug vehicle DMSO in a 384-well plate format. After 3 d of continuous culture in the presence of 17AAG, cell viability was determined by using the CellTiter-Glo (Promega), and the effect of compounds on the cellular response to 17AAG was estimated by comparing the viability data between drug- and vehicle-treated plates. After data normalization, we classified the effect of each compound upon 17AAG sensitivity according to fold change in cell viability. As shown by others, taxol (positive control) was found to sensitize Tsc2−/− MEF to 17AAG (15). Interestingly, the GLS inhibitor bis-2-(5-phenylacetamido-1,2,4-thiadiazol-2-yl)ethyl sulfide (BPTES) (16) caused the most significant effect (threefold increase) (Fig. 1B and Fig. S1A). GLS is the enzyme that generates glutamate from glutamine in the first step of a process termed glutamine anaplerosis, which plays

Fig. 1. A targeted small-molecule screen identifies GLS inhibition to sensitize Tsc2−/− MEFs to Hsp90 inhibition. (A) Phosphospecific intensities for PERK and S6K1 in Tsc2−/− MEFs treated with increasing concentrations of 17AAG as indicated for 72 h. The intensities were calculated by using the LiCOR-Odyssey infrared imaging system. The mean is shown; error bars represent SEM (n > 3). (B) Molecules identified to sensitize Tsc2−/− to 17AAG (0.3 μM) at a cutoff of twofold. (C) A diagram showing the enzymes involved in glutamine anaplerosis and GSH production and the inhibitors used in this study (see text for more details). (D) Glutamine consumption of Tsc2−/− MEFs after 72 h of treatment with DMSO, 17AAG (0.5 μM), BPTES (10 μM), or BPTES plus 17AAG. The mean is shown; error bars represent SEM (n > 3). (E) Cell viability of Tsc2−/− MEFs using the CellTiter-Glo. Relative luminescence was measured in Tsc2−/− MEFs after 72 h of treatment with increasing concentrations of 17AAG with or without BPTES as indicated. The mean is shown; error bars represent SEM (n > 3).
a major role in cancer (Fig. 1C) (17). Glutamine uptake rates were significantly reduced in BPTES-treated cells (Fig. 1D). Importantly, there is an increased sensitivity to BPTES at low concentration of 17AAG starting at 0.25 μM (Fig. 1E).

**Inhibition of Glutamine Anaplerosis and Hsp90 Causes Potent Apoptosis in Tsc2−/− Cells.** To assess the magnitude of 17AAG sensitivity in the presence of BPTES on cell viability, we examined cell morphology using microscopy analysis. Phase-contrast imaging confirmed decreased cell viability of Tsc2−/− MEFS treated with BPTES plus 17AAG by 48 h. This effect was more pronounced after 72-h treatment compared with single-drug-treated or vehicle-treated cells (Fig. 2A). Moreover, to elucidate the biological consequences of combined BPTES and 17AAG, we performed transmission electron microscopy (TEM). Within 24 h, BPTES plus 17AAG induced profound morphological changes of subcellular components (Fig. 2C). The dual-combination treatment induced an accumulation of lipid droplets, which are commonly found in cells undergoing apoptosis (Fig. 2B) (18). The ER and mitochondria can both act as a source of membranes for autophagosomes (19–21). In our study, BPTES plus 17AAG induced an accumulation of double-membrane autophagosomes (Fig. 2C), lysosomes (L), and late endosomes (LE) (Fig. 2D), suggesting that the autophagy-lysosomal system is active in cells treated with BPTES and 17AAG. Supporting this idea, we observed a decrease in the level of p62, a marker of autophagy (Fig. S2).

Targeted therapies are anticipated to be more effective in inducing selective death of cancer cells over normal cells. Thus, we compared the viability of Tsc2−/− MEFS with Tsc2−/− MEFS treated with increasing doses of 17AAG with or without BPTES. Strikingly, the treatment with combined BPTES and 17AAG was more toxic in Tsc2−/− MEFS than Tsc2−/− MEFS at lower doses of 0.25 and 0.5 μM (Fig. 3A). Similarly, decreased cell viability with BPTES (10 μM) plus 17AAG (0.25 and 0.5 μM) was also observed in ELT3 cells, but not in TSC2-reexpressing ELT3 cells (Fig. S3A). To note, single treatment with 17AAG at a concentration of 1 μM decreased the viability of Tsc2−/− MEFS and was not selective when combined with BPTES (Fig. 3A). This result is likely due to UPR-induced cell death as a result of excessive ER stress (10), as suggested by the large increase in P-PERK at this concentration (Fig. 1A). The increased cell death in Tsc2−/− MEFS treated with BPTES plus 17AAG nicely correlated with the cleavage of poly(ADP-ribose) polymerase (PARP), a robust and reliable apoptosis marker (22) (Fig. 3B and Fig. S3C). In contrast, no obvious cleavage of PARP was detected in Tsc2−/− MEFS at these doses (Fig. 3B), and the cleavage of PARP was also reduced in Tsc2−/−-reexpressing Tsc2 cells (Fig. S3 D and E). Another GLS inhibitor, molecule 968 (23), also increased sensitivity of Tsc2−/− MEFS to 17AAG (Figs. 3B and 4A). In addition, BPTES treatment yielded similar results in combination with either BIIB021 or AUY922, two structurally unrelated Hsp90 inhibitors (Fig. S3G). Finally, the RNA intereference (RNAi)-mediated knockdown of GLS in Tsc2−/− MEFS induced the cleavage of PARP in the presence of 17AAG (Fig. S3F). Together, our data demonstrate a clinically relevant role of GLS during Hsp90 inhibition in Tsc2−/− cells.

**Hsp90 Inhibition Reverses Rapamycin-Insensitive mTORC1 Phenotypes.** Rapamycin has been shown to promote tumor regression when combined with Hsp90 inhibitors in two models of Kras-driven tumors (24). This finding prompted us to assess the efficacy of this combination in our Tsc2−/− model in vitro. For this purpose, we treated Tsc2−/− MEFS with combined rapamycin (20 ng/mL) and 17AAG (1 μM) for 2 and 24 h. Interestingly, 17AAG was able to abrogate rapamycin-insensitive processes downstream of mTORC1 (Fig. 1C). Accordingly, prolonged rapamycin treatment (24 h) is not able to suppress the mTORC1-dependent phosphorylation of 4EBP1 in a variety of cell lines (25). We reproduced these observations in Tsc2−/− MEFS and the patient angiomyolipoma-derived TSC2−/− 621-101 cells (Fig. 3C and Fig. S3H). However, combined

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**Fig. 2.** The combined inhibition of GLS and Hsp90 results in decreased viability and morphological changes of Tsc2−/− MEFS. (A) Tsc2−/− MEFS were treated with DMSO, 17AAG (0.5 μM), rapamycin (20 ng/mL), and BPTES (10 μM) as indicated for 48 and 72 h. Phase microscopy was used to observe cell viability. (B) Transmission electron microscopy (TEM) images (9,300 × 1.4x) of Tsc2−/− MEFS after 24 h of treatment with DMSO, 17AAG (0.5 μM), BPTES (10 μM), or BPTES plus 17AAG. Black arrows indicate mitochondria, and asterisks indicate lipid droplets. (C) TEM image (23,000 × 1.4x) showing a double-membrane autophagosome in Tsc2−/− MEFS treated with combined BPTES (10 μM) and 17AAG (0.5 μM). (D) TEM images depicting a lysosome (L) and late endosome (LE) in Tsc2−/− MEFS treated with combined BPTES (10 μM) and 17AAG (0.5 μM).
17AAG and rapamycin exhibited reduced phosphorylation of 4EBP1 after 24 h of treatment (Fig. 3C and Fig. S3H). Moreover, rapamycin is known to mildly induce autophagy in mammalian cells (26). Similar to 4EBP1 de-repression, treatment of Tsc2<sup>−/−</sup> MEFs with 17AAG plus rapamycin for 24 h resulted in increased cleavage of LC3B (LC3B-II) and decreased p62, two major autophagy markers (Fig. 3D). Microscopy analysis revealed that, although proliferation of Tsc2<sup>−/−</sup> MEFs was decreased after 48 h of treatment with 17AAG plus rapamycin, cells did not grow, but remained viable after 72 h of treatment (Fig. 2A). Therefore, this combination seems unlikely to be effective in our model. However, we do not exclude the possibility that a different phenotype might occur in a different cell context or by using specific in vivo systems. Together, these observations support our approach that inducing proteotoxic stress in cells with mTORC1 ON will prove to be more effective at inducing toxicity than when inhibiting the mTORC1 pathway.

**Glutamate Dehydrogenase Inhibition Does Not Sensitize Cells to Hsp90 Inhibition.** Glutamate, the product of the GLS-catalyzed reaction, is deaminated to generate alpha-ketoglutarate (αKG) by the action of glutamate dehydrogenase (GDH) (Fig. 1C). We therefore tested whether epigallocatechin-3-gallate (EGCG), a GDH inhibitor (27), also increased sensitivity to Hsp90 inhibition. We recently validated the action of EGCG on GDH activity in Tsc2<sup>−/−</sup> MEFs (28). We found that the combination of EGCG and 17AAG neither affected the viability of Tsc2<sup>−/−</sup> nor induced a significant cleavage of PARP (Fig. 4A and B). Similar results were observed in GDH-depleted cells treated (6) with 17AAG (Fig. S44). Thus, our data show a dependency on glutamate to promote cell survival during Hsp90 inhibition. Accordingly, glutamate intracellular levels were significantly reduced in Tsc2<sup>−/−</sup> MEFs as a result of GLS inhibition or RNAi-mediated GLS silencing (Fig. 4C). The addition of glutamate to BPTES plus 17AAG-treated Tsc2<sup>−/−</sup> MEFs abrogated the induction of apoptosis, as assessed by PARP cleavage (Fig. 4E), thus confirming the role of glutamate in the dual-treatment–triggered apoptosis. Nonetheless, we noticed that the addition of glutamate did not completely rescue the viability of the cells (Fig. 4D and Fig. S4B), which could be due to the permeability and/or stability of glutamate. Alternatively, 17AAG plus BPTES may also affect cysteine and/or glyoxal metabolism or the key enzymes involved in the synthesis of GSH (see below) (Fig. S5B).

As stated above, glutamate serves as a carbon donor for αKG production, an intermediate of the tricarboxylic acid cycle, a metabolic hub with central importance in both energy production and biosynthesis. Importantly, pyruvate and dimethyl-αKG (DM-αKG), a cell-permeable form of αKG, were not able to abolish the cleavage of PARP in BPTES plus 17AAG-treated cells (Fig. 4F). Thus, our results suggest that a metabolite derived from glutamate other than αKG is involved in the modulation of cell viability as a result of combined BPTES and 17AAG treatment.

**Deregulated Redox Balance Is Responsible for the Apoptosis Induced by BPTES and 17AAG.** Besides the role in energy production and macromolecule synthesis, glutamine metabolism (via glutamate) buffers against oxidative damage through GSH, the major intracellular antioxidant (Fig. 5A). Glutamine deprivation or RNAi-mediated silencing of GLS suppresses GSH pools and results in increased reactive oxygen species (ROS) (29–31). In agreement with these observations, we found decreased GSH levels in Tsc2<sup>−/−</sup> MEFs treated with combined BPTES and 17AAG (Fig. 5B). The dual treatment also resulted in increased ROS levels compared with single treatments (Fig. 5C). ER stress triggers intraluminal calcium release, which promotes mitochondrial membrane depolarization and ROS production (32, 33). ROS further promotes protein misfolding, thereby enhancing ER...
viability of stressed cells was abolished by BPTES plus 17AAG on cell death (Fig. 5A). Most importantly, GSH (Fig. 5E) and vitamin C, was able to abrogate the cleavage of PARP with BPTES plus 17AAG (Fig. 5G). Glutamate concentrations used for the compounds were BPTES (10 μM), glutamate (4 mM), pyruvate (1 mM), and DMSO (0.25 M) for 72 h. A siRNA targeting GLS was used as a control. The mean is shown; error bars represent SEM (% Cell death) (Fig. 6A). Immunoblot analysis of cleaved PARP and α-tubulin in Tsc2−/− MEFs treated with the indicated compounds for 24 h. The concentrations used for the compounds were BPTES (10 μM), glutamate (4 mM), pyruvate (1 mM), and DM-αKG (7 mM).

**Dual Inhibition of GLS and Hsp90 Causes Regression of TSC2-Deficient ELT3 Cell Xenograft Tumors.** To evaluate the role of GLS and Hsp90 inhibition in cells with mTORC1 hyperactivation in vivo, we used an ELT3 cell-xenograft model. We treated mice bearing ELT3-luciferase-expressing xenograft tumors with BPTES and 17AAG as single treatments or in combination. Drug toxicity was first evaluated by body-weight changes, and no obvious effect was observed after the drug treatments (Fig. 6A). We next assessed the possible benefits of combination treatment on tumor development and found that xenograft tumor size for 3 wk significantly declined (Fig. 6B; P < 0.05). Subsequently, in comparison with the vehicle control, single treatment of either BPTES or 17AAG for 3 wk reduced tumor growth by ~0.35-fold in bioluminescence intensity (Fig. 6C and D; P < 0.05). More importantly, the combination of BPTES and 17AAG led to a >25-fold reduction in xenograft tumor size (Fig. 6D). Therefore, these results suggest that dual inhibition of GLS and Hsp90 may be a promising strategy for the treatment of TSC2-deficient tumors.

**GSH is synthesized through two reactions.** First, γ-glutamylcysteine is synthesized from glutamate and cysteine via the glutamate cysteine ligase (GCL). GCL is a heterodimeric enzyme composed of a catalytic (GCLC) and modulatory (GCLM) subunit. GCLC constitutes all of the enzymatic activity, whereas GCLM increases the catalytic efficiency of GCLC. Second, glycine is added to the C-terminal of γ-glutamylcysteine via the enzyme GSH synthetase (GSS) (Fig. 5A). The RNAi-mediated knockdown of either GCLC or GSS sensitized Tsc2−/− MEFs to Hsp90 inhibition, as evidenced by increased cleavage of PARP.

**Induction of apoptosis was prevented by treatment with GLS and Hsp90.** To evaluate the role of GLS and Hsp90 in the xenograft model, we treated mice bearing xenograft tumors with the indicated compounds for 72 h. The mean is shown; error bars represent SEM (% Cell death) (Fig. 6A). Immunoblot analysis of cleaved PARP and α-tubulin in Tsc2−/− MEFs treated with the indicated compounds for 24 h. The concentrations used for the compounds were BPTES (10 μM), glutamate (4 mM), pyruvate (1 mM), and DM-αKG (7 mM).

**Dual Inhibition of GLS and Hsp90 Causes Regression of TSC2-Deficient ELT3 Cell Xenograft Tumors.** To evaluate the role of GLS and Hsp90 inhibition in cells with mTORC1 hyperactivation in vivo, we used an ELT3 cell-xenograft model. We treated mice bearing ELT3-luciferase-expressing xenograft tumors with BPTES and 17AAG as single treatments or in combination. Drug toxicity was first evaluated by body-weight changes, and no obvious effect was observed after the drug treatments (Fig. 6A). We next assessed the possible benefits of combination treatment on tumor development and found that xenograft tumor size for 3 wk significantly declined (Fig. 6B; P < 0.05). Subsequently, in comparison with the vehicle control, single treatment of either BPTES or 17AAG for 3 wk reduced tumor growth by ~0.35-fold in bioluminescence intensity (Fig. 6C and D; P < 0.05). More importantly, the combination...
treatment of BPTES and 17AAG for 3 wk completely suppressed xenograft tumor progression and resulted in tumor regression (Fig. 6 C and D; *P* < 0.05). Immunohistochemical staining showed that the combination treatment of BPTES and 17AAG resulted in lower levels of cell-proliferation marker proliferating cell nuclear antigen (PCNA), indicating the reduced growth in tumors relative to either agent alone (Fig. 6E). We also showed that the combination treatment induces increased apoptotic cell death compared with single-agent treatment using the TUNEL assay (Fig. 6F).

**Discussion**

mTORC1 is an evolutionarily conserved serine/threonine kinase complex that controls cell growth and metabolism in response to nutrients, growth factors, and cellular energy levels. Because numerous oncogenes and tumor suppressors control the activation of mTORC1, deregulation of this pathway is frequently observed in cancers and other diseases. Therefore, targeting the mTORC1 pathway became an attractive therapeutic approach. Preclinical and clinical studies have shown that the mTORC1 inhibitor rapamycin and its analogs are cytostatic rather than cytotoxic. Although rapamycin treatment resulted in decreased tumor size, tumors quickly returned to their initial size shortly after treatment withdrawal (3, 4). Thus, there is an urgent need to develop an alternative way to rapidly induce death in cells with activated mTORC1. In this study, we took an approach by focusing on the observation that cells with activated mTORC1 via the loss of TSC1/2 are highly sensitive to proteotoxic stress. Our primary goal was to identify new determinants of cell sensitivity.
Interestingly, through our small-molecule screen, we found that inhibition of GLS sensitizes Tsc2−/− cells to Hsp90 inhibition by decreasing the intracellular antioxidant GSH and further increasing the oxidative stress. Hsp90 inhibition is considered a potential anticancer strategy, and to date, there are 17 distinct Hsp90 inhibitors in clinical trials. Studies have shown that tumors stopped growing when Hsp90 inhibitors were given to animals bearing human tumors. However, similar to rapamycin therapy, the tumors regained their growth capacity after the Hsp90-inhibitor treatment was stopped (36). Therefore, Hsp90 inhibitors or rapamycin may have limited use as a monotherapy. On the contrary, combination therapies offer potential benefits for inhibiting multiple targets and signaling pathways to effectively kill cancer cells and preventing/delaying the emergence of drug resistance (36, 37). The use of combination therapies is supported by recent observations demonstrating that HER2+ breast cancer tumors are more responsive to Hsp90 inhibition when tanespimycin (a geldanamycin analog) was combined with trastuzumab. The effectiveness of this combination could be due to potent target degradation, and it may overcome or delay the initial resistance to trastuzumab (38).

De Raedt et al. demonstrated that the combination of rapamycin and Hsp90 inhibitors induced tumor regression in Kras-driven tumor models (24); however, this combination did not seem to be effective in our cell system (Fig. 2). Clinical trials are testing the efficacy of geldanamycin analogs in combination with other chemotherapeutics agents (clinicaltrials.gov/show/NCT01362400). One other major pitfall with the Hsp90-targeted therapeutics is that Hsp90 also participates in normal cellular physiology, and high doses of these compounds may result in toxicity due to

**Fig. 6.** Dual inhibition of GLS and Hsp90 causes a regression of xenograft tumor development. (A) Female CB17-scid mice were inoculated with ELT3-luciferase cells s.c. Mice were treated with vehicle, BPTES, 17AAG, or combined BPTES and 17AAG for 3 wk. Body weight was measured every week. (B) Tumor area was measured weekly by using a digital caliper. The left y axis indicates the relative fold growth of tumor size vs. the baseline measurement before drug treatment. (C and D) Bioluminescent intensity in xenograft tumors was recorded and quantified weekly. The left y axis indicates the relative tumor growth vs. the baseline quantification before drug treatment. (E) Representative images of immunohistochemical staining of cell proliferation marker PCNA in tumors from mice treated with vehicle, BPTES, 17AAG, or combined BPTES and 17AAG. Percentage of cells with nuclear immunoreactivity of PCNA was scored from four to six random fields per section. **P < 0.01, Student t test. (F) Representative images of tumor sections labeled by TUNEL, a method for detecting apoptotic cell death, in tumors from mice treated vehicle, BPTES, 17AAG, or BPTES plus 17AAG.
off-target or increased on-target effects (39). In our combination study, we used low doses of Hsp90 inhibitor that were able to mildly induce the activation of the ER-stress pathway (Fig. 1), while not affecting the viability of the cells. This method could be potentially translated to a better response in the clinic by reducing the toxicity that was previously associated with single treatment. There are several chemically unrelated Hsp90 inhibitors that have unproven toxicity profiles in the clinical development (39). Thus, it will be interesting to test these structurally distinct inhibitors when combined with GLS inhibitors. Moreover, it will also be worthwhile to identify other specific GLS inhibitors and test their efficacy to sensitize cells to different Hsp90 inhibitors. Glutamine anaplerosis inhibition has recently been shown to result in decreased mTORC1 signaling in the human osteosarcoma cell line U2OS (40). Such an effect was not observed in Tsc2−/− MEFs given their constitutive activation of mTORC1 (Fig. 3B). However, GLS inhibition resulted in decreased P-S6K1 in Tsc2−/− MEFs, which were less sensitive to dual GLS and Hsp90 inhibition (Fig. 3 A and B), thus supporting the notion that maintaining mTORC1 ON is an effective way to induce cell death. Finally, other agents that enhance ER stress, stimulate ROS production, or inhibit GSH synthesis could also be explored.

We have demonstrated a promising synthetic lethality strategy by targeting Hsp90 and GLS in vitro and in a TSC-xenograft tumor model. Although we used Tsc2−/− deficient cells as a tool to study cells with hyperactive mTORC1, future studies should assess the efficacy of the combination of GLS and Hsp90 inhibitors in a broader spectrum of cancer cells. We anticipate that this combination approach may have significant benefits in slowing mTORC1-driven tumor progression, including those characterized by the loss- or gain-of-function mutations of upstream regulators of mTORC1, including PTEN and PIK3CA, respectively. Further investigation is warranted to evaluate the therapeutic efficacy.

**Experimental Procedures**

**Cell Lines and Culture.** Tsc2−/− p53−/− and Tsc2−/− p53−/− MEFs were kindly provided by Brendan Manning and David Kwiatkowski (Harvard Medical School, Boston). ELT3 cells were provided by Cheryl Walker, Texas A&M Health Science Center Institute of Biosciences and Technology, Houston. ELT3-luciferase cells were described (40). MEFs and ELT3 cells were cultured in DMEM supplemented with 10% (vol/vol) FBS (Invitrogen) and dialyzed for experiments (Gibco). All extra energetic additives that are often added to some DMEM formulations such as sodium pyruvate and succinate were excluded. The human renal angiomylolipoma-derived cells (621-101 cells) were cultured in IIA complete medium, 50/50 mixture of Dulbecco’s modified Eagle medium/Ham F12 (Sigma D8062) supplemented with sodium selenite (5 × 10−8 mol/L), insulin (25 μg/mL), hydrocortisone (2 × 10−7 mol/L), transferrin (10 μg/mL), triiodothyronine (1 × 10−3 mol/L), vasopressin (10 μU/mL), cholesterol (1 × 10−8 mol/L), ferrous sulfate (1.6 × 10−6 mol/L), epidermal growth factor (10 ng/mL), and 10% FBS.

**Small-Molecule Screening.** Tsc2−/− p53−/− MEFs were cultured in DMEM with 10% FBS and penicillin/streptomycin. After overnight culturing, pin-transfer of the small-molecule library was performed at the Institute of Chemistry and Cell Biology-Longwood screening facility. At 72 h after compound addition, the plates were allowed to equilibrate to room temperature for 1 h. Then, 30 μL of CellTiter-Glo (Promega) was added to each well. The plates were allowed to sit for 1 min before being read on the Envision multilabel plate reader (Perkin-Elmer).

**GSH Measurement.** Cells grown in 6- or 12-well plates were harvested and trypsinized. The trypsinized cells were then resuspended in 0.5 mL of PBS containing 1% FBS and incubated with 40 μM monobromobimane (Biochemika) for 10 min at room temperature. After incubation, cells were placed on ice, and the fluorescence at 485 nm (blue spectra) was measured by flow cytometry.

**Animal Studies.** All animal work was performed in accordance with protocols approved by the Children’s Hospital Boston Institutional Animal Care and Use Committee.

**Statistics.** Data were expressed as average ± SEM of at least three independent experiments. An unpaired, two-tailed Student t test was used to determine differences between two groups. ANOVA test was used for the analysis of tumor regression among treatment groups.

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**REFERENCES**


Supporting Information

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SI Experimental Procedures

Antibodies and Chemicals. The antibodies used for this study are as follows: 389(P) p70, 240/244(P) S6, α-tubulin, 4E-BP1, 51(P) eIF2α, 981(P) PERK, cleaved PARP, and PCNA were all purchased from Cell Signaling Technologies. Antibodies to GLS and GDH were purchased from Abcam. P62/SQSTM1 antibody was purchased from Sigma. GAPDH antibody was purchased from Ambion. LC2 antibody was from Novus Biological. The following chemicals were used in this study: rapamycin (Calbiochem), EGCG (Calbiochem), 17AAG (Sigma), DMSO (Sigma), DM-α-ketoglutaric acid (Sigma), sodium pyruvate (Sigma), BSO (Sigma), GSH-MEE (Sigma), glutamate (Sigma), vitamin C (Sigma), NAC (Sigma), monobromobimane (Biochemika), dichlorofluorescein diacetate (DCFDA) (Invitrogen-Molecular Probes), 968 (Specs), AUY922 (Selleckchem), BIBO21 (Selleckchem), and BPTES (provided by Takashi Tsukamoto, Johns Hopkins, Baltimore).

Cell Viability Measurements. All cell viability experiments were conducted with PI exclusion assay as described (1). For all phase images, the Nikon Eclipse TE300 camera was used, and images were taken at the indicated time points.

Determination of ROS. Before treating the cells with the small molecules, cells were rinsed with 1x PBS. Cells were incubated with 1x PBS containing 10 μM DCFDA (Invitrogen–Molecular Probes) for 5 min at 37 °C. PBS was removed, and small molecules dissolved in experimental medium were added if necessary. Once the experimental time was achieved, medium was collected in a 15-cm conical tube. Cells were then washed with saline solution and collected in the same tube. Trypsin (500 μL for a 10-cm² well) was added, cells were incubated on ice for 5 min, and trypsin was collected in the same 15-cm tube. The wells were washed with saline solution and collected in the 15-cm tube. Samples were centrifuged (180 × g, 4 °C) for 3 min. Cell pellets were resuspended in 300 μL of 1x PBS containing 0.3% BSA (filtered in 0.2 or 0.45 μm). Lastly, test samples were analyzed by FACS with a FITC filter.

Cell Lysis and Immunoblotting. Cells were washed once with cold PBS and solubilized on ice either in a regular lysis buffer [40 mM Hepes (pH 7.4), 1 mM EDTA, 120 mM NaCl, 10 mM β-glycerophosphate, 1 mM NaF, 1 mM Na3VO4, and 0.3% CHAPS] or in a low-salt lysis buffer [40 mM Hepes (pH 7.4), 1 mM EDTA, 10 mM β-glycerophosphate, 1 mM NaF, 1 mM Na3VO4, and 0.3% CHAPS] supplemented with protease inhibitors (250 μM PMSF, 5 μg/mL Pepstatin A, 10 μg/mL Leupeptin, and 5 μg/mL Aprotinin). Cleared cell lysates were obtained by centrifugation at 16,000 × g for 10 min at 4 °C and analyzed by immunoblotting.

Intracellular Glutamate Level Measurements. To measure glutamate levels, we used the Amplex Red Glutamic Acid/Glutamate Oxidase Assay from Invitrogen–Molecular Probes (MP12221) according to the manufacturer’s instructions.

TEM. Cells were fixed with 2% glutaraldehyde/2% formaldehyde in cacodylate buffer, followed by 1% osmium tetroxide. The samples were embedded in epoxy resin and viewed with a FEI Tecnai 12 transmission electron microscope operated at 80 kV.

siRNA Transfections. Twenty-five nM siRNAs were transfected in cells right after being seeded at a density of 30–50% confluency depending on experiments by using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer’s protocols. Cells were harvested 36–60 h after transfection as described in the figure legends. All siRNAs used were obtained from Dharmacon.

Quantitative RT-PCR Analysis. Total cellular RNA was purified from cultured cells by using the RNeasy mini kit (Qiagen) following the manufacturer’s protocol. For quantitative real-time PCR (qRT-PCR), RNA was reverse-transcribed by using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer’s instructions. The resulting cDNA was analyzed by qRT-PCR using the QuantiTect SYBR Green qPCR System (Qiagen). A QuantiTect Primer Assay was used to amplify the target gene (GCLC catalog no. Q70074300 and GSS catalog no. Q70015304) and normalization control gene (GAPDH catalog no. Q701658692) (Qiagen). All reactions were run on an ABI 7900HT Fast Real-Time PCR instrument with a 15-min hot start at 95 °C followed by 40 cycles of a three-step thermocycling program: denaturation, 15 s at 94 °C; annealing, 30 s at 55 °C; and extension, 30 s at 70 °C. Melting curve analysis was performed at the end of every run to ensure that a single PCR product of the expected melting temperature was produced in a given well. A total of three biological replicates × four technical replicates was performed for each treatment group. Data were analyzed by using the comparative Ct method (ΔΔCt method).

Metabolite Analysis of Spent Medium. Glutamine concentrations were measured in fresh and spent medium (after 72 h of culture in the presence or absence of drugs) by using a Yellow Springs Instruments 7100. Glutamine levels were normalized to cell numbers. The medium used for these experiments did not contain pyruvate and were supplemented with 10% dialyzed FBS.

Animal Studies. All animal work was performed in accordance with protocols approved by the Children’s Hospital Boston Institutional Animal Care and Use Committee. Female intact CB17-SCID mice were used as described (2, 3). For xenograft tumor establishment, 2 × 106 cells were inoculated bilaterally into the posterior back region of mice. Five weeks after cell inoculation, mice bearing s.c. tumors were randomized into four groups: vehicle control (n = 5; 10% DMSO in corn oil, 100 μL/d, oral gavage in 0.2% methyl cellulose), 17-AAG (n = 5; 80mg/kg per day; oral gavage in 0.2% methyl cellulose), BPTES (n = 5; 40 mg/kg per day, oral gavage in 0.2% methyl cellulose), and 17-AAG plus BPTES (n = 5; 80 mg/kg per day, 40 mg/kg per day, oral gavage in 0.2% methyl cellulose). Drug treatment was initiated 5 wk after cell inoculation. Tumor area (width × length) was measured weekly by using a calipers. Tumor volume was calculated by the formula: V = (width)² × length/2.

Bioluminescent Reporter Imaging

Ten minutes before imaging, animals were injected with luciferin (Xenogen) (120 mg/kg, i.p.). Bioluminescent signals were recorded using the Xenogen IVIS System. Total photon flux of tumors was analyzed (2).

Immunohistochemical Staining

Histology sections were prepared from mouse tumors after 10% formalin fixation and cutting into 10-μm sections. Slides were deparaffinized, and antigen retrieval was performed by using Dako Target Retrieval Solution, pH 6. Immunohistochemical staining was performed by using antibodies against...
PCNA (Cell Signaling Technologies) for cell proliferation, TACS 2 TdT-DAB in Situ Apoptosis Detection Kit (Trevigen) for cell death, and a Histostain-Plus Detection Kit (Life Technologies). After staining, images were captured by using an Olympus FluoView FSX100 microscope (Fig. 6 E and F) (sbars, 50 μM).


Fig. S1. A targeted small-molecule screen identifies GLS inhibition to sensitize Tsc2−/− MEFs to Hsp90 inhibition. (A) Cell viability of Tsc2−/− MEFs using the CellTiter-Glo. Relative luminescence was measured in Tsc2−/− MEFs after 72 h of treatment with small molecules and 17AAG (0.3 μM). The mean is shown; error bars represent SEM. (B) Immunoblot analysis of PERK, P-S6K1, S6K1, P-eIF2α, and GAPDH in Tsc2−/− MEFs treated with increasing concentrations of 17AAG as indicated for 72 h.

Fig. S2. The combined inhibition of GLS and Hsp90 results in decreased viability and morphological changes of Tsc2−/− MEFs. Immunoblot analysis of p62 in Tsc2−/− MEFs treated with DMSO, 17AAG (0.5 μM), and BPTES (10 μM) as indicated for 24 h.
Fig. S3. (Continued)
Fig. S3. Inhibition of glutamine anaplerosis and Hsp90 causes potent apoptosis in Tsc2−/− cells; Hsp90 inhibition reverses rapamycin insensitive mTORC1 phenotypes. (A) Cell death of ELT3 and TSC2-reexpressing ELT3 cells after 96 h of treatment with 17AAG (0.25 and 0.5 μM) and with or without BPTES (10 μM) was measured via PI exclusion assay. The mean is shown; error bars represent SEM (n > 3). (B) Tsc2−/− MEFs and WT MEFs were treated with DMSO, 17AAG (0.25 and 0.5 μM), and BPTES (10 μM) as indicated for 72 h. Phase microscopy was used to observe cell viability. (C) Relative intensity of cleaved PARP was measured in Tsc2−/− MEFs treated with the indicated compounds for 24 h. (D) Immunoblot analysis of TSC2, cleaved PARP, S6, P-S6, and α-tubulin in Tsc2−/− MEFs and Tsc2−/− +Tsc2 MEFs treated with DMSO, 17AAG (0.5 μM), and BPTES (10 μM) for 24 h. (E) Relative intensity of cleaved PARP was measured in Tsc2−/− MEFs and Tsc2−/− +Tsc2 MEFs treated with the indicated compounds for 24 h. (F) Immunoblot analysis of cleaved PARP and α-tubulin in Tsc2−/− MEFs treated with the indicated compounds (Hsp90 inhibitors: BIIB021, AUY922, and BPTES) for 24 h. (H) Immunoblot analysis of P-4EBP1, 4EBP1, S6K1, P-S6, S6, and GAPDH in 621-101 cells treated with rapamycin (20 ng/mL), 17AAG (1 μM), or the combination of both for the indicated time points.
GDH inhibition does not sensitize cells to Hsp90 inhibition. (A) Immunoblot analysis of cleaved PARP, GLS, GDH, and GAPDH in Tsc2−/− MEFs after RNAi-mediated knockdown of GLS or GDH with the indicated compounds. (B) Cell death of Tsc2−/− MEFs treated with DMSO, 17AAG (0.5 μM), BPIES (10 μM), glutamate (4 mM) and vitamin C (100 μM) for 48 h.
Fig. S5. Deregulated redox balance is responsible for the apoptosis induced by BPTES and 17AAG. (A) Immunoblot analysis of cleaved PARP, p-eIF2α and GAPDH in Tsc2−/− MEFs after RNAi-mediated knockdown of GCLC or GSS with the indicated compounds. (B) Intracellular GSH levels were measured in Tsc2−/− MEFs treated with DMSO, BPTES (10 μM), 17AAG (0.5 μM), BPTES plus 17AAG, or BPTES plus 17AAG plus glutamate for 24 h.

Table S1. Small-molecule library

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<tr>
<th>Compound</th>
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<th>BPTES</th>
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Li et al. www.pnas.org/cgi/content/short/1417015112