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Therapeutics, Targets, and Chemical Biology

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The Antidepressant Sertraline Inhibits Translation Initiation by Curtailing Mammalian Target of Rapamycin Signaling

Chen-Ju Lin[1], Francis Robert[1], Rami Sukarieh[1], Stephen Michnick[3], and Jerry Pelletier[1,2]

Abstract

Sertraline, a selective serotonin reuptake inhibitor, is a widely used antidepressant agent. Here, we show that sertraline also exhibits antiproliferative activity. Exposure to sertraline leads to a concentration-dependent decrease in protein synthesis. Moreover, polysome profile analysis of sertraline-treated cells shows a reduction in polysome content and a concomitant increase in 80S ribosomes. The inhibition in translation caused by sertraline is associated with decreased levels of the eukaryotic initiation factor (eIF) 4F complex, altered localization of eIF4E, and increased eIF2α phosphorylation. The latter event leads to increased REDD1 expression, which in turn impinges on the mammalian target of rapamycin (mTOR) pathway by affecting TSC1/2 signaling. Sertraline also independently targets the mTOR signaling pathway downstream of Rheb. In the *Eμ-myc* murine lymphoma model where carcinogenesis is driven by phosphatase and tensin homologue (PTEN) inactivation, sertraline is able to enhance chemosensitivity to doxorubicin. Our results indicate that sertraline exerts antiproliferative activity by targeting the mTOR signaling pathway in a REDD1-dependent manner. *Cancer Res; 70(8); 3199–208. ©2010 AACR.*

Introduction

Deregulated protein synthesis is emerging as a key driver of oncogenesis (1, 2) by selectively affecting translation of mRNAs whose products are required for cell growth, proliferation, differentiation, and cellular homeostasis (3). The recruitment of ribosomes to the 5′ end of mRNAs during translation initiation in eukaryotes is generally thought to be the rate-limiting step of protein synthesis and is stimulated by eukaryotic initiation factor (eIF) 4F, a heterotrimeric complex consisting of three subunits: eIF4E, which interacts directly with the mRNA cap structure; eIF4A, an RNA helicase that prepares the mRNA template for ribosome binding; and eIF4G, a large molecular scaffold that mediates binding of the mRNA to the 43S preinitiation complex (4).

A second node of translational control is at the level of ternary complex formation. The ternary complex is formed on binding of Met-tRNA$_i^{Met}$, GTP, and eIF2, which in turn binds to 40S ribosomes (in conjunction with eIF1, eIF1A, and eIF3) to form 43S preinitiation complexes. Following one round of initiation, eIF2-bound GTP is hydrolyzed to GDP and the resulting eIF2-GDP complex must be recycled. Phosphorylation of the eIF2α subunit inhibits recycling, decreases the amount of 43S preinitiation complexes, and is an important component of the unfolded protein response and environmental cellular adaptive mechanism (5). The resulting reduction in eIF2 activity inhibits general protein synthesis but stimulates translation of a subset of mRNAs (6).

Both translational control mechanisms are intimately linked to the mammalian target of rapamycin (mTOR) protein kinase signaling pathway. mTOR functions by integrating extracellular signals (growth factors and hormones) with amino acid availability and intracellular energy status to control translation rates and additional metabolic processes (7). The two best-characterized targets of mTOR phosphorylation are the eIF4E binding proteins (4E-BP; of which there are three and the most studied one is 4E-BP1) and ribosomal protein S6 kinase (S6K) 1 and S6K2 (p70S6K1/2). Hypophosphorylated 4E-BP1 inhibits cap-dependent translation initiation by competing with eIF4G for binding to eIF4E, and mTOR-mediated phosphorylation of 4E-BP1 liberates eIF4E from this inhibitory complex, enabling it to bind eIF4G and enter the eIF4F complex (8). S6K1 directly phosphorylates the 40S ribosomal protein S6 and a known tumor suppressor gene product, programmed cell death 4 (PDCD4), the latter of which binds eIF4A (9) to inhibit cap-dependent protein synthesis. When PDCD4 becomes phosphorylated, it is degraded to release eIF4A for assembly into eIF4F (10). Therefore, mTOR regulates translation initiation by controlling eIF4F assembly and hence ribosome recruitment.

The phosphorylation status of eIF2α can affect Akt/mTOR signaling. It has recently been reported that ATF4 regulates expression of *REDD1* (11), a hypoxia-inducible factor-1–responsive gene induced upon hypoxia and eIF2α phosphorylation (12). REDD1 competes with TSC2 for binding to 14-3-3

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proteins, leading to increased suppression of mTOR activity due to enhanced TSC1/2 signal integration (11). It remains to be established whether compounds that induce phosphorylation of eIF2α modulate mTOR activity through an ATF4-dependent increase in REDD1 expression.

The mTOR signaling pathway is aberrantly activated in a substantial number of human cancers, making it an especially promising drug target (13). Rapamycin, a specific mTOR inhibitor, inhibits cap-dependent translation (14) and induces apoptosis in many tumor cell lines (15). Rapamycin is found to synergistically enhance chemotherapy-induced cytotoxicity in genetically modeled tumors containing lesions in the Akt/mTOR signaling pathway (16). As well, targeting the translation initiation pathway using antisense oligonucleotides to eIF4E (2) or a small-molecule inhibitor of eIF4F activity (17, 18) curtails tumor growth in xenograft and the Eμ-myc lymphoma models, respectively. CCI-779, an analogue of rapamycin, has shown promising anticancer activity against many tumor types, including renal cell carcinoma, breast cancer, glioma, and endometrial cancer (19).

Sertraline, a selective serotonin reuptake inhibitor, is a widely used antidepressant agent. It has been shown to downregulate expression of translationally controlled tumor protein (TCTP), and this has been associated with antiproliferative activity against MDA-MB-231 cancer cells *in vitro* and in a xenograft setting (20). A high-content protein fragment complementation assay screen aimed at identifying hidden phenotypes of drugs revealed that sertraline shared assay responses with clotrimazole (21), an antiproliferative compound that causes phosphorylation of eIF2α (22, 23). Sertraline has recently been shown to arrest transformed cells in G_0-G_1 and induce apoptosis (24) and has been identified in a chemical screen as a chemical sensitizer to tamoxifen in MCF-7 cells (25). We report herein that sertraline downregulates mTOR-dependent translation initiation through a REDD1-dependent mechanism. Sertraline inhibits translation initiation by targeting two translational checkpoints: assembly of the eIF4F complex and inducing phosphorylation of eIF2α.

Materials and Methods

Cell lines and cell culture. MCF-7 cells obtained from the American Type Culture Collection were cultured in DMEM/F12 (Cellgro Mediatech) supplemented with 10% fetal bovine serum (FBS), 2 mmol/L L-glutamine, and 100 μg/mL penicillin-streptomycin. *TSC2*$^{+/+}$*p53*$^{-/-}$ and *TSC2*$^{-/-}$*p53*$^{-/-}$ mouse embryonic fibroblasts (MEF; ref. 26) were kindly provided by Dr. David Kwiatkowski (Brigham and Women's Hospital, Boston, MA) and cultured in DMEM supplemented with 10% FBS, 2 mmol/L L-glutamine, and 100 μg/mL penicillin-streptomycin.

Generation of stable cell lines overexpressing Rheb (S16H). The retroviral vectors MSCV/IRES-GFP and MSCV/Rheb(S16H)-IRES-GFP were transiently transfected into the Phoenix packaging cell line using the calcium phosphate method. Retroviruses were harvested 48 to 60 h after transfection and used to infect *TSC2*$^{+/+}$*p53*$^{-/-}$ MEFs in the presence of 5 μg/mL polybrene. Infection frequencies based on green fluorescent protein (GFP) expression were 60%. Cell populations were obtained by fluorescence-activated cell sorting.

Western blot analysis. Cells were harvested in radioimmunoprecipitation assay (RIPA) lysis buffer [50 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 1 mmol/L DTT, 0.1% SDS, 1% NP40, 0.5% sodium deoxycholate, 0.1 mmol/L phenylmethylsulfonyl fluoride, 1 μg/mL each of leupeptin, pepstatin, and aprotinin]. Protein concentrations were quantified using a Bio-Rad protein assay. Total protein lysates (30 μg) were resolved by SDS-PAGE, and proteins were transferred to polyvinylidene difluoride membranes (Millipore) and analyzed using the indicated antisera and enhanced chemiluminescence detection (Amersham).

Cell growth assays. Cells were seeded in 96-well plates (20,000 per well) 24 h before sertraline treatment. Cells were treated with the indicated concentrations of sertraline in DMEM/F12 containing 10% FBS or with vehicle (DMSO) only as control for 24 h. Cell growth was determined using the sulforhodamine B (SRB) assay (27).

Metabolic labeling. MCF-7 cells (5×10^4) were seeded in 24-well plates 1 d before the experiment. Cells were incubated with the indicated concentrations of sertraline for 24 h. For protein labeling, cells were cultured for 15 min in methionine-free medium, followed by 15 min in [^{35}S]methionine-containing medium supplemented with 10% dialyzed FCS, washed, and lysed in RIPA buffer. Proteins were trichloroacetic acid precipitated on 3 MM paper, and the amount of incorporated radioactivity was quantitated by scintillation counting. Values were normalized with respect to total protein levels as determined by the Bradford assay.

Animal treatment studies. The generation of *Pten*$^{+/-}$*Eμ-Myc, Eμ-Myc/Bcl-2*, and *Eμ-Myc/eIF4E* lymphomas has been described elsewhere (16, 28). A total of 2×10^6 secondary lymphoma cells were injected into the tail vein of 6- to 8-wk-old female C57BL/6 mice. On development of well-palpable tumors (auxiliary and inguinal lymph nodes), mice were injected i.p. with doxorubicin (once at 10 mg/kg), rapamycin (4 mg/kg daily for 5 d), and sertraline (20 mg/kg daily for 5 d). Rapamycin, doxorubicin, and sertraline were diluted in 5.2% PEG400/5.2% Tween 80 immediately before i.p. injection. In combination studies, sertraline or rapamycin was administered once daily for 5 consecutive days, whereas doxorubicin was administered on day 2. Tumor-free survival is defined as the time between disappearance and reappearance of a palpable lymphoma following treatment. For terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) assays, 6- to 8-wk-old C57BL/6 mice bearing well-palpable tumors were treated once with sertraline (30 mg/kg) and, the next day, treated again with or without doxorubicin (10 mg/kg). Six hours later, tumors were removed and fixed in 10% neutral-buffered formalin overnight and embedded in paraffin. Tumor sections were used in TUNEL assays using *In Situ* Cell Death Detection Kit, POD (Roche) according to the manufacturer's protocol. For Ki67 staining, the rabbit monoclonal Ki67 (clone SP6) antibody was purchased from Thermo Scientific. Antigen retrieval

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was performed by boiling samples for 15 min in 10 mmol/L citrate buffer (pH 6.5). Sections were incubated with primary antibody for 1 h at room temperature. The UltraVision Detection System Anti-Rabbit, HRP/DAB (Thermo Scientific) was used according to the manufacturer's instructions.

RNA interference. Short interfering RNA (siRNA) duplexes targeting human REDD1 were purchased from siGENOME SMARTpool (M-010855-01). Control siRNA (eIF4AIII inverted) was a kind gift from Dr. Nahum Sonenberg (McGill University, Montreal, Quebec, Canada; ref. 29). Transfections with siRNA were performed using Lipofectamine 2000 according to the manufacturer's instructions (Invitrogen).

Results

Sertraline inhibits cell growth and translation initiation in MCF-7 human breast cancer cells. Sertraline (Fig. 1A, left) has been shown to downregulate expression of TCTP and exhibit antiproliferative activity against MDA-MB-231 cancer cells (20). To determine if this could be a consequence of protein synthesis inhibition, MCF-7 cells were exposed to increasing concentrations of sertraline for 24 hours and found to show a dose-dependent inhibition of cell growth with an IC_{50} of ~25 μmol/L (Fig. 1A, right). Metabolic labeling studies with [^{35}S]methionine revealed that exposure of MCF-7 cells to sertraline led to a reduction in protein synthesis, with a maximal inhibition of 55% at 20 μmol/L (Fig. 1B). Moreover, exposure of MCF-7 cells to sertraline caused a reduction in the abundance of heavy polysomes and an accumulation of free 80S ribosomes, consistent with sertraline exerting partial inhibition of translation initiation (Fig. 1C). The reduction in translation was not a consequence of sertraline-induced apoptosis, which was assessed by Annexin V/propidium iodine staining

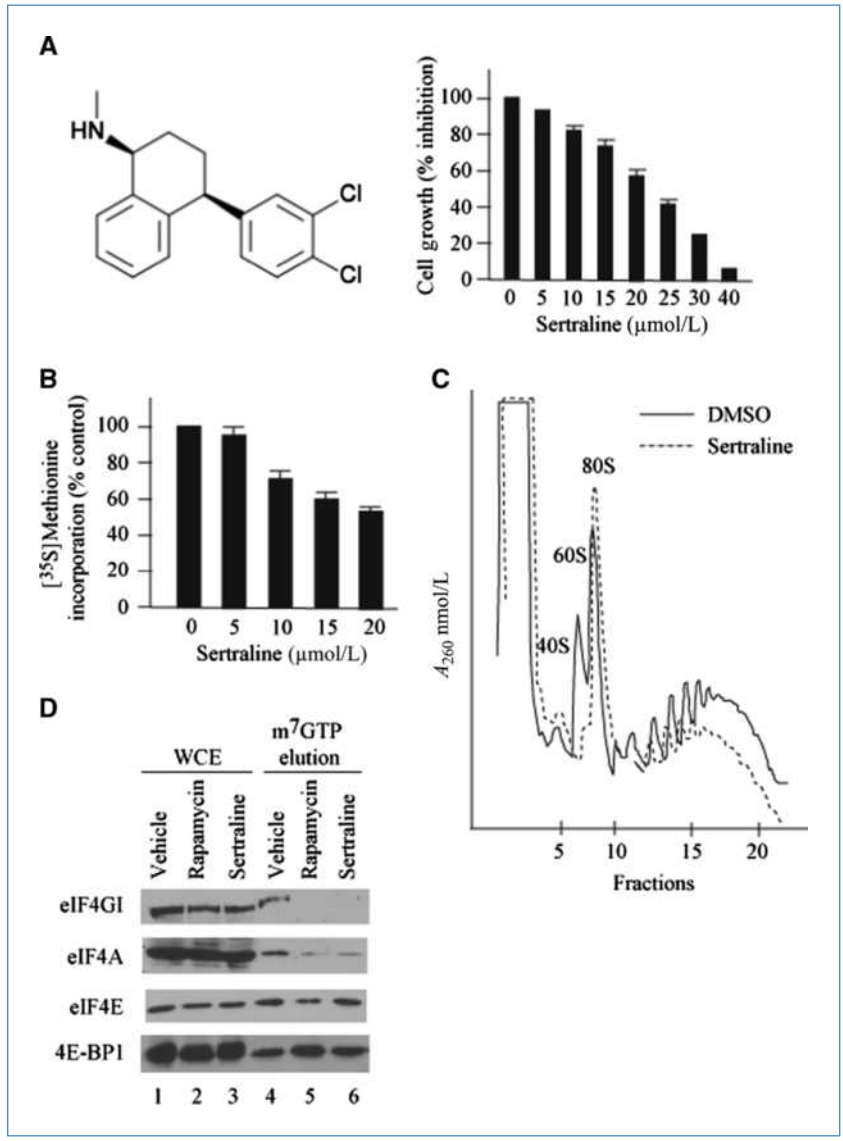

Figure 1. Effect of sertraline on cell growth, protein synthesis, and polysome distribution in MCF-7 breast cancer cells. A, left, chemical structure of sertraline; right, extent of cell growth of MCF-7 cells treated with the indicated concentrations of sertraline for 24 h by the SRB assay. Results are expressed relative to vehicle controls (set at 100%) and represent the average three experiments in triplicate with error bars (n = 9). B, MCF-7 cells were incubated with the indicated concentrations of sertraline for 20 h followed by [^{35}S]methionine labeling for 15 min. Columns, mean of triplicate samples from three independent experiments; bars, SE. C, MCF-7 cells were exposed to 20 μmol/L sertraline or DMSO for 2 h. Polysome analysis was performed as described in Supplementary Materials and Methods. D, extracts prepared from MCF-7 cells treated with vehicle, 20 nmol/L rapamycin, or 10 μmol/L sertraline were incubated with m^{7}GTP-coupled Sepharose resin, washed, and eIF4F eluted with m^{7}GDP. Aliquots of the eluted fractions (25 μL) were analyzed by Western blotting with the indicated antibodies.

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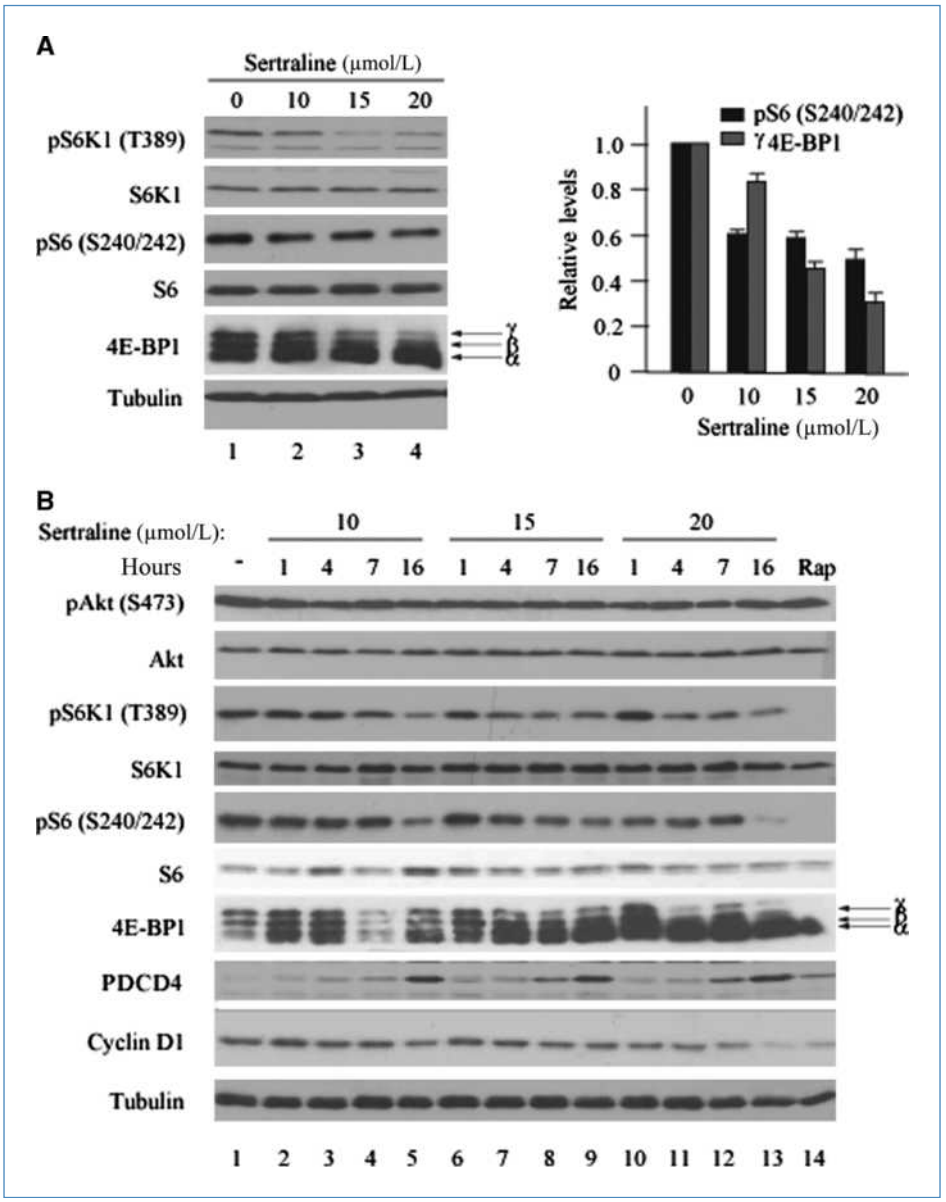

Figure 2. Sertraline inhibits mTOR signaling in a dose- and time-dependent manner. A, MCF-7 cells were treated with the indicated doses of sertraline for 24 h. Cells were harvested and lysates were resolved on 13% SDS-PAGE gels. Western blot analysis was carried out using the indicated antibodies. The percentage of 4E-BP1 protein present in the γ form and the levels of pS6 relative to total S6 were both quantitated by densitometry by ImageJ software. Columns, mean of three independent experiments; bars, SE. B, MCF-7 cells were incubated with 10, 15, and 20 μmol/L sertraline at the indicated time points. MCF-7 cells were also treated with 20 nmol/L rapamycin (Rap) for 16 h as a positive control. Equal amounts of protein were analyzed by SDS-PAGE, and Western blots were immunoblotted with the indicated antibodies.

(Supplementary Fig. S1). Long-term exposure to sertraline also did not induce apoptosis on nontransformed cells (Supplementary Fig. S1). Because sertraline has recently shown to also inhibit Akt activation (25, 30), we next determined if the sertraline-mediated decrease in translation was associated with altered levels of the eIF4F complex. To this end, we performed m^7GTP pull-down assays and analysis of the m^7GTP eluents revealed that, like rapamycin, sertraline caused a reduction in eIF4E-associated eIF4G and eIF4A and an increase in eIF4E-associated 4E-BP1 (Fig. 1D). These results indicate that exposure of cells to sertraline leads to decreased levels of eIF4F complex.

Sertraline inhibits mTOR signaling. To examine if sertraline could be affecting translation by targeting the mTOR signaling pathway, we performed Western blot analysis to assess the phosphorylation status of two downstream targets of mTOR: S6K1/2 and 4E-BP1. In MCF-7 cells, sertraline treatment led to a dose-dependent decrease in the phosphorylation of S6K1 and its target, S6 (Fig. 2A). Sertraline also caused a dose-dependent decrease in 4E-BP1 phosphorylation, reducing the levels of the hyperphosphorylated form (labeled γ) and increasing the hypophosphorylated form (labeled α; Fig. 2A). The inhibitory effect of sertraline on mTOR activity was observed as early as 1 hour at 10 μmol/L and continued through 16 hours of treatment (Fig. 2B). The serine/threonine protein kinase Akt is a major regulator of mTOR, and we noted that phospho-Akt status was unchanged upon sertraline treatment in MCF-7 cells (Fig. 2B). We did not detect changes in levels of caspase-3–dependent poly(ADP-ribose) polymerase cleavage in MCF-7 cells treated

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with 20 μmol/L sertraline for 24 hours (Supplementary Fig. S2), indicating the observed sertraline-mediated mTOR inhibition is not an indirect consequence of cell death. We also observed inhibition of mTOR activity in HeLa cells upon sertraline treatment, thus excluding that the effects documented here are cell line specific (data not shown).

Because sertraline treatment inhibited activation of the mTOR/p70S6K pathway, we also examined its effects on PDCD4 expression, a tumor suppressor gene product whose stability is under mTOR/S6K1 control (10). Treatment of MCF-7 cells with sertraline caused an increase in PDCD4 protein, prominently at 16 hours, whereas rapamycin caused only a slight increase in PDCD4 levels (Fig. 2B). Moreover, expression of cyclin D1, an eIF4F-responsive transcript, was inhibited after 16 hours of sertraline treatment and most notably at 20 μmol/L concentration (Fig. 2B). Consistently, sertraline treatment at 20 μmol/L induced G_1 cell cycle arrest (76.6% versus 55.4% in control) with a reduction of cells in S phase (12.9% versus 25.3%) and G_2-M phase (10.4% versus 19.3%; Supplementary Fig. S3), indicating that sertraline may inhibit cell proliferation in a cyclin D1–dependent manner.

Previous studies have shown that nuclear 4E-BPs sequester eIF4E in the nucleus in MEFs in a manner that is dependent on the phosphorylation status of 4E-BP1—a reduction in phospho-4E-BP1 status being associated with increased nuclear retention of eIF4E (31). Because our data indicate that sertraline causes hypophosphorylation of 4E-BP1, we expected that it should increase the nuclear content of eIF4E. Cells were treated with either rapamycin or sertraline for 6 hours, and the amount of cytoplasmic/nuclear eIF4E was quantified in MCF-7 using immunofluorescence (Supplementary Fig. S4A and B). eIF4E relocalized from the cytoplasm to the nucleus upon sertraline and rapamycin treatment (Supplementary Fig. S4), consistent with nuclear 4E-BP1 phosphorylation status being affected by sertraline treatment and the reduction in eIF4F levels that we observed (Fig. 1E).

Sertraline acts downstream of TSC2 and Rheb. To elucidate the mechanism of action by which sertraline inhibits mTOR activity, we examined the effect of sertraline on established *Tsc2*$^{+/+}$ and *Tsc2*$^{-/-}$ MEFs. In *Tsc2*$^{+/+}$ MEFs, exposure to sertraline decreased phosphorylation of S6K1, S6, and 4E-BP1 in response to serum and insulin (Fig. 3A). As expected, rapamycin also led to a decrease in phosphorylation of S6K1 and S6 and a shift from hyperphosphorylated 4E-BP1 to the hypophosphorylated forms (Fig. 3A). In *Tsc2*$^{-/-}$ MEFs, mTOR activity is elevated relative to *Tsc2*$^{+/+}$ cells, as judged by levels of phospho-S6K1, phospho-S6, and phospho-4E-BP1 (26). Sertraline was still capable of reducing phospho-S6K1, phospho-S6, and phospho-4E-BP1 levels in *Tsc2*$^{-/-}$ MEFs (Fig. 3A). The results indicated that exposure to sertraline is influencing the mTOR pathway downstream of the TSC1/TSC2 complex. We therefore assessed the ability of sertraline to block the activity of Rheb(S16H), a Rheb mutant that exhibits gain-of-function properties in activating mTORC1 signaling (Fig. 3B). Relative to wild-type Rheb, Rheb(S16H) is more active at promoting the phosphorylation of the mTOR effectors S6K1 and 4E-BP1 (32). *Tsc2*$^{+/+}$ MEF cells were infected with MSCV/IRES-GFP or MSCV/Rheb(S16H)IRES-GFP retroviruses, and Western blotting was performed to confirm elevated expression of Rheb(S16H) in MSCV/Rheb(S16H)IRES-GFP–infected cells (Fig. 3B). As expected, phospho-S6K1, phospho-S6, and phospho-4E-BP1 levels were elevated in the presence or absence of serum in MSCV/Rheb(S16H)IRES-GFP–infected cells compared with MSCV/IRES-GFP–infected cells (Fig. 3B). Exposure of serum-stimulated MSCV/Rheb(S16H)IRES-GFP–infected

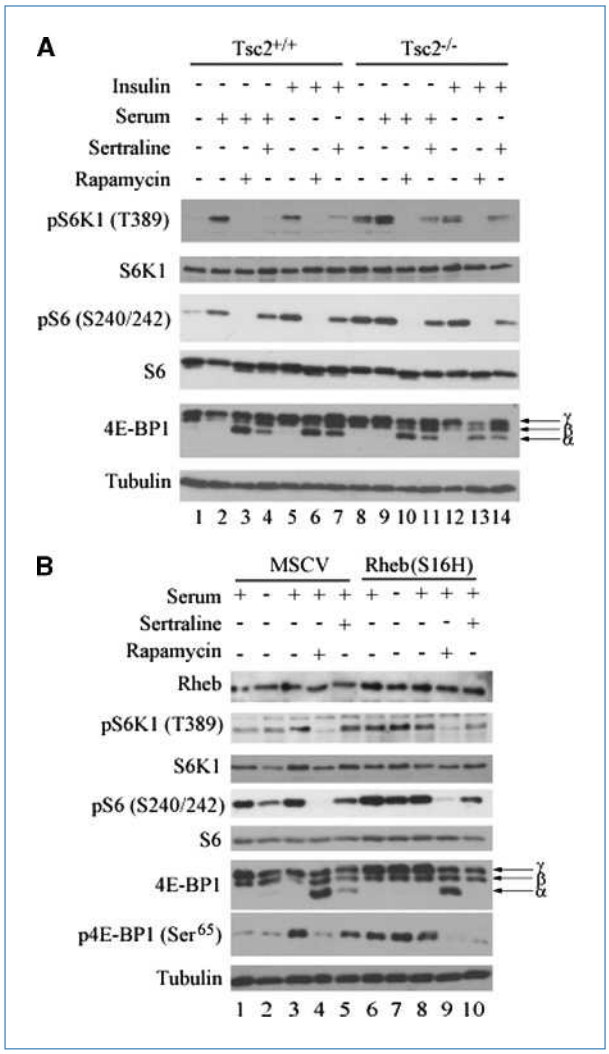

Figure 3. Sertraline inhibits mTOR signaling by acting downstream of TSC2 and Rheb. A, *Tsc2*$^{+/+}$ and *Tsc2*$^{-/-}$ MEFs were serum starved for 24 h, exposed to 10 μmol/L sertraline or 20 nmol/L rapamycin for another 16 h, and stimulated with either 10% serum or 100 nmol/L insulin for 30 min. Total cell extracts were subjected to SDS-PAGE followed by immunoblotting with antibodies against the indicated proteins. B, proliferating *Tsc2*$^{+/+}$ MEF cells were infected with MSCV/IRES-GFP or MSCV/Rheb(S16H)-IRES-GFP retroviruses. Cells were serum starved for 5 h, exposed to 20 μmol/L sertraline or 20 nmol/L rapamycin for another 1 h, and, where indicated, stimulated with 10% serum for 30 min before harvesting. Total cell extracts were subjected to SDS-PAGE followed by immunoblotting with antibodies against the indicated proteins.

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cells to sertraline or rapamycin reduced the phosphorylation status of S6K1, S6, and 4E-BP1 (Fig. 3B), indicating that sertraline was acting downstream of Rheb to inhibit mTOR signaling.

Sertraline enhances phosphorylation of eIF2α and induces REDD1 expression. The results described above do not exclude an effect of sertraline on other components of the translation apparatus, and we therefore examined the phosphorylation status of eIF2α, a well-known checkpoint for translational control. Western blotting of extracts prepared from sertraline-treated MCF-7 cells revealed increased phosphorylation of eIF2α upon exposure to sertraline (Fig. 4A). The eIF2α downstream target ATF4 is necessary and sufficient for stress-induced upregulation of REDD1 expression

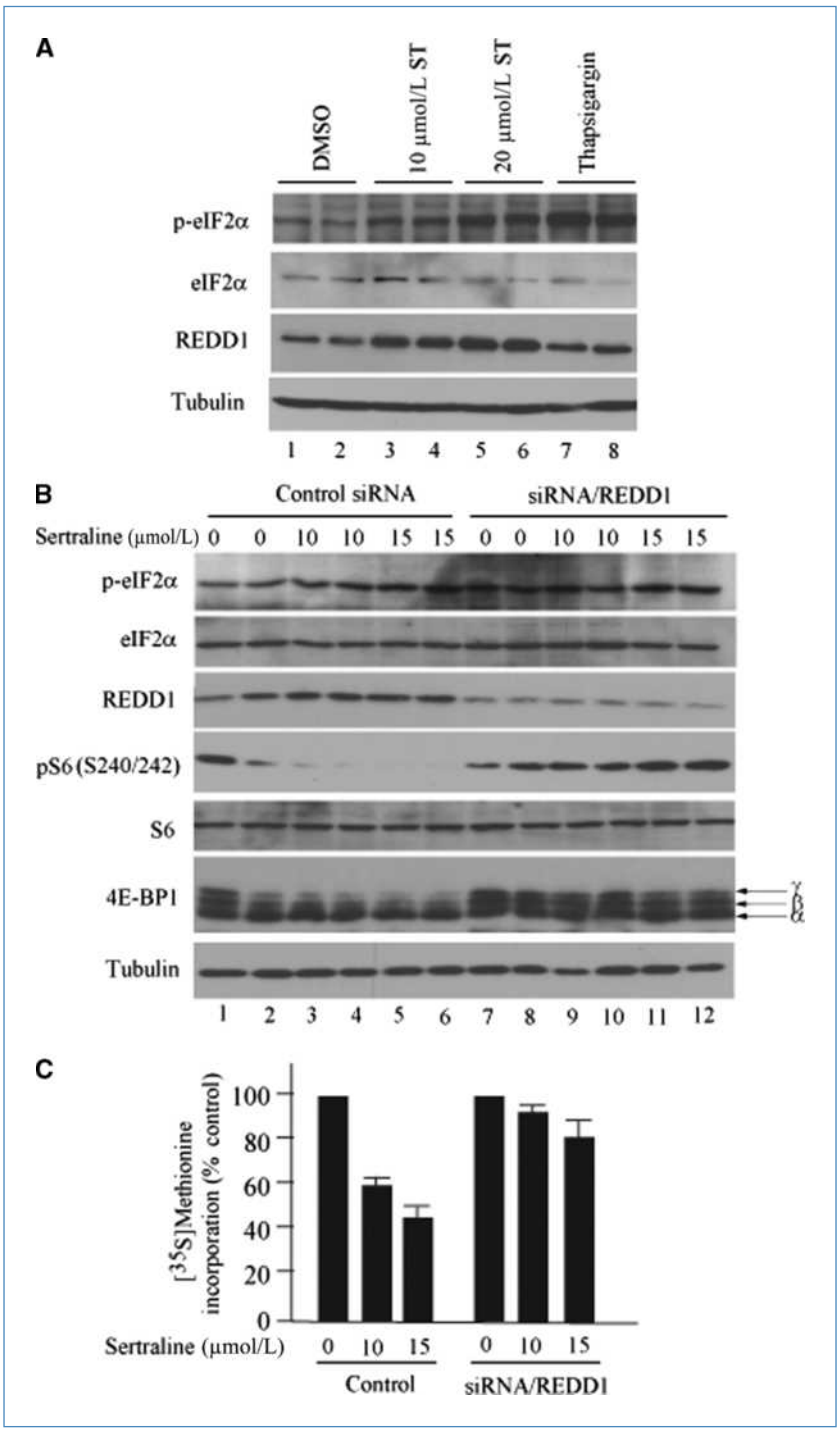

Figure 4. Sertraline causes increased phosphorylation of eIF2α and induces REDD1 expression. A, MCF-7 cells were treated with DMSO, 10 μmol/L or 20 μmol/L sertraline (ST), or 1.5 μmol/L thapsigargin for 4 h. Total cell extracts were subjected to SDS-PAGE followed by immunoblotting with antibodies against the indicated proteins. MCF-7 cells were transfected with either control or REDD1 siRNA for 48 h followed by 10 or 15 μmol/L sertraline treatment for 16 h and labeled with [^{35}S]methionine for 15 min. Cells lysates were prepared and the indicated protein levels were measured by Western blot analysis (B) or used to measure [^{35}S]methionine incorporation (C) by scintillation counting. C, columns, mean of triplicate samples from three independent experiments (n = 3); bars, SE.

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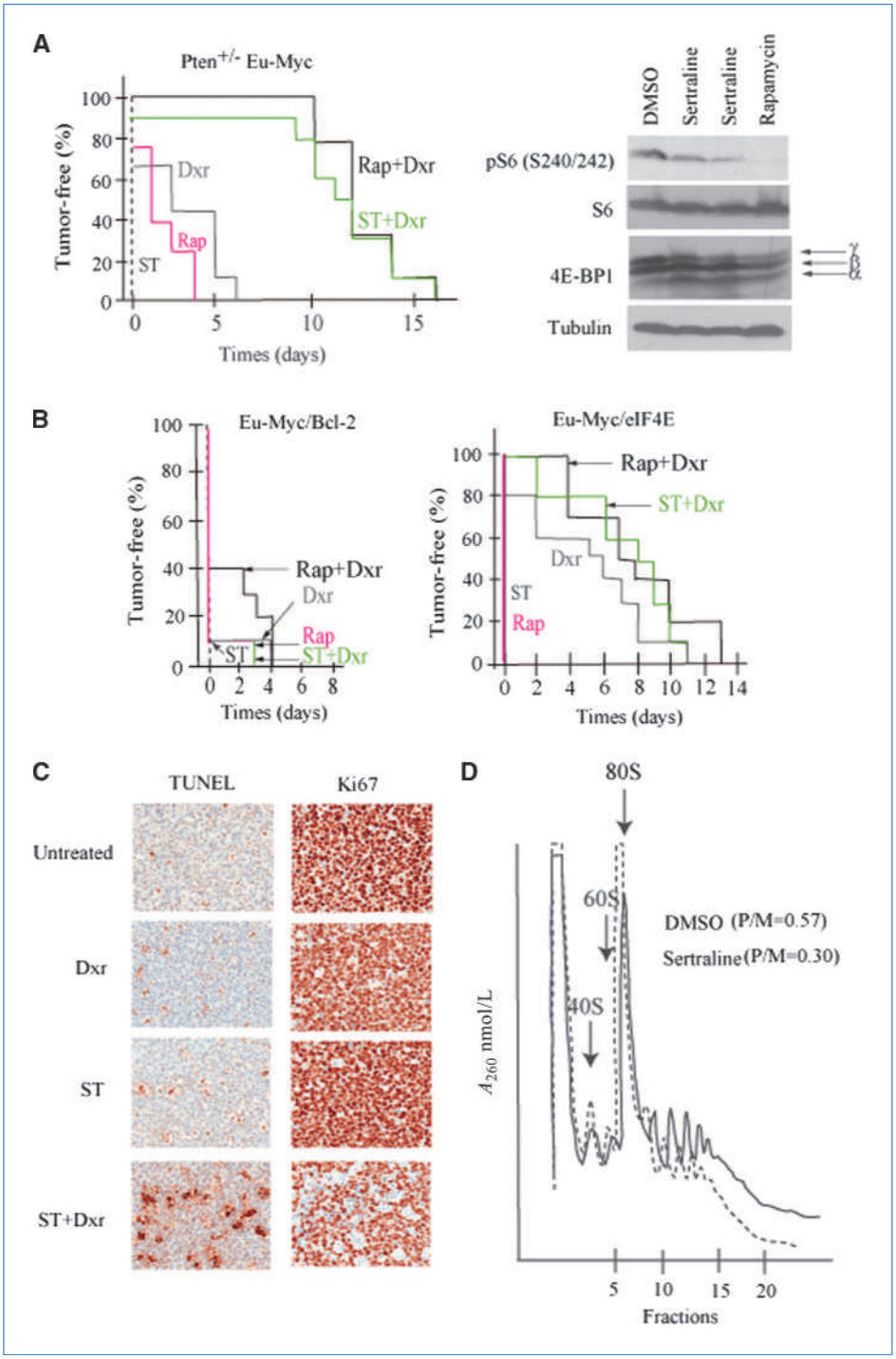

Figure 5. Sertraline alters chemosensitivity in *Pten*$^{+/-}$Eμ-*Myc* tumors *in vivo*. A, sertraline sensitizes *Pten*$^{+/-}$Eμ-*Myc* tumors to the effects of doxorubicin (Dxr) *in vivo*. Kaplan-Meier plot showing tumor-free survival of mice bearing *Pten*$^{+/-}$Eμ-*Myc* tumors following treatment with doxorubicin (solid gray line; $n = 9$), rapamycin (solid red line; $n = 8$), rapamycin and doxorubicin (solid black line; $n = 9$), sertraline (dotted black line; $n = 10$), or sertraline + doxorubicin (solid green line; $n = 10$). $P < 0.001$, combination treatments compared with single-agent treatments. The Western blotting to the right panel represents *Pten*$^{+/-}$Eμ-*Myc* tumors treated with sertraline (30 mg/kg) or rapamycin (4 mg/kg) for 4 h. B, Kaplan-Meier plot showing tumor-free survival of mice ($n = 10$) bearing Eμ-*Myc/Bcl-2* tumors (left) or Eμ-*Myc/eIF4E* tumors (right) following treatment with rapamycin, rapamycin + doxorubicin, doxorubicin, sertraline, or sertraline + doxorubicin. C, representative micrographs of *Pten*$^{+/-}$Eμ-*Myc* lymphomas following drug treatments. Mice were injected once with sertraline (30 mg/kg) and then again 20 h later with sertraline ± doxorubicin. Six hours later, tumors were processed for TUNEL analysis and immunohistochemistry for Ki67. D, sertraline inhibits translation initiation in *Pten*$^{+/-}$Eμ-*Myc* tumors *in vivo*. Mice bearing *Pten*$^{+/-}$Eμ-*Myc* tumors were injected with sertraline (30 mg/kg). Cytoplasmic extracts were prepared from excised tumors 4 h later, and polysome analysis was performed as described in Supplementary Materials and Methods.

(33), and we found a concomitant increase in REDD1 protein levels (Fig. 4A). To assess whether REDD1 was necessary for the repression of protein synthesis and inhibition of mTOR signaling observed with sertraline, siRNAs directed against the human REDD1 were used to reduce its expression in MCF-7 cells. Administration of REDD1 siRNA caused a reduction in REDD1 protein expression compared with control siRNA cells (Fig. 4B). Transfection of REDD1 siRNA did not cause a reduction in sertraline-mediated phosphorylation of S6 and 4E-BP1, suggesting that this compound represses mTOR signaling through enhanced expression of REDD1 (Fig. 4B). Moreover, we found that cells transfected with REDD1 siRNA were more resistant to inhibition of protein synthesis by sertraline as assessed in metabolic labeling studies with [^{35}S]methionine incorporation

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(Fig. 4C). Next, to determine the extent to which phosphorylation of eIF2α on Ser^{51} contributes to the translation inhibition observed *in vivo* by sertraline, we compared protein synthesis in wild-type MEFs with that of MEFs derived from homozygous $eIF2\alpha^{S51A/S51A}$ "knock-in" mouse embryos (34). We found that cells derived from $eIF2\alpha^{S51A/S51A}$ mice were more resistant to inhibition of protein synthesis by sertraline (Supplementary Fig. S5).

Sertraline reverses chemoresistance mediated by PTEN inactivation in Eμ-Myc lymphomas. Because sertraline inhibits mTOR signaling and subsequent downstream translation initiation, we tested its ability to alter chemosensitivity in the *Eμ-Myc* lymphoma model (16). Doxorubicin and rapamycin synergized in mice bearing $Pten^{+/-}$*Eμ-Myc* tumors and extended tumor-free survival to 10 to 16 days (Fig. 5A, left; $P < 0.001$, rapamycin plus doxorubicin versus rapamycin or doxorubicin alone), as previously reported (28). As a single agent, sertraline showed no activity against $Pten^{+/-}$*Eμ-Myc* lymphomas. However, in conjunction with doxorubicin, clear synergy was observed with all animals achieving remissions that lasted up to 16 days (Fig. 5A, left; $P < 0.001$, sertraline plus doxorubicin versus sertraline or doxorubicin). Western blot analysis confirmed decreased phosphorylation of S6 and 4E-BP1 in animals harboring $Pten^{+/-}$*Eμ-Myc* tumors treated with sertraline (Fig. 5A, right). No synergy between sertraline and doxorubicin was observed in *Eμ-Myc/Bcl-2* lymphomas, implying that the observed effect is genotype specific (Fig. 5B, left). *Eμ-Myc/Bcl-2* lymphomas are also refractory to the combination of rapamycin and doxorubicin treatment (Fig. 5B, left), consistent with previous studies (16). *Eμ-Myc/eIF4E* lymphomas, which overcome the inhibitory effects of rapamycin on mTOR by overexpressing eIF4E, did not respond to sertraline alone or to the combination of sertraline and doxorubicin (Fig. 5B, right). Enhanced drug sensitivity in $Pten^{+/-}$*Eμ-Myc* tumors was associated with increased apoptosis and decreased proliferation for sertraline/doxorubicin and rapamycin/doxorubicin combinations compared with single-agent treatments with doxorubicin, rapamycin, or sertraline (Fig. 5C; data not shown). To ensure that the concentration of sertraline achieved *in vivo* was sufficient to inhibit translation, we analyzed the polysome profile of tumors extracted from sertraline-treated $Pten^{+/-}$*Eμ-Myc* mice (Fig. 5D). Sertraline caused a reduction of polysomes and a concomitant increase in 80S ribosomes compared with that from nontreated tumors (Fig. 5D), indicating that sertraline was targeting the translation process *in vivo*.

Discussion

The phosphatidylinositol 3-kinase (PI3K)/Akt signaling is a major pathway altered in human cancers and shown to play key physiologic roles in regulating cell growth, cell cycle regulation, protein synthesis and degradation, cell migration, and survival (35). Therefore, interdicting mTOR as a downstream kinase in the PI3K/Akt pathway is an attractive therapeutic target for cancer therapy, as exemplified by current clinical trials of three rapamycin analogues. Herein, we report that sertraline has significant antiproliferative activity by targeting two translation initiation checkpoints, eIF2α phosphorylation (Fig. 4A) and assembly of eIF4F, the latter being achieved through downregulation of mTOR signaling (Fig. 2).

We find that the effect of sertraline on translation initiation seems to converge with phosphorylation of eIF2α, leading to upregulation of REDD1 (Fig. 4A), a hypoxia-induced gene product implicated in the mTOR response to hypoxia (36). Increased levels of REDD1 are associated with endoplasmic reticulum stress and activation of ATF4, a downstream target of increased eIF2α phosphorylation levels (33). Blockage of REDD1 expression by siRNA resulted in restoration of phosphorylation of S6 and 4E-BP1 protein and rescued inhibition of protein synthesis in sertraline-treated MCF-7 cells, indicating that REDD1 is necessary for these processes (Fig. 4B and C). Interestingly, REDD1 has been shown to have a very short half-life (5 minutes) and, on inhibition of translation, is quickly eliminated from cells (37). We clearly do not see this phenomenon (Fig. 4A), and this might be a cell-dependent effect or dependent on the mode by which translation is inhibited (initiation versus elongation). REDD1 has recently been implicated in RAS-mediated transformation, and those results would seem to suggest that increased expression of REDD1 is an undesired approach to exert antiproliferative effects (38). However, that study did not address whether the REDD1/mTOR connection was maintained and may suggest a stratification approach to determining tumor responsiveness to increased phospho-eIF2α levels.

Targeting eIF2α phosphorylation by sertraline would have therapeutic potential to target breast cancer. Double-stranded RNA-activated kinase, PKR, an upstream eIF2a kinase, has been implicated as a tumor suppressor (39), and a nonphosphorylated form of eIF2α has been shown to be oncogenic *in vitro* (40). Compounds that induce phosphorylation of eIF2α, such as clotrimazole, have been explored as chemotherapeutic agents (22, 23), but because these agents exert additional effects (such as depletion of intracellular Ca^{2+} stores), it has been difficult to attribute the antiproliferative effects solely to inhibition of translation.

Ectopic overexpression of eIF4E and eIF4GI is also oncogenic *in vitro* (41, 42), and eIF4E cooperates with c-Myc during lymphomagenesis *in vivo* (16). eIF4E is also a genetic modifier of rapamycin resistance (28). One mechanism to explain the transforming potential of eIF4F has been attributed to translational remodeling of the oncoproteome, resulting in a subsequent blockade of proapoptotic stimuli (43, 44). Increased levels of the eIF4F complex preferentially increase translation of "weak" mRNAs, which encode growth factors and proto-oncogenes such as vascular endothelial growth factor, c-Myc, cyclin D1, and ornithine decarboxylase (45). These mRNAs have lengthy, G+C-rich, highly structured 5′ untranslated region that requires a higher dependency on eIF4F for ribosome loading. We show here that cyclin D1 expression was preferentially decreased (Fig. 2B) when eIF4F complex levels were downregulated in sertraline-treated cells (Fig. 1E). The sertraline-mediated decrease in cyclin D1 protein expression was associated

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with G_1 cell cycle arrest (Supplementary Fig. S3) and mTOR inhibition with a decrease in phosphorylation of 4E-BP1 (Fig. 2). This is consistent with the findings that expression of constitutively active 4E-BP1 in MCF-7 cells leads to cell cycle arrest, which is associated with downregulation of cyclin D1 (46).

In our study, we found that treatment of MCF-7 cells with sertraline led to decreased phosphorylation of S6K (Thr^{389}) and a corresponding increase in PDCD4 protein levels (Fig. 2B), indicating that sertraline also affects assembly of eIF4A into the eIF4F complex and thus inhibits translation initiation. *PDCD4* is a tumor suppressor gene product whose levels are reduced in human lung, renal, and glial tumors (47). PDCD4 has been shown to inhibit tumor promoter–induced neoplastic transformation in the murine JB6 cell model (48). Moreover, overexpression of PDCD4 in the epidermis delays tumor onset and progression in a chemically induced murine skin tumor model (49). A recent study has shown that PDCD4 downregulation significantly reduced the sensitivity of MCF-7 cells to tamoxiften and geldanamycin (47). Therefore, therapeutic strategies to upregulate PDCD4 expression with geldanamycin and that target other aspects of eIF4F assembly, such as sertraline, in combination, may offer promise to target breast cancer.

We note that when sertraline is used as an antidepressant, the serum levels range from 16 to 78 ng/mL (0.05–0.26 μmol/L; ref. 50). The concentrations used in our study *in vitro* are approximately 75 to 100 times higher than these levels. Testing of a small number of sertraline analogues revealed that some of these seem to retain the ability of exerting the same biological effects as sertraline, suggesting that the core scaffold has yet to be modified to achieve optimal inhibition of protein synthesis (data not shown). We note that relatively high doses of sertraline seemed well tolerated *in vivo* in mice, where the compound exerted antiproliferative activity in conjunction with doxorubicin and was able to inhibit protein synthesis at these higher doses (Fig. 5).

In our study, we used the genetically defined *Eμ-Myc* murine lymphoma model to study the drug response of sertraline *in vivo*. Previous studies have shown that activation of Akt signaling in this preclinical model accelerates tumorigenesis and promotes chemoresistance (16). As a single agent, sertraline was ineffective against Eμ-Myc lymphomas harboring PTEN lesions or overexpressing Bcl-2 and eIF4E (Fig. 5A and C). However, in combination with doxorubicin, sertraline was effective against $Pten^{+/-}$*Eμ-Myc* lymphomas, whereas *Eμ-Myc/Bcl-2* and *Eμ-Myc/eIF4E* lymphomas remained insensitive to this drug combination (Fig. 5A and C). Sertraline/doxorubicin treatment in these chemoresistant $Pten^{+/-}$*Eμ-Myc* tumors induced an apoptotic response and decreased cell proliferation as determined by TUNEL and Ki67 analysis, respectively (Fig. 5D). We speculate that the partial inhibition of protein synthesis exerted on sertraline administration *in vivo* (Fig. 5E) curtails prosurvival signaling to allow proapoptotic triggering by doxorubicin. Our findings may provide a rationale for the development of sertraline analogues as antiproliferative compounds.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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