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Natural Product-Derived Antitumor Compound Phenethyl Isothiocyanate Inhibits mTORC1 Activity via TSC2

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ABSTRACT: Phenethyl isothiocyanate (**1**) is a natural dietary phytochemical with cytostatic, cytotoxic, and antitumor activity. The effects of **1** were investigated on the activity of mTOR, a kinase that enhances the translation of many RNAs encoding proteins critical for cancer cell growth, including the angiogenesis regulator HIF1α. Compound **1** effectively blocked *HIF1α* RNA translation in MCF7 breast cancer cells, and this was associated with reduced phosphorylation of 4E-BP1 and p70 S6K, well-characterized downstream substrates of the mTOR-containing mTORC1 complex. Compound **1** also inhibited mTORC1 activity in mouse embryonic fibroblasts (MEFs). The **1**-mediated inhibition of mTORC1 activity appeared to be independent of the upstream regulators PTEN, AKT, ERK1/2, and AMPK. By contrast, **1**-mediated inhibition of mTORC1 activity was dependent on the presence of TSC2, part of a complex that regulates mTORC1 activity negatively. TSC2-deficient MEFs were resistant to **1**-mediated inhibition of p70 S6K phosphorylation. TSC2-deficient MEFs were also partially resistant to **1**-mediated growth inhibition. Overall, the present results confirm that **1** inhibits mTORC1 activity. This is dependent on the presence of TSC2, and inhibition of mTORC1 contributes to optimal **1**-induced growth inhibition. Inhibition of RNA translation may be an important component of the antitumor effects of phenethyl isothiocyanate.

Phenethyl isothiocyanate (**1**) is a dietary phytochemical that has received considerable attention for its potential cancer chemopreventive activity.[1−3] This compound interferes with carcinogen activation and decreases carcinogen-induced cancer development in vivo. Compound **1** also exerts direct effects against established cancer cells via induction of apoptosis, inhibition of cell-cycle progression, suppression of angiogenesis, and/or decreased migration/invasion. Consistent with this, **1** exerts therapeutic effects in xenograft and genetically induced tumors in in vivo models where carcinogens are not thought to play a role. Phenethyl isothiocyanate, along with other related isothiocyanates, is thought to be an important player in mediating the potential anticancer benefits of diets rich in cruciferous vegetables.[4,5] The chemopreventive/chemotherapeutic effects of **1** are currently being explored in clinical studies in low-grade B-cell lymphoma and lung cancer (NCT00968461, NCT00691132; http://clinicaltrials.gov/).

Phenethyl isothiocyanate (**1**) is an electrophilic compound that reacts readily with cellular thiols. Following uptake into cells, the predominant initial reaction of **1** is with glutathione (GSH), the major intracellular antioxidant.[6,7] Resultant **1**-conjugates are exported from cells via efflux pumps. However, extracellular hydrolysis of **1**-conjugates results in liberation of **1**, which is free to re-enter the cell. The net effect of this cycle is a rapid depletion of intracellular GSH and a ∼100-fold accumulation of intracellular **1**. GSH depletion results in increased accumulation of reactive oxygen species (ROS); the production of intracellular ROS may also increase due to **1**-mediated inhibition of mitochondrial oxidative phosphorylation.[8,9] Free intracellular **1** is also able to react with cysteinyl thiols of cellular proteins, potentially leading to altered protein function.[10−14]

Our own work on phenethyl isothiocyanate (**1**) has focused on angiogenesis, the development of new blood vessels from an existing vasculature and one of the key "hallmarks" of cancer.[1,15] The HIF1 transcription factor is a central regulator of angiogenesis and is often overexpressed in cancer cells.[16,17] Hypoxia prevents proteolysis of HIF1α, allowing it to accumulate to relatively high levels. HIF1α then interacts with its constitutive binding partner HIF1β and activates a battery of target genes involved in control of angiogenesis, survival, and metabolism. Our studies have demonstrated that **1** interferes with both the hypoxia-dependent accumulation of HIF1α and the expression of endogenous HIF1 target genes.[18] This was independent of changes in *HIF1α* RNA expression and did not require the canonical protein degradation machinery. It was suggested that inhibition of HIF1α expression by **1** is due to decreased translation of *HIF1α* RNA.[18]

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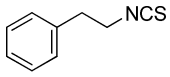
The *HIF1α* RNA has a complex 5′-untranslated region (UTR), and its translation is highly dependent on the activity of the mTORC1 complex, one of two complexes containing the mTOR kinase.[19,20] Thus, treatment of cells with rapamycin, a selective mTORC1 inhibitor, results in rapid down-modulation of *HIF1α* RNA translation.[21,22] The mTORC1 complex is activated commonly by growth factor signaling downstream of the PI3K/AKT and the ERK1/2 MAP kinase pathways. PI3K activation leads to PDK1-mediated activation of AKT, via phosphorylation of AKT T^{308}, which, in turn, causes phosphorylation and inactivation of the TSC1/2 complex, the major negative regulator of mTORC1 activity. Once activated, mTORC1 phosphorylates 4E-BP1 on multiple residues, preventing it from binding to and inhibiting the function of eIF4E, which is required particularly for translation of RNAs with complex 5-UTRs.[23] Another substrate for mTORC1 is T^{389} of p70 S6K. The mechanisms that regulate the second mTOR complex, mTORC2, are less well understood. However, one key target for mTORC2 is S^{473} of AKT, and this phosphorylation is required for optimal activation of AKT.[24] Other targets for mTORC2 include SGK1, which, in turn, phosphorylates T^{346} of NDRG1.[25]

Phenethyl isothiocyanate (**1**) has been linked previously to inhibition of mTOR activity. Our group and others have shown that treatment of human MCF7 (breast cancer), HCT116 (colon cancer), or PC3 (prostate cancer) cells leads to decreased phosphorylation of the mTORC1 substrate 4E-BP1.[18,26] Given that mTORC1 plays a key role in *HIF1α* RNA translation, it was reasoned that inhibition of mTORC1 activity by **1** may be responsible for down-modulation of HIF1α expression. In the present work, human MCF7 breast cancer cells and genetically engineered mouse embryonic fibroblasts (MEFs) were used to examine the effect of **1** on mTOR signaling.



(**1**)

RESULTS AND DISCUSSION

We previously demonstrated that phenethyl isothiocyanate (**1**) decreases expression of HIF1α in hypoxia-treated MCF7 cells.[18] Since **1** did not alter the levels of *HIF1α* RNA and also decreased HIF1α protein expression in VHL-deficient cells, it was reasoned that this compound was likely to act via inhibition of *HIF1α* RNA translation. To test this directly, metabolic labeling was performed with [^{35}S]-labeled amino acids followed by HIF1α immunoprecipitation. Incubation of MCF7 cells with **1** effectively prevented *HIF1α* RNA translation (Figure 1A). Quantitation of multiple experiments demonstrated that the IC_{50} for inhibition of *HIF1α* RNA translation was ∼7.5 μM, and, at 20 μM, **1** inhibited *HIF1α* RNA translation to the same extent as the positive control, cycloheximide, which inhibits overall protein synthesis (Figure 1B).

Translation of *HIF1α* RNA is highly dependent on the activity of mTORC1.[21,22] Therefore, the effect of **1** was investigated on mTORC1 activity by analyzing phosphorylation of two mTORC1 substrates, p70 S6K and 4E-BP1 (Figure 2A,B). p70 S6K phosphorylation was analyzed using a phospho-T^{389} specific antibody. 4E-BP1 is subject to multisite

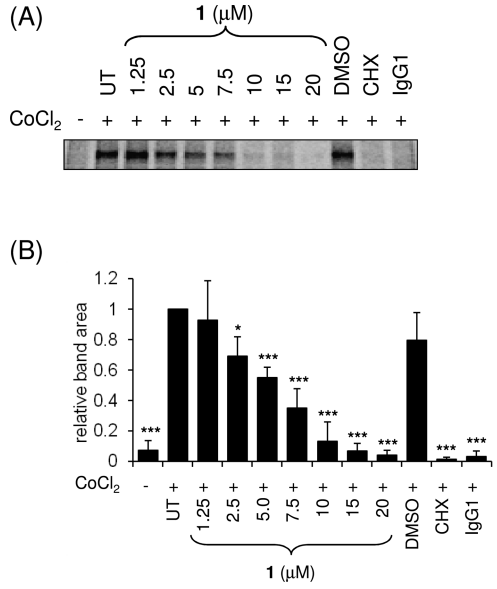


Figure 1. Effect of phenthyl isothiocyanate (**1**) on *HIF1α* RNA translation. MCF7 cells were pretreated with the indicated concentrations of **1**, DMSO, and cycloheximide (CHX; 10 μg/mL) or left untreated (UT) as a control for 1 h prior to metabolic labeling for 2 h. $CoCl_2$ was included to inhibit HIF1α degradation as indicated. Cells were lysed, and HIF1α protein was immunoprecipitated and analyzed by SDS-PAGE. IgG1 indicates a sample immunoprecipitated using an isotype control antibody. (A) Representative gel image; (B) quantitative analyses (means ± SD of three independent experiments). Statistically significant differences between untreated and treated cells are indicated (repeated measures ANOVA with Tukey's multiple comparison test; $^{**}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$). All other comparisons were not statistically significant.

phosphorylation by mTORC1, and 4E-BP1 phosphorylation was analyzed by quantifying the abundance of more slowly migrating isoforms relative to total 4E-BP1 expression detected in immunoblots using a total 4E-BP1 antibody. Treatment of MCF7 cells with **1** caused a statistically significant reduction in p70 S6K T^{389} phosphorylation.

Consistent with previous studies,[18,26] **1** also reduced the phosphorylation of 4E-BP1. Therefore, **1** probably inhibits mTORC1 signaling in MCF7 cells. Time-course experiments showed that, at 20 μM, **1** decreased p70 S6K phosphorylation within 15 min (Figure 2C). Similar experiments were performed in MEFs, focusing on p70 S6K T^{389} phosphorylation as a read-out of mTORC1 activity. Similar to MCF7 cells, **1** also effectively decreased p70 S6K phosphorylation in control MEFs ($PTEN^{+/+}$ in Figure 3A and $p53^{-/-}TSC2^{+/+}$ in Figure 5B,C). However, MEFs appeared to be more sensitive to the inhibitory effects of **1**, with strongly reduced p70 S6K T^{389} phosphorylation observed at 2.5−5 μM.

To determine the mechanism by which **1** inhibited mTORC1 activity, the effects of this compound were investigated on key upstream regulators. AKT is a major regulator of mTORC1 since AKT-mediated phosphorylation of TSC2 leads to inactivation of TSC2 and mTORC1 activation.[27,28] The AKT pathway is itself regulated negatively by PTEN, a lipid phosphatase that dephosphorylates PIP3, thereby interfering with PIP3-dependent activation of PI3K.[29] To determine the potential role of PTEN/AKT in **1**-mediated mTORC1 inhibition, it was first determined whether PTEN status influences the ability of **1** to inhibit mTORC1 activity by

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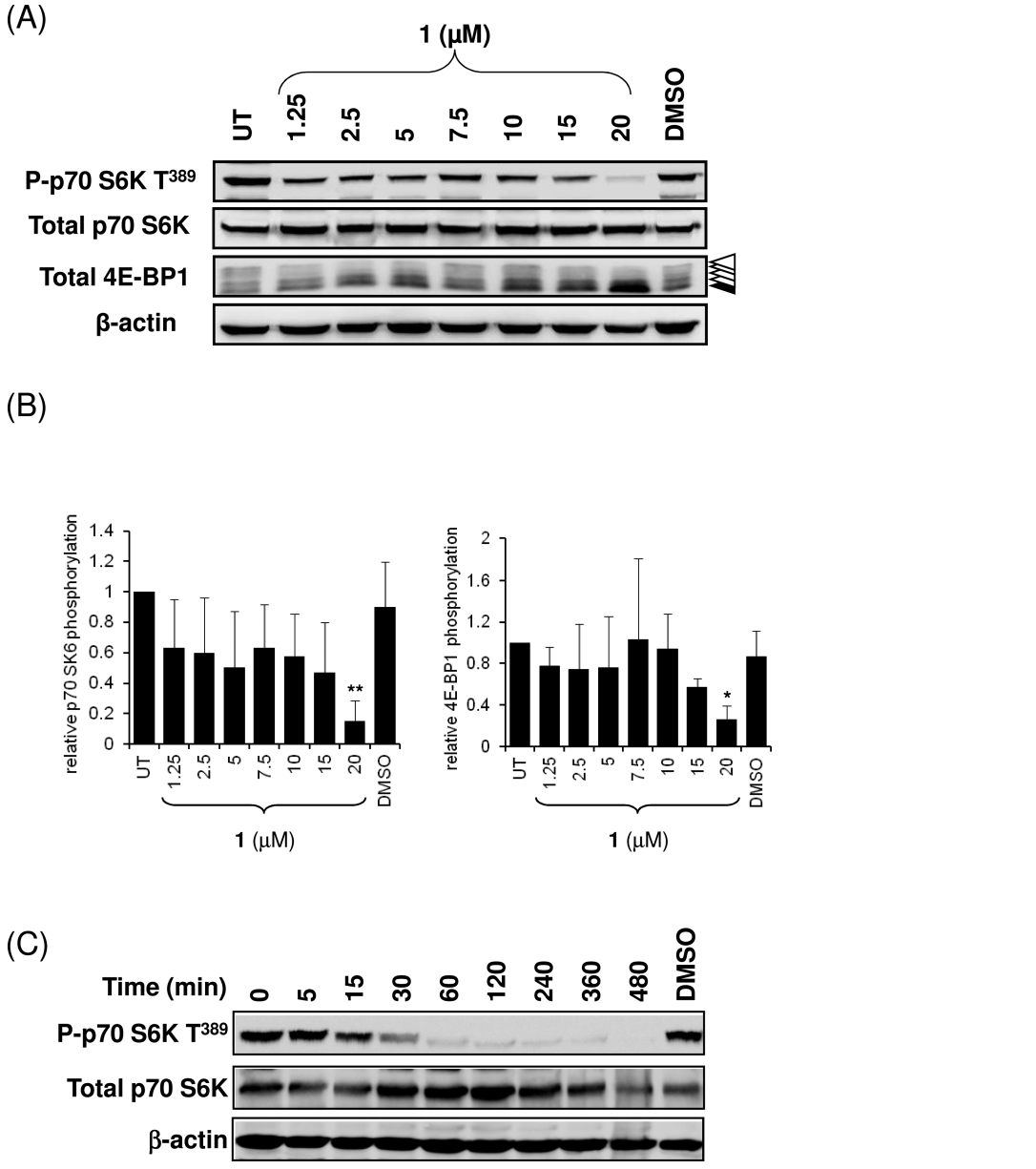

Figure 2. Effect of phenthyl isothiocyanate (**1**) on mTORC1 signaling in MCF7 cells. (A, B) MCF7 cells were treated with indicated concentrations of **1** or DMSO for 3 h or left untreated as a control (UT). Expression of total p70 S6K, phospho-p70 S6K T^{389}, total 4E-BP1, and β-actin was analyzed by immunoblotting. (A) Representative immunoblots. Open and closed arrow heads indicate phosphorylated and unphosphorylated 4E-BP1, respectively. (B) Quantitation; means ± SD derived from three independent experiments. For p70 S6K phosphorylation the phospho-specific signal was normalized using the intensity of total p70 S6K expression. For 4E-BP1 phosphorylation, the proportion of slower migrating bands was quantified as a proportion of total 4E-BP1 expression. The level of phosphorylation in untreated cells was set to 1.0. Statistically significant differences between untreated and treated cells are indicated (repeated measures ANOVA with Tukey's multiple comparison test; $*p < 0.05$; $**p < 0.01$). All other comparisons were not statistically significant. (C) MCF7 cells were treated with 20 μM **1** or DMSO for the indicated time points. Expression of total p70 S6K, phospho-p70 S6K, and β-actin was analyzed by immunoblotting.

comparing the effects of this compound on p70 S6K T^{389} phosphorylation in PTEN-deficient and control MEFs (Figure 3A). Similar to control MEFs, **1** also inhibited p70 S6K phosphorylation in PTEN-deficient MEFs, demonstrating that PTEN is not required for modulation of mTORC1 activity. Consistent with this, it was shown also that **1** (20 μM) did not decrease phosphorylation of AKT T^{308} in MCF7 cells (Figure 3B), indicating that **1**-mediated inhibition of mTORC1 activity is not associated with inhibition of PI3K→AKT signaling. In fact, there was a ∼3-fold increase in AKT T^{308} relative to total AKT in **1**-treated cells (Figure 3C), indicating activation of AKT signaling.

The effects of **1** were then characterized on other upstream regulators of mTORC1, ERK1/2, and AMPK. ERK1/2 can enhance mTORC1 activity via phosphorylation of TSC2, either directly or via $p90^{RSK}$s, which are activated by ERK1/2,[30,31] whereas AMPK, activated in response to ATP depletion, inhibits mTORC1 activity via phosphorylation of TSC2 on T^{1227} and S^{1345}.[32] Treatment of MCF7 cells with **1** resulted in an increase in ERK1/2 phosphorylation and did not affect AMPK phosphorylation (Figure 4A,B). Therefore, these

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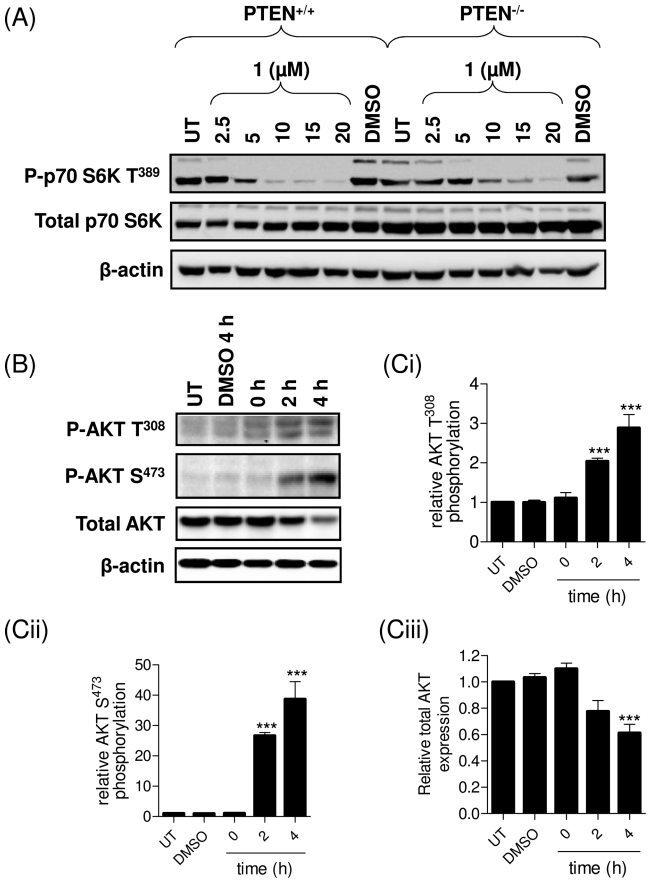

Figure 3. Effect of phenthyl isothiocyanate (**1**) in wild-type and PTEN-deficient MEFs and modulation of AKT phosphorylation in MC7 cells. (A) $PTEN^{+/+}$ and $PTEN^{-/-}$ MEFs were treated with indicated concentrations of **1** or DMSO, or left untreated as a control (UT), for 3 h. Expression of phospho-p70 S6K T^{389}, total p70 S6K, and β-actin was analyzed by immunoblotting. (B, C) MCF7 cells were treated with 20 μM **1** or DMSO for up to 4 h. Expression of phospho-AKT T^{308}, phospho-AKT S^{473}, total AKT, and β-actin (loading control) was analyzed by immunoblotting. (B) Representative immunoblots. (C) Quantitation; means ± SD derived from three independent experiments. For AKT phosphorylation phospho-specific signals were normalized using the intensity of total AKT expression. For total AKT the signal was normalized using the intensity of β-actin expression. Statistically significant differences between untreated and DMSO/**1**-treated cells are indicated (repeated measures ANOVA with Tukey's multiple comparison test; $***p < 0.001$). All other comparisons were not statistically significant.

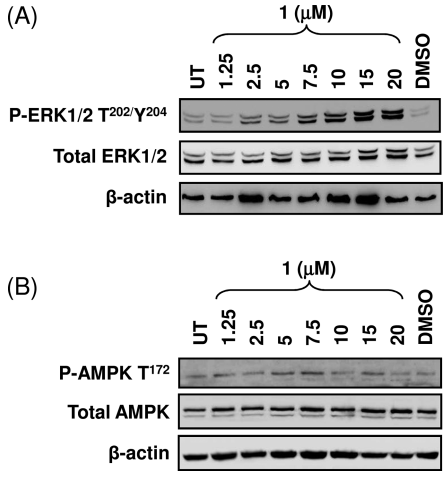

Figure 4. Effect of phenthyl isothiocyanate (**1**) on AKT, ERK1/2, and AMPK phosphorylation in MCF7 cells. MCF7 cells were treated with indicated concentrations of **1** or DMSO (equivalent to 20 μM **1**), or left untreated as a control (UT), for 3 h. Expression of phospho-ERK1/2 T^{202}/Y^{204}, total ERK1/2, phospho-AMPK T^{172}, total AMPK, and β-actin (loading control) was analyzed by immunoblotting.

pathways are unlikely to be involved in mediating the mTORC1 inhibition by **1**.

The TSC complex is the major negative regulator of mTORC1 since TSC2 stimulates the intrinsic GTPase activity of Rheb, a Ras homologue that activates mTORC1 when it is in GTP-bound state.[32,33] To determine whether **1**-mediated mTORC1 inhibition was dependent on the TSC complex, the effects of **1** were compared in wild-type and TSC2-deficient MEFS. (Note that $p53^{-/-}TSC2^{-/-}$ and control $p53^{-/-}TSC2^{+/+}$ MEFs were used since TSC2 deficiency results in premature senescence that can be rescued by deletion of p53.[34]) As expected, the basal phosphorylation of p70 S6K T^{389} was higher in the $TSC2^{-/-}$ cells and was constitutive (i.e., independent of serum) in TSC2-deficient cells. This demonstrates that mTORC1 is effectively uncoupled from upstream negative regulation in TSC2-deficient cells (Figure 5A). Similar to previous experiments in $PTEN^{+/+}$ control MEFs, **1** caused a significant reduction in p70 S6K T^{389} phosphorylation in control $p53^{-/-}TSC2^{+/+}$ MEFs. In contrast, the decrease in p70 S6K T^{389} phosphorylation was blunted significantly in TSC2-deficient cells (Figure 5B,C). Therefore, TSC2 is required for **1**-mediated inhibition of mTORC1 activity. Compound **1** is likely to effect either the function of the TSC1/2 complex directly or some upstream regulatory component. Phenethyl isothiocyanate is unlikely to have a major effect on the function of Rheb and/or mTORC1 components directly, since, in this case, it would have been expected to observe equivalent inhibitory effects of **1** in TSC2-deficient and wild-type MEFs.

The TSC2-deficient and control MEFs were used to investigate the effects of **1** on mTORC2 activity (Figure 6A). In control MEFs, there was readily detectable basal phosphorylation of both AKT S^{473} and NDRG T^{346}, and this was largely unaffected following addition of **1**. Basal levels of AKT S^{473} and NDRG1 phosphorylation were reduced greatly in TSC2-deficient cells, presumably as a consequence of mTORC1-mediated inhibition of mTORC2 activity,[35] and were increased modestly following treatment with **1**. These data suggest that **1** may increase mTORC2 activity, and this is independent of TSC2. Consistent with this, it was observed also that **1** increased AKT S^{473} phosphorylation in MCF7 cells (Figure 3B). This suggests that **1** increased mTORC2 activity in a TSC2-independent manner. The mechanisms controlling mTORC2 activity are not well understood; however, similar observations have been made in cells treated with the mTORC1 inhibitor rapamcyin, which can also induce increased phosphorylation of AKT S^{473} and NDRG1.[35] Compound **1**-mediated p70 S6K inhibition could interfere with the ability of p70 S6K to inhibit PI3K via effects on IRS1.[36,37] Alternatively, mTORC1 can also regulate negatively mTORC2 via phosphorylation of Rictor by p70 S6K.[35,38] Thus, increased phosphorylation of AKT S^{473} and NDRG1 in **1**-treated cells may be a downstream consequence of mTORC1 inhibition.

Finally, the role of TSC2 was investigated in **1**-induced growth inhibition using the CellTiter 96 assay. Whereas **1** inhibited growth of wild-type MEFs with an IC_{50} of 4.7 ± 1.3 μM, this compound inhibited growth of TSC2-deficient MEFs

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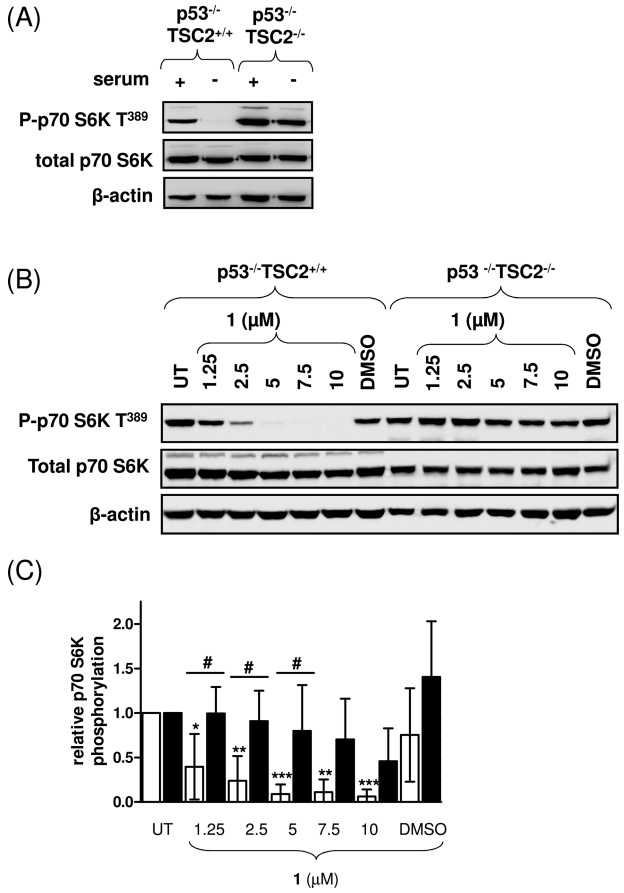

Figure 5. Effect of phenthyl isothiocyanate (**1**) on p70 S6K phosphorylation in TSC2-deficient MEFs. (A) $p53^{-/-}TSC2^{+/+}$ and $p53^{-/-}TSC2^{-/-}$ MEFs were serum starved for 24 h and then cultured in the presence or absence of serum for 2 h. Expression of phospho-p70 S6K T^{389}, total p70 S6K, and β-actin (loading control) was analyzed by immunoblotting. (B, C) $p53^{-/-}TSC2^{+/+}$ and $p53^{-/-}TSC2^{-/-}$ MEFs (grown in standard serum-containing medium) were treated with indicated concentrations of **1** or DMSO (equivalent to 20 μM **1**), or left untreated as a control (UT), for 3 h. Expression of phospho-p70 S6K T^{389}, total p70 S6K, and β-actin was analyzed by immunoblotting. (B) Representative immunoblots. (C) Quantitation; means ± SD derived from three independent experiments (□ $p53^{-/-}TSC2^{+/+}$; ■ $p53^{-/-}TSC2^{-/-}$). Statistically significant differences between untreated and treated cells for each cell line (repeated measures ANOVA with Tukey's multiple comparison test; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$) and between cell lines for the same treatment (*t* test; $^{\#}p < 0.05$) are indicated. All other comparisons were not statistically significant.

with an IC_{50} of 8.0 ± 0.8 μM (mean ± SD derived from three independent experiments; $p = 0.013$, Student's *t* test for difference in **1** IC_{50}'s). Taken together, the present data demonstrate that **1** inhibits mTORC1 activity. TSC2 is required for mTORC1 inhibition by this compound, and TSC2-deficient cells are partially resistant to its growth inhibitory effects.

The current study appears to eliminate several candidates that might have mediated the inhibitory effects of **1** on signaling to mTORC1. Compound **1**-mediated mTORC1 inhibition was not influenced by PTEN status, and this substance actually increased AKT T^{308} phosphorylation at 2–4 h. Previous studies have shown that **1** can either inhibit or increase AKT phosphorylation.[39−42] One variable may be the time of

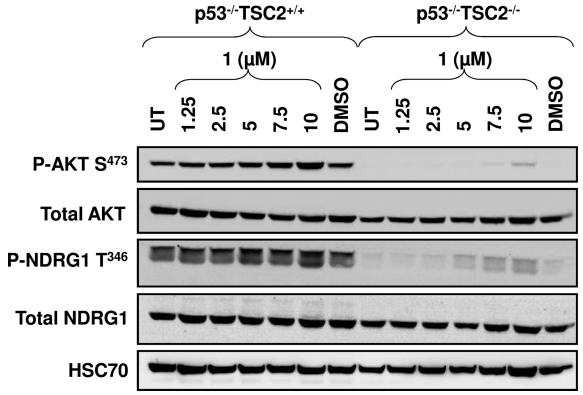

Figure 6. Effect of phenthyl isothiocyanate (**1**) on mTORC2 activity in TSC2-deficient MEFs. $p53^{-/-}TSC2^{+/+}$ and $p53^{-/-}TSC2^{-/-}$ MEFs (grown in standard serum-containing medium) were treated with indicated concentrations of **1** or DMSO (equivalent to 20 μM **1**), or left untreated as a control (UT), for 3 h. Expression of phospho-AKT S^{473}, total AKT, phospho-NDRG1 T^{346}, total NDRG1, and HSC70 (loading control) was analyzed by immunoblotting.

exposure since, in MM.1S multiple myeloma cells, short-term (2 h) exposure to **1** increased AKT phosphorylation, whereas long-term (12 h) exposure decreased AKT phosphorylation.[41] Since decreased p70 S6K phosphorylation was detected within minutes following treatment with **1** and was maintained for up to 8 h, these data suggest that inhibition of AKT signaling was not responsible for **1**-mediated inhibition of mTORC1 activity.

Similarly, **1** increased ERK1/2 T^{202}/Y^{204} phosphorylation (i.e., activation), which is also associated with TSC2 inactivation.[19,20] Compound **1** also does not seem to act via ATP depletion since phosphorylation of AMPK was not altered in **1**-treated cells. Another potential regulator of mTORC1 activity is REDD1, which is transcriptionally induced in response to oxidative and endoplasmic reticulum stress and inhibits mTORC1 activity via TSC2.[43−46] However, since **1**-mediated mTORC1 inhibition is very rapid, it is very unlikely to involve a transcriptionally induced intermediate. Recent studies have shown that decreased pH can lead to TSC2-dependent inhibition of mTORC1 and that this appears to be independent of any previously characterized pathways of TSC2 regulation.[47] Since **1** has been shown recently to inhibit oxidative phosphorylation and to increase extracellular acidification, such a pathway may also act to couple this compound to mTORC1 inhibition.[9]

mTORC1 is a key regulator of RNA translation, especially of RNAs containing complex 5′-UTRs. Many of the targets for mTORC1 are critical for cancer cell survival (MCL1), proliferation (e.g., MYC, CCND2), and angiogenesis (HIF1α), suggesting that mTORC1 inhibition is likely to be important for **1**-mediated antitumor effects. Indeed, the studies of our group have shown that **1** does effectively suppress translation of *HIF1α* RNA, which is known to be highly dependent on mTORC1. Consistent with the idea that inhibition of mTORC1 activity may play an important role in the antitumor effects of **1**, overexpression of eIF4E, the downstream target for unphosphorylated 4E-BP1, protected cells from **1**-mediated growth inhibition.[26] Moreover, our own data demonstrate that TSC2-deficient MEFs are also protected from **1**-mediated growth inhibition. However, effects in these experiments were partial, demonstrating that **1** also affects other pathways required for cancer cell survival and

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proliferation. However, inhibition of mTORC1 activity does appear to significantly contribute to phenethyl isothiocyanate-mediated cancer cell growth inhibition.

EXPERIMENTAL SECTION

Cell Lines, Chemicals, and Reagents. MCF7 human breast cancer cells were obtained from American Type Culture Collection (Manassas, VA, USA). PTEN-deficient ($PTEN^{-/-}$) and matched control MEFs ($PTEN^{+/+}$) were a kind gift of Dr. V. Stambolic (University of Toronto, Canada).[48] TSC2-deficient ($p53^{-/-}TSC2^{-/-}$) and matched control MEFs ($p53^{-/-}TSC2^{+/+}$) were kindly provided by Drs. A Tee (Cardiff University, UK) and D. Kwiatkowski (Brigham and Women's Hospital, Boston, MA, USA).[34] Cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM; Lonza Group Ltd., Basel, Switzerland) supplemented with 10% (v/v) fetal calf serum (FCS; PAA Laboratories, Yeovil, UK), 1 mM L-glutamine, and penicillin/streptomycin (Lonza Group Ltd.). Phenethyl isothiocyanate (**1**; 99% purity by HPLC), cobalt chloride ($CoCl_2$), cycloheximide (CHX), and staurosporine (STS) were from Sigma Chemicals (Poole, UK). Dimethylsulfoxide (DMSO) was used as a solvent control and was added at a dilution equivalent to the highest concentration of **1** tested in each assay.

Growth Assays. Cells were plated at a density of 1000 cells per well of a 96-well plate in 50 μL of complete growth medium. The following day, cells were treated with **1** or DMSO as a solvent control or were left untreated. After 6 days, relative cell number was determined using the CellTiter 96 AQ_{ueous} One Solution Reagent (Promega, Southampton, UK), according to the manufacturer's instructions. Relative cell number was calculated as a percentage of untreated cells, and IC_{50} values were determined using GraphPad Prism (GraphPad Software Inc., La Jolla, CA, USA; version 4.03).

Metabolic Labeling. MCF7 cells were seeded at a density of 1×10^6 cells per well of a six-well dish. The following day cells were washed three times in DMEM lacking L-glutamine, L-cysteine, and L-methionine (MP Biomedicals, Illkirch, France) before being incubated in DMEM without L-glutamine, L-cysteine, and L-methionine supplemented with 10% (v/v) dialyzed FCS and 2 mM glutamine for 1 h. Cells were then pretreated with **1**, CHX (10 μg/mL), or DMSO for 1 h before addition of $TRAN^{35}S$−LABEL No−Thaw Metabolic Labeling Reagent (0.75 MBq per mL; >37.0 TBq/mmol; MP Biomedicals). Labeling was performed in the presence of $CoCl_2$ (100 μM) to block HIF1α degradation. Cells were lysed in 1× RIPA buffer containing protease and phosphatase inhibitor cocktails (Sigma Chemicals). Lysates were incubated overnight with anti-HIF1α monoclonal antibody (1 μg; BD Biosciences, Oxford, UK) or isotype control antibody at 4 °C, and immune complexes were collected using protein G-coupled Sepharose beads (GE Healthcare UK Ltd., Amersham, UK) before analysis by SDS-PAGE and phosphor imaging.

Immunoblotting. Immunoblots were performed using rabbit polyclonal antibodies specific for 4E-BP1, total AKT, total AMPK, total NDRG1, total p70 S6K, phospho-S^{308} AKT, phospho-T^{473} AKT, phospho-T^{172} AMPK, phospho-T^{202}/Y^{204} ERK1/2, phospho-T^{346} NDRG1, phospho-T^{389} p70 S6K (all Cell Signaling Technology, Beverley, MA, USA) and β-actin (Sigma Chemicals), and mouse monoclonal antibodies specific for total ERK1/2 (Cell Signaling Technologies), HIF1α (BD Biosciences), and HSC70 (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Horseradish peroxidase conjugated secondary antibodies were from GE Healthcare, and bound immunocomplexes were detected using SuperSignal West Pico chemiluminescent reagents (Perbio Science UK Ltd., Cramlington, UK). Immunoblot signals were quantified using Quantity One image analysis software (BioRad). For p70 S6K and AKT phosphorylation the phospho-specific signal was normalized using the intensity of total p70 S6K/AKT expression. For 4E-BP1 phosphorylation, the proportion of slower migrating bands was quantified as a proportion of total 4E-BP1 expression. The levels of phosphorylation in untreated cells was set to 1.0.

Statistics. Statistical analysis was performed using Student's paired *t* test or repeated measures ANOVA with Tukey's multiple comparison test, as indicated in the figure legends. Statistical comparisons were performed using GraphPad Prism.

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Notes

The authors declare no competing financial interest.

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REFERENCES

(1) Cavell, B. E.; Syed Alwi, S. S.; Donlevy, A.; Packham, G. *Biochem. Pharmacol.* **2011**, *81*, 327−336.
(2) Cheung, K. L.; Kong, A. N. *AAPS J.* **2010**, *12*, 87−97.
(3) Hayes, J. D.; Kelleher, M. O.; Eggleston, I. M. *Eur. J. Nutr.* **2008**, *47*, 73−88.
(4) Kim, M. K.; Park, J. H. *Proc. Nutr. Soc.* **2009**, *68*, 103−110.
(5) Higdon, J. V.; Delage, B.; Williams, D. E.; Dashwood, R. H. *Pharmacol. Res.* **2007**, *55*, 224−236.
(6) Zhang, Y. *Carcinogenesis* **2000**, *21*, 1175−1182.
(7) Zhang, Y. *Carcinogenesis* **2001**, *22*, 425−431.
(8) Trachootham, D.; Zhou, Y.; Zhang, H.; Demizu, Y.; Chen, Z.; Pelicano, H.; Chiao, P. J.; Achanta, G.; Arlinghaus, R. B.; Liu, J.; Huang, P. *Cancer Cell* **2006**, *10*, 241−252.
(9) Xiao, D.; Powolny, A. A.; Moura, M. B.; Kelley, E. E.; Bommareddy, A.; Kim, S. H.; Hahm, E. R.; Normolle, D.; Van Houten, B.; Singh, S. V. *J. Biol. Chem.* **2010**, *285*, 26558−26569.
(10) Mi, L.; Wang, X.; Govind, S.; Hood, B. L.; Veenstra, T. D.; Conrads, T. P.; Saha, D. T.; Goldman, R.; Chung, F. L. *Cancer Res.* **2007**, *67*, 6409−6416.
(11) Xu, K.; Thornalley, P. J. *Biochem. Pharmacol.* **2001**, *61*, 165−177.
(12) Mi, L.; Xiao, Z.; Hood, B. L.; Dakshanamurthy, S.; Wang, X.; Govind, S.; Conrads, T. P.; Veenstra, T. D.; Chung, F. L. *J. Biol. Chem.* **2008**, *283*, 22136−22146.
(13) Li, W.; Kong, A. N. *Mol. Carcinog.* **2009**, *48*, 91−104.
(14) Khor, T. O.; Yu, S.; Kong, A. N. *Planta Med.* **2008**, *74*, 1540−1547.
(15) Hanahan, D.; Weinberg, R. A. *Cell* **2011**, *144*, 646−674.
(16) Rankin, E. B.; Giaccia, A. J. *Cell Death Differ.* **2008**, *15*, 678−685.
(17) Weidemann, A.; Johnson, R. S. *Cell Death Differ.* **2008**, *15*, 621−627.
(18) Wang, X. H.; Cavell, B. E.; Syed Alwi, S. S.; Packham, G. *Biochem. Pharmacol.* **2009**, *78*, 261−272.
(19) Zoncu, R.; Efeyan, A.; Sabatini, D. M. *Nat. Rev. Mol. Cell. Biol.* **2011**, *12*, 21−35.
(20) Dancey, J. *Nat. Rev. Clin. Oncol.* **2010**, *7*, 209−219.
(21) Treins, C.; Giorgetti-Peraldi, S.; Murdaca, J.; Semenza, G. L.; Van Obberghen, E. *J. Biol. Chem.* **2002**, *277*, 27975−27981.
(22) Opdenaker, L. M.; Farach-Carson, M. C. *J. Cell. Biochem.* **2009**, *107*, 473−481.
(23) Koromilas, A. E.; Lazaris-Karatzas, A.; Sonenberg, N. *EMBO J.* **1992**, *11*, 4153−4158.
(24) Breuleux, M.; Klopfenstein, M.; Stephan, C.; Doughty, C. A.; Barys, L.; Maira, S. M.; Kwiatkowski, D.; Lane, H. A. *Mol. Cancer Ther.* **2009**, *8*, 742−753.

(25) Garcia-Martinez, J. M.; Alessi, D. R. *Biochem. J.* **2008**, *416*, 375−385.
(26) Hu, J.; Straub, J.; Xiao, D.; Singh, S. V.; Yang, H. S.; Sonenberg, N.; Vatsyayan, J. *Cancer Res.* **2007**, *67*, 3569−3573.
(27) Manning, B. D.; Tee, A. R.; Logsdon, M. N.; Blenis, J.; Cantley, L. C. *Mol. Cell* **2002**, *10*, 151−162.
(28) Tee, A. R.; Fingar, D. C.; Manning, B. D.; Kwiatkowski, D. J.; Cantley, L. C.; Blenis, J. *Proc. Natl. Acad. Sci. U. S. A.* **2002**, *99*, 13571−13576.
(29) Hollander, M. C.; Blumenthal, G. M.; Dennis, P. A. *Nat. Rev. Cancer* **2011**, *11*, 289−301.
(30) Ma, L.; Chen, Z.; Erdjument-Bromage, H.; Tempst, P.; Pandolfi, P. P. *Cell* **2005**, *121*, 179−193.
(31) Roux, P. P.; Ballif, B. A.; Anjum, R.; Gygi, S. P.; Blenis, J. *Proc. Natl. Acad. Sci. U. S. A.* **2004**, *101*, 13489−13494.
(32) Inoki, K.; Zhu, T.; Guan, K. L. *Cell* **2003**, *115*, 577−590.
(33) Tee, A. R.; Manning, B. D.; Roux, P. P.; Cantley, L. C.; Blenis, J. *Curr. Biol.* **2003**, *13*, 1259−1268.
(34) Zhang, H.; Cicchetti, G.; Onda, H.; Koon, H. B.; Asrican, K.; Bajraszewski, N.; Vazquez, F.; Carpenter, C. L.; Kwiatkowski, D. J. *J. Clin. Invest.* **2003**, *112*, 1223−1233.
(35) Julien, L. A.; Carriere, A.; Moreau, J.; Roux, P. P. *Mol. Cell. Biol.* **2010**, *30*, 908−921.
(36) Tremblay, F.; Jacques, H.; Marette, A. *Curr. Opin. Clin. Nutr. Metab. Care* **2005**, *8*, 457−462.
(37) Um, S. H.; D'Alessio, D.; Thomas, G. *Cell Metab.* **2006**, *3*, 393−402.
(38) Treins, C.; Warne, P. H.; Magnuson, M. A.; Pende, M.; Downward, J. *Oncogene* **2010**, *29*, 1003−1016.
(39) Gao, N.; Budhraja, A.; Cheng, S.; Liu, E. H.; Chen, J.; Yang, Z.; Chen, D.; Zhang, Z.; Shi, X. *Cell Death Dis.* **2011**, *2*, e140.
(40) Satyan, K. S.; Swamy, N.; Dizon, D. S.; Singh, R.; Granai, C. O.; Brard, L. *Gynecol. Oncol.* **2006**, *103*, 261−270.
(41) Jakubikova, J.; Cervi, D.; Ooi, M.; Kim, K.; Nahar, S.; Klippel, S.; Cholujova, D.; Leiba, M.; Daley, J. F.; Delmore, J.; Negri, J.; Blotta, S.; McMillin, D. W.; Hideshima, T.; Richardson, P. G.; Sedlak, J.; Anderson, K. C.; Mitsiades, C. S. *Haematologica* **2011**, *96*, 1170−1179.
(42) Xiao, D.; Singh, S. V. *Cancer Res.* **2007**, *67*, 2239−2246.
(43) Shoshani, T.; Faerman, A.; Mett, I.; Zelin, E.; Tenne, T.; Gorodin, S.; Moshel, Y.; Elbaz, S.; Budanov, A.; Chajut, A.; Kalinski, H.; Kamer, I.; Rozen, A.; Mor, O.; Keshet, E.; Leshkowitz, D.; Einat, P.; Skaliter, R.; Feinstein, E. *Mol. Cell. Biol.* **2002**, *22*, 2283−2293.
(44) Whitney, M. L.; Jefferson, L. S.; Kimball, S. R. *Biochem. Biophys. Res. Commun.* **2009**, *379*, 451−455.
(45) Jin, H. O.; Seo, S. K.; Woo, S. H.; Kim, E. S.; Lee, H. C.; Yoo, D. H.; An, S.; Choe, T. B.; Lee, S. J.; Hong, S. I.; Rhee, C. H.; Kim, J. I.; Park, I. C. *Free Radical Biol. Med.* **2009**, *46*, 1158−1167.
(46) Brugarolas, J.; Lei, K.; Hurley, R. L.; Manning, B. D.; Reiling, J. H.; Hafen, E.; Witters, L. A.; Ellisen, L. W.; Kaelin, W. G. *Genes Dev.* **2004**, *18*, 2893−2904.
(47) Balgi, A. D.; Diering, G. H.; Donohue, E.; Lam, K. K.; Fonseca, B. D.; Zimmerman, C.; Numata, M.; Roberge, M. *PLoS One* **2011**, *6*, e21549.
(48) Stambolic, V.; Suzuki, A.; de la Pompa, J. L.; Brothers, G. M.; Mirtsos, C.; Sasaki, T.; Ruland, J.; Penninger, J. M.; Siderovski, D. P.; Mak, T. W. *Cell* **1998**, *95*, 29−39.