High-Throughput Drug Screen Identifies Chelerythrine as a Selective Inducer of Death in a TSC2-null Setting

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Abstract

Tuberous sclerosis complex (TSC) is an autosomal dominant syndrome associated with tumors of the brain, heart, kidney, and lung. The TSC protein complex inhibits the mammalian or mechanistic target of rapamycin complex 1 (mTORC1). Inhibitors of mTORC1, including rapamycin, induce a cytostatic response in TSC tumors, resulting in temporary disease stabilization and prompt regrowth when treatment is stopped. The lack of TSC-specific cytotoxic therapies represents an important unmet clinical need. Using a high-throughput chemical screen in TSC2-deficient patient-derived cells, we identified a series of molecules antagonized by rapamycin and therefore selective for cells with mTORC1 hyperactivity. In particular, the cell-permeable alkaloid chelerythrine induced reactive oxygen species (ROS) and depleted glutathione (GSH) selectively in TSC2-null cells based on metabolic profiling. N-acetylcysteine or GSH cotreatment protected TSC2-null cells from chelerythrine’s effects, indicating that chelerythrine-induced cell death is ROS dependent. Induction of heme-oxygenase-1 (HMOX1/HO-1) with hemin also blocked chelerythrine-induced cell death. In vivo, chelerythrine inhibited the growth of TSC2-null xenograft tumors with no evidence of systemic toxicity with daily treatment over an extended period of time. This study reports the results of a bioactive compound screen and the identification of a potential lead candidate that acts via a novel oxidative stress–dependent mechanism to selectively induce necroptosis in TSC2-deficient tumors.

Implications: This study demonstrates that TSC2-deficient tumor cells are hypersensitive to oxidative stress–dependent cell death, and provide critical proof of concept that TSC2-deficient cells can be therapeutically targeted without the use of a rapalog to induce a cell death response. Mol Cancer Res; 13(1); 50–62. ©2014 AACR.

Introduction

Tuberous sclerosis complex (TSC) is an autosomal dominant, hamartomatous syndrome characterized by seizures, autism, and tumors of the brain (subependymal giant cell astrocytomas), heart (rhabdomyomas), kidney (angiomyolipomas), and skin (angiofibromas). Multiple bilateral renal angiomyolipomas occur in the majority of both children and adults with TSC. At least 30% of women with TSC develop lymphangioleiomyomatosis (LAM), a destructive cystic lung disease (1, 2). LAM also occurs in a sporadic form, in which somatic TSC2 mutations are found in the distinctive pulmonary LAM cells and in renal angiomyolipomas, which occur in approximately 50% of patients with sporadic LAM.

TSC is caused by germline loss-of-function mutations in either the TSC1 or TSC2 gene, which encode the proteins hamartin and tuberin. The TSC protein complex negatively regulates the activity of the mammalian or mechanistic target of rapamycin (mTOR) kinase via the small GTPase Rheb (3–5). mTOR is a serine/threonine protein kinase complex that regulates autophagy, cell growth, cell proliferation, cell motility, protein synthesis, transcription, and cell survival (6, 7). Two distinct mTOR kinase complexes have been identified: mTORC1, which includes mTOR, Raptor, MLST8, DEPTOR, and Pras40, and mTORC2, which contains mTOR, Rictor, MSIN1, and Gβl (8).

Clinical trials have demonstrated the efficacy of mTORC1 inhibitors in TSC and LAM. Rapamycin (sirolimus), which inhibits mTORC1, slows further loss-of-lung function in LAM and partially decreases the size of TSC–associated kidney and brain tumors (9, 10). Everolimus, a sirolimus analogue or ‘rapalog,’ also induces a partial decrease in tumor size and is FDA approved for the treatment of angiomyolipomas and subependymal astrocytomas (11, 12). Rapalogs appear to induce a primarily cytostatic effect in TSC-deficient cells. Tumors regrow and lung function declines when treatment is discontinued (9, 10), with documented regrowth of a subependymal giant cell astrocytoma to its original size within 6 weeks after discontinuation of rapamycin (13). Therefore, continuous therapy appears to be necessary in

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both adults and children with TSC-associated tumors and/or LAM. The adverse effects of rapalogs include oral mucositis, fatigue, hyperlipidemia, interstitial pneumonitis, electrolyte imbalance, and immune suppression, further underscoring the unmet clinical need for therapeutic strategies that induce a cytostatic rather than cytotoxic response in cells with hyperactive mTORC1, thereby inducing more complete and durable clinical responses.

To identify compounds that induce death in mTORC1-hyperactive cells, we performed a high-throughput screen of 6,700 "known bioactive" compounds using human angiomyolipoma-derived cells that carry biallelic inactivation of the TSC2 gene (14). The screen was performed in the presence and absence of rapamycin, allowing the identification of compounds that selectively inhibit proliferation in the setting of hyperactive mTORC1, which we refer to as rapamycin antagonists. Thirty-two compounds were antagonized by rapamycin by at least 2-fold, thereby meeting the criterion of acting selectively in the presence of hyperactive mTORC1. Chelerythrine chloride demonstrated the highest-fold rapamycin antagonism. Chelerythrine chloride is a plant-derived benzophenanthridine alkaloid that was first identified as a protein kinase C (PKC) inhibitor (15). However, it has since been found to induce rapid release of cytochrome c (16, 17), decrease Bcl-xL and increase Bax expression (18), induce reactive oxygen species (ROS; refs. 19, 20), and activate RAF/MEK/ERK signaling (21). Here, we demonstrate that chelerythrine chloride treatment depletes glutathione levels and induces ROS production in TSC2-null cells, leading to necrotic cell death. These data support the hypothesis that the metabolic vulnerabilities of TSC2-deficient cells can be therapeutically targeted by single agents, without the use of mTORC1 inhibitors.

Materials and Methods

Cell culture and reagents

621-101, ELT-V3/T3, and mouse embryonic fibroblasts (MEF) were maintained in high-glucose DMEM supplemented with 10% FBS and 1% pen/strep. The V3/T3 cell media was additionally supplemented with 2 μg/mL of puromycin (Invitrogen). Chelerythrine chloride, Mitotempo, N-acetylcysteine (NAC), and Necrostatin-1 were purchased from Sigma-Aldrich.

High-throughput screen

621-101 cells were plated at a density of 1,200 cells per well in 384-well format and incubated overnight at 3% CO2 and 37°C. Sixteen hours later, cells were pretreated with either DMSO or 20 mmol/L rapamycin for 2 hours. Compound libraries were then transferred to a 621-101 supernatant was dried and samples were reconstituted in methanol extraction. The supernatant was dried and samples were reconstituted in methanol and shaking for 2 hours. Methanol was evaporated in vacuo and the sample was resuspended in water and solubilized by the addition of methanol and shaking for 10 minutes. Absorbance was read at 540 nm.

Immunoblotting

Cells were lysed on ice in lysis buffer (1% NP-40, 25 mmol/L Tris pH 7.4, and 150 mmol/L NaCl) and centrifuged at 14,000 rpm for 15 minutes. The supernatants were boiled, proteins separated by SDS-PAGE, and transferred onto nitrocellulose. Membranes were incubated with the following antibodies from Cell Signaling Technology: Actin, phospho-S6 (Ser235/36 and Ser240/44), phospho-p70S6K, PARP and cleaved PARP, TSC2, p-AKT (Ser473) HO-1, and p-ERK (p42/44); Abcam: HO-1; Sigma: p62; and Bach1 and SP1: Santa Cruz Biotechnology. Chemiluminescent signal was captured using a Syngene G-BOX iChem iXT imager.

Preparation of nuclear and cytoplasmic extracts

Nuclear extracts for Bach-1 were prepared from the cells using a nuclear extraction kit (Active Motif). Following treatment, cells were collected in PBS in the presence of phosphatase inhibitors. The cells were resuspended in hypotonic buffer (supplied with the kit) and incubated on ice for 15 minutes. Cytoplasmic fractions were collected after adding detergent (supplied with the kit) and centrifuging the cells. The nuclear pellets were resuspended in complete lysis buffer and incubated on ice for 30 minutes. The nuclear fractions were collected by centrifuging the cells for 10 minutes.

Receptor tyrosine kinase array

Tsc2+/+ and Tsc2−/− MEFs were treated with chelerythrine chloride (2 μmol/L, 60 minutes). Analysis of phosphorylated antibodies was performed as suggested by the manufacturer (R&D Systems). Chemiluminescent signal was captured using a Syngene G-BOX iChem iXT imager, and densitometry was performed using Syngene GeneTools software.

Cellular ROS analysis

Five thousand cells per well were plated in 96-well format. Sixteen hours later, a 100 mmol/L solution of DFCDA was diluted 1:2000 in phenol red free DMEM and 100 μL was added to each well. Cells were incubated for 45 minutes, followed by the indicated treatments. Fluorescence was measured at an excitation of 485 nm and emission of 535 nm using a BioTek Synergy HT plate reader.

Metabolic profiling

Tsc2+/+ and Tsc2−/− MEFs were treated with chelerythrine chloride (1 μmol/L, 4 hours). Metabolites were isolated via cold methanol extraction. The supernatant was dried and samples were run on an AB/SCIEX 5500 QTRAP triple quadrupole instrument. Raw data were normalized to vehicle-treated cells (n = 3). Normalized data were analyzed using MetaboAnalyst (http://www.metaboanalyst.ca/MetaboAnalyst/).

Animal studies

All animal experiments were performed in accordance with approved protocols by the Institutional Animal Care and Use Committee of Boston Children's Hospital. Female intact nude mice (Charles River) were used. For xenograft tumors, 2 × 10⁶ cells were inoculated bilaterally into the posterior flank. Six weeks post cell injection, mice bearing subcutaneous tumors were randomized and treated with 5 mg/kg chelerythrine or vehicle daily for 2 weeks. All animal experiments were performed in accordance with the Institutional Animal Care and Use Committee of Boston Children's Hospital.
tumors were randomized into two groups: Vehicle control ($n = 7$, 10% DMSO in PBS, 100 μL/day, i.p.) and chelerythrine chloride ($n = 9$, 10 mg/kg/day, i.p.). Tumor area was measured weekly using a digital Caliper. Tumor volume was calculated: $(\text{width}^2 \times \text{length})/2$.

Bioluminescent reporter imaging

Ten minutes before imaging, mice were injected with D-luciferin (Xenogen; 120 mg/kg, i.p.). Bioluminescent signals were recorded using a Xenogen IVIS System. Total photon flux was analyzed for each tumor (22).

Results

A high-throughput screen for rapamycin antagonists identifies chelerythrine chloride as an inhibitor of angiomyolipoma-derived 621-101 cells

To discover single agents that inhibit TSC2-null, mTORC1-hyperactive cells, we performed a high-throughput drug screen in 621-101 cells which were derived from a renal angiomyolipoma in a woman with the sporadic form of LAM and carry biallelic TSC2 inactivation (14, 23). The collections that were screened include approximately 6,700 agents that are FDA approved, have been in clinical trials, and/or have a known mechanism of action. To identify agents that act via an mTORC1-dependent mechanism, rapamycin (20 nmol/L), an allosteric mTORC1 inhibitor, was added 2 hours before pin transfer of the compounds. ATP levels were measured using CellTiter Glo after 48 hours of incubation (Fig. 1A).

Thirty-two compounds showed a greater than 2-fold inhibition of ATP levels when used alone versus in combination with rapamycin, indicating selectivity for cells with hyperactive mTORC1 (Supplementary Table S1), representing several distinct drug classes (Fig. 1B). The top hit, based on fold change, was chelerythrine chloride, a plant-derived benzophenanthridine alkaloid (Fig. 1C). Interestingly, multiple selective serotonin reuptake inhibitors (SSRI) and other neuroactive agents were identified in the screen, including...
paroxetine, which showed the second highest antagonism to rapamycin (Fig. 1D).

**Chelerythrine chloride inhibits the growth of TSC2-null cells**

To confirm that chelerythrine chloride, the top hit from the screen, selectively inhibits mTORC1-hyperactive cells, 621-101 cells were treated for 24 hours with DMSO, rapamycin (20 μmol/L), chelerythrine chloride (2 μmol/L), or rapamycin (2-hour pretreatment) plus chelerythrine chloride (Fig. 2A). Chelerythrine chloride inhibited growth when used alone, but not in combination with rapamycin, consistent with the results from the screen. Paroxetine, the second highest hit in terms of
fold-change of ATP levels with and without rapamycin, inhibited the growth of TSC2-deficient ELT3-V3 cells, but not TSC2-expressing ELT3-T3 cells (Supplementary Fig. S1), further confirming the integrity of the screen.

To define the dose–response of chelerythrine chloride, we analyzed ATP levels using five concentrations of chelerythrine chloride (100 μmol/L to 10 μmol/L) in 621-101 cells (Fig. 2B). The IC50 (the concentration that inhibited 50% of ATP levels) in 621-101 cells was 1.8 μmol/L without rapamycin versus 3.75 μmol/L with rapamycin. To determine if the effects of chelerythrine are TSC2-dependent, we tested Tsc2−/− and Tsc2+/+ MEFs (Fig. 2C). The IC50 for Tsc2-null MEFs was 1.5 μmol/L versus 2.5 μmol/L for the Tsc2+/+ MEFs. A significant decrease in cell survival, measured using crystal violet staining, was seen in the Tsc2−/− MEFs when compared with the Tsc2+/+ MEFs, consistent with the ATP analyses (Fig. 2D). These data indicate that chelerythrine chloride–induced changes in cell number are TSC2 dependent.

Chelerythrine chloride induces cell death in Tsc2-null cells

The major goal of the screen was to identify agents that induce a cytoidal, rather than cytostatic, response in Tsc2-null cells, because of the potential therapeutic benefit in TSC and LAM. To determine whether chelerythrine chloride induced cell death, we treated Tsc2-null and Tsc2+/+ MEFs with chelerythrine chloride (2 μmol/L) or paroxetine (10 μmol/L) for 4 hours and analyzed PARP levels by Western blot (Fig. 3A). Chelerythrine chloride induced PARP cleavage in Tsc2-null MEFs but not Tsc2+/+ expressing MEFs. Interestingly, paroxetine, the second-highest hit from the screen, did not induce PARP cleavage at this timepoint. Together with the data in Fig. 2, this indicates that chelerythrine chloride induces cell death in a TSC2-dependent manner. Chelerythrine also induced PARP cleavage in TSC2-null ELT-V3 cells but not in TSC2-expressing ELT-T3 cells, further confirming the TSC2 dependence of chelerythrine’s effects (Fig. 3B). To determine whether chelerythrine chloride affects the mTORC1 signaling pathway, Tsc2-null and Tsc2+/+ MEFs were treated with DMSO or chelerythrine chloride (2 μmol/L) for 2 hours (Fig. 3C). Chelerythrine chloride treatment induced PARP cleavage, as expected. Chelerythrine chloride did not inhibit the phosphorylation of p70-S6K or ribosomal protein S6, suggesting that its primary mechanism of action does not involve mTORC1 inhibition. Chelerythrine blocked the phosphorylation of Akt (S473) and induced cleavage of p62/sequestosome 1, a signaling adaptor and autophagy substrate that is essential for the in vivo growth of TSC2-deficient cells (6).

Chelerythrine chloride activates EGFR–MEK–ERK signaling in TSC2-null cells, but this is not required for induction of cell death

To identify signaling pathways that are altered in TSC2-null cells treated with chelerythrine chloride, Tsc2-null and Tsc2+/+ MEFs were treated with chelerythrine chloride for 1 hour and lysates were applied to a phospho-receptor tyrosine kinase array. Phospho-EGFR (2.7-fold) and phospho-HER2 (6.5-fold) were among most differentially upregulated phosphoproteins in chelerythrine-treated Tsc2-null MEFs compared with chelerythrine-treated Tsc2+/+ MEFs after normalization to vehicle control (Fig. 4A). These findings are consistent with prior work showing that chelerythrine-induced apoptosis is mediated by the MEK–ERK pathway in osteosarcoma-derived cells (21). To determine whether induction of MEK signaling mediates chelerythrine’s TSC2-dependent effects, 621-101 cells and Tsc2-null MEFs were treated with chelerythrine chloride with or without pretreatment with the MEK inhibitor CI-1040 (1 μmol/L). CI-1040 pretreatment did not abrogate the effects of chelerythrine chloride in 621-101 cells or Tsc2-null MEFs (Fig. 4B and C). Furthermore, neither CI-1040 or PD98059 was sufficient to prevent chelerythrine chloride–induced PARP cleavage (Fig. 4D), despite inhibiting the phosphorylation of ERK (Supplementary Fig. S2), suggesting that the effects of chelerythrine are MEK independent, in contrast to prior work in osteosarcoma-derived cells in which chelerythrine-induced apoptosis is mediated by the MEK–ERK pathway (21). On the basis of the kinases that were most upregulated on the phospho-kinase array, we tested the EGFR/HER2 inhibitor Afatinib, the Axl inhibitor R428, and the Trk-beta inhibitor TrikIII. None of these agents was sufficient to protect Tsc2-null cells from chelerythrine chloride–induced death (Fig. 4E).
Figure 4.
Chelerythrine chloride induces EGFR and HER2 signaling in Tsc2-null cells. A, phospho-receptor tyrosine kinase array of Tsc2−/− and Tsc2+/+ MEFs treated with 1 μmol/L chelerythrine chloride for 1 hour. The bar graph shows densitometric analysis of relative changes in chelerythrine-treated Tsc2−/− versus Tsc2+/+ cells after normalization to vehicle control. B and C, 621-101 cells and Tsc2−/− MEFs were pretreated with the MEK inhibitor CI-1040 (1 μmol/L) for 16 hours followed by the addition of 5 μmol/L chelerythrine chloride (621-101) or 2 μmol/L chelerythrine (MEFs) for 2 hours. Cell proliferation was measured by crystal violet staining. MEK inhibition did not block the chelerythrine-induced effects in either cell type. D, Immunoblot analysis of Tsc2−/− and Tsc2−/− MEFs pretreated with the ERK inhibitor PD98059 (50 μmol/L) or the MEK inhibitor CI-1040 (1 μmol/L) for 16 hours followed by chelerythrine chloride (1 μmol/L) for 3 hours. E, Phase contrast images of Tsc2-null MEFs treated with chelerythrine chloride (2 μmol/L) and/or the EGFR inhibitor Afatinib (300 nmol/L), the Axl inhibitor R428 (300 nmol/L), or the Trk-beta inhibitor TrkIII (300 nmol/L). Inhibitors were chosen based on results from A. Images were captured 24 hours post addition of chelerythrine.
Chelerythrine chloride–induced decrease in glutathione levels is required for cell death induction in TSC2-null cells

To further investigate chelerythrine’s chloride mechanism of action in TSC2-null cells, we performed steady-state metabolite analysis of Tsc2−/− and Tsc2+/+ MEFs treated with chelerythrine chloride (1 μmol/L) for 4 hours. Interestingly, glutathione (GSH) was the second most decreased metabolite when comparing Tsc2-null–treated cells with Tsc2+/+–treated cells (Fig. 5A); only 6-phosphogluconate showed a higher-fold reduction. To determine whether this reduction in glutathione is required for chelerythrine chloride–induced death, we treated cells with chelerythrine chloride, GSH, or both chelerythrine and GSH (Fig. 5B). GSH abrogated the chelerythrine chloride–induced cell death in both Tsc2-null MEFs and 621-101 cells, suggesting that glutathione depletion is a critical component of chelerythrine chloride’s mechanism of action in TSC2-null cells.

Induction of reactive oxygen species and necroptosis is necessary for chelerythrine chloride–induced death in Tsc2-null, mTORC1-hyperactive cells

On the basis of the glutathione results, we hypothesized that chelerythrine selectively induces ROS levels in TSC2-null cells. To test this hypothesis, we used the cell-permeant reporter compound 2,7-dichlorodihydrofluorescein diacetate (DCFDA). ROS levels at baseline were approximately 1.5-fold higher in the Tsc2−/− MEFs compared with Tsc2+/+ MEFs, consistent with prior findings (24–26). After 20 hours of chelerythrine chloride treatment, ROS levels were further increased in Tsc2-null MEFs by 1.6-fold relative to untreated cells, with no change in Tsc2+/+ MEFs (Fig. 6A). Furthermore, NAC, a ROS scavenger, restored ROS levels to baseline in chelerythrine chloride–treated cells, but had no significant impact on ROS levels in Tsc2+/+ cells (Fig. 6A).

To determine whether chelerythrine-induced ROS production is necessary for the induction of cell death, Tsc2-null MEFs were treated with the combination of chelerythrine chloride and NAC. NAC was sufficient to protect cells from chelerythrine chloride–induced death (Fig. 6B). Intracellular ROS can induce cell death via programmed necrosis, also known as necroptosis (27). Necroptosis is mediated by receptor-interacting serine/threonine-protein kinase 1 (RIPK1), which interacts with RIPK3 to activate the necrosome (28, 29). To determine whether chelerythrine chloride induces cell death in TSC2-null cells via necroptosis, we used the RIPK1 inhibitor Necrostatin-1 (30). Necrostatin-1 blocked the anti-proliferative effects of chelerythrine chloride in Tsc2-null MEFs (Fig. 6B). NAC or necrostatin also protected 621-101 cells from chelerythrine chloride–induced death as measured by crystal violet staining (Fig. 6C). To assess the relative contribution of mitochondrial-derived ROS to these phenotypes, we used the mitochondrial ROS scavenger Mito-tempo, which partially rescued the effects of chelerythrine chloride on Tsc2−/− MEFs (Fig. 6D).

Finally, to further verify that NAC and Necrostatin-1 protect Tsc2-null cells from chelerythrine chloride–induced death, PARP levels were monitored by immunoblot (Fig. 6E). Chelerythrine chloride induced PARP cleavage selectively in the Tsc2-null cells, as shown earlier. PARP cleavage was prevented by cotreatment with NAC or Necrostatin-1. On the basis of these data, we conclude that chelerythrine chloride induces TSC2-dependent necroptosis via an ROS-dependent mechanism.
Induction of HO-1 protects Tsc2-null cells from chelerythrine chloride–induced cell death

To further investigate the mechanism through which chelerythrine chloride induces ROS-dependent death in Tsc2-null cells, we examined the expression of three antioxidant response genes: heme oxygenase 1 (Hmox1), superoxide dismutase (SOD), and thioredoxin (TXN1). Chelerythrine chloride induced a 3-fold increase in Hmox1 mRNA levels in Tsc2⁺/⁺ MEFs, but did not induce Hmox1 in Tsc2-null MEFs (Fig. 7A). To better understand the mechanism of this difference between Tsc2⁺/⁺ and Tsc2⁻/⁻ MEFs, we investigated the nuclear and cytoplasmic localization of Bach-1, a negative regulator of HO-1 expression (31–33), in Tsc2⁺/⁺ and Tsc2⁻/⁻ MEFs treated with chelerythrine chloride or vehicle alone. Following treatment, the nuclear and cytoplasmic fractions were isolated. Upon chelerythrine chloride treatment, Bach-1 was increased in the nuclear fraction and reduced in the cytoplasmic fraction of Tsc2⁻/⁻ MEFs (Fig. 7B and C), whereas there was no significant change in Bach-1 levels between the nuclear and cytoplasmic fractions of Tsc2⁺/⁺ MEFs upon chelerythrine chloride treatment. These findings suggest that chelerythrine chloride treatment selectively promotes nuclear translocation of Bach-1 in Tsc2-null cells, leading to downregulation of Hmox1 and HO-1 expression in Tsc2-null versus Tsc2-expressing cells. Consistent with this model, induction of HO-1 using...
Figure 7.
Induction of HO-1 is sufficient to rescue Tsc2-null cells from chelerythrine chloride–induced death. A, Tsc2+/+ and Tsc2−/− MEFs were treated with chelerythrine chloride (2 μmol/L, 2 hours). Hmox1, SOD2, and TXN1 levels were measured by quantitative RT-PCR and normalized to β-actin. *, P < 0.05. B, Tsc2+/+ and Tsc2−/− null MEFs were treated with chelerythrine chloride (2 μmol/L) or vehicle alone. Cells were harvested after 6 hours, the nuclear and cytoplasmic fractions were isolated from these cells, and the expression of Bach-1 was quantified by Western blot analysis. Expression of SP-1 and GAPDH was used to assess the purity of the nuclear and cytoplasmic fractions, respectively. C, densitometry was used to quantitate the results of three independent experiments. *, P < 0.05, two-sided t-test. D, Tsc2+/+ and Tsc2−/− MEFs were treated for 20 hours with DMSO or Hemin (10 μmol/L) in replicate cultures; chelerythrine chloride (2 μmol/L, 2 hours) was added for the final 2 hours to the indicated wells. Phase contrast images (4×) show rescue of chelerythrine chloride–induced death by Hemin in the Tsc2−/− cells. E, immunoblot showing induction of HO-1 by Hemin.
Hemin was sufficient to block chelerythrine chloride–induced death in Tsc2-null MEFs (Fig. 7D and E). These results indicate that chelerythrine chloride–induced death in Tsc2-null cells is HO-1 dependent.

Chelerythrine chloride inhibits the progression of Tsc2-null cell xenograft tumors

To evaluate the efficacy of chelerythrine chloride in vivo, mice bearing TSC2-null, ELT3-luciferase–expressing xenograft tumors (22) were treated daily with chelerythrine chloride or vehicle control for four weeks. No effect on body weight or other evidence of toxicity was observed (Fig. 8A). Chelerythrine chloride significantly reduced tumor volume by 44% at week 3 and 57% week 4, compared with vehicle treatment (Fig. 8B). Chelerythrine chloride also significantly decreased the bioluminescence intensity of the xenograft tumors (Fig. 8C and D; \( P < 0.05 \)).

Discussion

The TSC proteins form a complex that inhibits mTORC1. Rapamycin and its analogues decrease the size of TSC-associated angiomyolipomas and subependymal giant cell astrocytomas and decrease the rate of lung function decline in women with TSC-associated and sporadic LAM. However, rapalogs only partially decrease tumor size in patients with TSC, with most studies demonstrating a 20% to 40% decrease in tumor volume. Furthermore, continuous therapy is required, because tumors regrow and lung function deteriorates when therapy is discontinued, consistent with the known cytostatic effects of rapalogs. One strategy to achieve a more durable clinical response in TSC and LAM would be to identify compounds that induce cytocidal effects in TSC2-deficient cells. To achieve this goal, we screened 6,700 compounds in patient-derived TSC2-deficient cells in the presence and absence of...
We focused on agents that are antagonized by rapamycin because they are predicted to be selective for cells with mTORC1 hyperactivation and may therefore represent a step toward single-agent cytoidal therapeutic strategies for TSC and LAM. Thirty-two compounds exhibited at least 2-fold rapamycin antagonism (i.e., at least 2-fold lower ATP levels when the drug was used alone vs. in combination with rapamycin). The top two hits were chelerythrine chloride and paroxetine, both of which selectively inhibited the proliferation of TSC2-deficient cells when compared with TSC2-expressing cells. Chelerythrine chloride, a benzophenanthridine alkaloid, induced cell death in TSC2-deficient cells, thereby achieving the major goal of the screen: to identify compounds that induce a selective cytoidal effect in TSC2-deficient cells.

To identify the mechanisms through which chelerythrine chloride affects cells with hyperactive mTORC1, we performed a kinase array and found that chelerythrine chloride induced the phosphorylation of multiple kinases (including EGFR, Her2, Axl, and Trk-beta) more strongly in TSC2-deficient versus TSC2-expressing cells, after normalization for baseline differences in phosphorylation in vehicle-treated cells. However, inhibition of these kinases was not sufficient to block chelerythrine chloride–induced death. Metabolite profiling revealed a selective decrease in glutathione levels after chelerythrine treatment of TSC2-null cells when compared with TSC2-expressing cells. Glutathione was sufficient to block chelerythrine chloride–induced death, indicating that oxidative stress pathways are essential to chelerythrine's selectivity for TSC2-null cells.

NAC and hemin, which induces heme oxygenase 1 (HO-1), also blocked chelerythrine chloride–induced cell death, further supporting a model in which ROS induction by chelerythrine chloride is required for its cytoidal effects on TSC2-deficient cells. In vivo, chelerythrine inhibited the growth of TSC2-deficient cells with no evidence of toxicity. Therefore, our screen for rapamycin antagonists has identified chelerythrine chloride as a novel potential single-agent therapeutic strategy for TSC and LAM that acts via a TSC2-dependent and ROS-dependent mechanism.

The TSC proteins have been previously shown to participate in the regulation of ROS levels via multiple mechanisms. Rheb localizes to the mitochondrial outer membrane where it promotes mitophagy in growth conditions that favor oxidative phosphorylation (34), leading us to hypothesize that mitochondria are the source of the increased ROS induced by chelerythrine chloride. However, the mitochondrial ROS scavenger, mito-tempo, only partially blocked chelerythrine chloride–induced cell death, in contrast to NAC which completely blocked cell death, suggesting that both mitochondrial and nonmitochondrial sources of ROS contribute to chelerythrine chloride–induced cell death. The peroxisome, to which the TSC protein complex was recently found to localize (35), may represent another source of chelerythrine-induced ROS. The TSC proteins also regulate the cellular response to ROS. Regulation of T-cell quiescence is regulated by ROS levels in a TSC-dependent manner (24, 36) and ATM signals to TSC2 to regulate mTORC1 in response to ROS (37, 38).

ROS can activate RIP1 and RIP3 kinase, which are components of the necrosome. We found that chelerythrine chloride–induced cell death is abrogated by cotreatment with Necrostatin-1, an inhibitor of RIP1 kinase. mTOR inhibition has been linked to imbalances in ROS leading to necroptosis in a renal cell carcinoma cell line (39), but this is the first time that the necrosome has been linked to TSC. Further work will be required to determine whether TSC2-deficient cells are hypersensitive to necroptosis induced by other stimuli.

Although we chose to focus on chelerythrine chloride because it induced apoptosis, it is remarkable that our screen identified four SSRIs with at least 2-fold rapamycin antagonism, including paroxetine, the second strongest rapamycin antagonist based on fold change. SSRIs have been previously shown to exert antiproliferative effects in tumor cells, including Burkitt's lymphoma (40, 41), but to our knowledge no prior links to the mTOR signaling pathway have been identified. Unexpectedly, four of the other top hits were also psychoactive drugs. We speculate that the strikingly high proportion of neuroactive agents identified in the screen reflects the cell of origin of angiomyolipomas, from which the cell line used in the screen was derived. Although the cell of origin of angiomyolipomas is unknown, it is speculated that angiomyolipomas and LAM arise from the neural crest lineage because they express many neural crest lineage markers including the majority of melanoma-associated antigens (2). The fact that these neuroactive agents are rapamycin antagonists may reflect fundamental effects of the TSC complex on cellular differentiation, consistent with previous work demonstrating that the TSC proteins regulate Notch-dependent cell fate decisions in the Drosophila external sensory organ and in mammalian cells (42, 43).

In summary, we report the results of a high-throughput screen for molecules that are antagonized by rapamycin. The top hit, chelerythrine chloride, acts via a novel oxidative stress-dependent mechanism to selectively induce necroptosis in TSC2-deficient cells. Our data provide proof of concept that the consequences of mTORC1 hyperactivation can be therapeutically targeted by a single agent, without the use of a rapalog, to generate a cytoidal response. We hypothesize that compounds that induce cell death in TSC2-deficient cells will lead to more complete and durable clinical responses when compared with rapalogs, with substantial clinical benefit for patients with TSC and LAM and for potentially also patients with sporadic malignancies harboring mutations in the TSC genes, including bladder cancer and renal cell carcinoma.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors' Contributions
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