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Novel mTOR inhibitory activity of ciclopirox enhances parthenolide antileukemia activity

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Abstract

Ciclopirox, an antifungal agent commonly used for the dermatologic treatment of mycoses, has been shown recently to have antitumor properties. Although the exact mechanism of ciclopirox is unclear, its antitumor activity has been attributed to iron chelation and inhibition of the translation initiation factor eIF5A. In this study, we identify a novel function of ciclopirox in the inhibition of mTOR. As with other mTOR inhibitors, we show that ciclopirox significantly enhances the ability of the established preclinical antileukemia compound, parthenolide, to target acute myeloid leukemia. The combination of parthenolide and ciclopirox demonstrates greater toxicity against acute myeloid leukemia than treatment with either compound alone. We also demonstrate that the ability of ciclopirox to inhibit mTOR is specific to ciclopirox because neither iron chelators nor other eIF5A inhibitors affect mTOR activity, even at high doses. We have thus identified a novel function of ciclopirox that might be important for its antileukemic activity.

Despite several recent advances, acute myelogenous leukemia (AML) remains a fatal disease and most patients die despite achieving initial complete remission. Unfortunately, standard therapy has changed little over the past several decades, and new approaches are needed to improve these dismal outcomes [1–3]. AML is thought to be initiated and maintained by a relatively rare, chemotherapy-resistant subpopulation of cells known as *leukemia stem cells* (LSCs) [4,5]. These cells have properties similar to normal hematopoietic stem cells (HSCs), including the capacity for self-renewal, proliferation, and differentiation into leukemic blasts. Phenotypically delineated compartments enriched in LSCs have been described in patient samples that are distinct from normal HSC compartments given the presence or absence of cell surface markers [6–10]. The observation has been made that patients with a higher proportion of LSCs (defined as

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CD34⁺CD38⁻) demonstrate significantly poorer relapse-free survival than do patients with low proportions of LSCs. In addition, LSCs can also contribute to multidrug resistance, further complicating the treatment [11,12].

In our efforts to identify agents that target LSCs, we previously demonstrated that the naturally occurring sesquiterpene lactone parthenolide (PTL) can ablate LSCs by inhibiting NF- κ B and induction of reactive oxygen species (ROS) [13]. PTL has relatively poor pharmacologic properties that can limit its use as a therapeutic agent. Thus, a chemical analog with equal anti-LSC properties, improved bioavailability, and solubility was generated (DMAPT/LC-1) [14–16]. However, treatment of AML cells with PTL or DMAPT/LC-1 has been shown to induce cytoprotective responses that can reduce the potency of PTL [17]. Increasing efforts have been made in different tumor systems to identify agents that can synergize with PTL or DMAPT/LC-1 by different mechanisms, including abrogation of ROS-induced cytoprotective responses [17–23]. In this study, we describe a new agent that enhances the antileukemic potential of PTL, the antifungal drug ciclopirox. In a previous study, ciclopirox was shown to reduce the viability of several AML cell lines and reduce tumor burden in a mouse model of leukemia [24]. In addition, ciclopirox also has been shown to synergize with imatinib [25]. In the current study, we show that ciclopirox acts as an inhibitor of mTOR and enhances the antileukemic effect of PTL by inhibiting the PTL-induced activation of mTOR.

Methods

Cell lines, primary AML samples, and compounds

Kasumi-1 cell line was purchased from the American Type Culture Collection (Manassas, VA, USA) and grown in RPMI 1640 (Gibco-Invitrogen, Carlsbad, CA, USA), supplemented with 20% fetal bovine serum (Gibco-Invitrogen, Carlsbad, CA, USA). Cryopreserved primary AML samples were obtained with informed consent and institutional review board approval. Samples were thawed and cultured as described previously [26,27]. Cells were cultured for 1 hour before treatment with PTL (Enzo Life Sciences, Farmingdale, NY, USA), ciclopirox, GC-7, deferoxamine, ferric ammonium citrate (Sigma-Aldrich, St. Louis, MO, USA), ortemsirolimus (LC Labs, Woburn, MA, USA).

Antibodies and immunoblots

Primary AML cells or Kasumi-1 cells were treated with parthenolide, ciclopirox, temsirolimus, GC-7, and deferoxamine at the indicated doses. Six hours after treatment, cells were collected and whole cell lysates were subjected to immunoblotting with antibodies to phospho-p65 (S536), phospho-p70S6K (T421/S424), phospho-p70S6K (T389), phospho-Akt (S473), phospho-4E-BP1 (T37/46), total Akt, total 4E-BP1, total p70S6K (Cell Signaling Technology, Danvers, MA, USA), and α -actin (Sigma-Aldrich).

Short interfering RNA transfection

Kasumi-1 cells were transfected with 1 μ mol/L of either scrambled, Raptor, or Rictor short interfering RNA (siRNA; Thermo Scientific, Waltham, MA, USA), by electroporation using the Neon Transfection System (Life Technologies, Grand Island, NY, USA), according to the manufacturer's protocol. At 48 hours after transfection, cells were treated with 5 μ mol/L PTL, and the 24-hour viability was evaluated with flow cytometry using annexin V and 7-aminoactinomycin. To determine the changes in phospho-S6 ribosomal protein, cells were fixed and permeabilized using BD Cytofix/Cytoperm (BD Biosciences, San Jose, CA, USA) buffer according to the manufacturer's protocol; afterward, they were stained with antibody against phospho-S6 ribosomal protein (Cell Signaling Technology). Knockdown of Raptor

and Rictor was confirmed by immunoblotting with antibodies to Raptor (Cell Signaling Technology), and Rictor (Novus Biologicals, Littleton, CO, USA), respectively.

In vitro kinase assay

The enzyme-linked immunosorbent assay–based K-LISA mTOR activity kit (EMD Millipore, Billerica, MA, USA) was used for the kinase assay, and the protocol followed was according to the manufacturer's instructions. Recombinant mTOR (treated with the drugs or solvent control) was incubated in the presence of assay buffer in the wells of a glutathione-coated plate containing p70S6 kinase-GST fusion protein as a specific mTOR substrate. The phosphorylated p70S6 kinase was then incubated with anti-p70S6 kinase-T389 primary, followed by detection with horseradish peroxidase–antibody conjugate and TMB Substrate. The relative activity was determined by reading the absorbance at dual wavelengths of 450 and 595 nm.

Flow cytometry

Apoptosis assays were performed as described [13]. After 18–24 hours of treatment, primary AML cells were stained for the surface antibodies CD38-allophycocyanin, CD34-PECy7, CD123-phycoerythrin (PE; BD Biosciences) for 15 minutes. Cells were washed in cold phosphate-buffered saline and resuspended in 200 μ L of annexin-V buffer (0.01 mol/L HEPES/NaOH, 0.14 mol/L NaCl, 2.5 mmol/L CaCl_2) annexin-V–fluorescein isothiocyanate or annexin V-PE (BD Biosciences) and 7-aminoactinomycin D (Molecular Probes, Eugene, OR, USA) were added, and the tubes were incubated at room temperature for 15 minutes and then analyzed with a BD LSRII flow cytometer. To analyze human cell engraftment in the NOD/SCID xenotransplant model, BM cells were blocked with the anti-Fc receptor antibody 2.4G2 and 25% human serum and later labeled with anti-human CD45-PE antibody (BD Biosciences). To determine the changes in phospho-S6 ribosomal protein, Kasumi-1 cells were fixed and permeabilized using BD Cytofix/Cytoperm (BD Biosciences) buffer according to the manufacturer's protocol, after which they were stained with antibody against phospho-S6 ribosomal protein (Cell Signaling Technology).

Methylcellulose colony-forming assay

AML, BM, or CB cells were cultured for 18 hours in the presence or absence of compounds. Cells were plated at 50,000 cells/mL in Methocult GFH4534 (Stem Cell Technologies, Vancouver, Canada) supplemented with 3 U/mL erythropoietin and 50 ng/mL G-CSF. Colonies were scored after 10–14 days of culture.

Nonobese diabetic–severe combined immunodeficient mouse assays

Nonobese diabetic–severe combined immunodeficient (NOD/SCID) mice were sublethally irradiated with 270 rad using a Rad-Source-2000 x-ray irradiator before transplantation. Cells to be assayed were injected via the tail vein (5–10 million cells) in a final volume of 0.2 mL of phosphate-buffered saline with 0.5% fetal bovine serum. After 6–8 weeks, animals were sacrificed, and BM was analyzed for the presence of human cells by flow cytometry.

Statistical analysis

Statistical analyses and graphs were performed using GraphPad Prism software (GraphPad Software, San Diego, CA, USA). For statistical analysis, the data were analyzed using one-way analysis of variance followed by a Tukey post hoc test. For two group comparisons, significance was determined with paired *t* tests.

Results

Ciclopirox augments the activity of parthenolide

We sought to determine whether the combination of PTL and ciclopirox was more effective compared with each individual compound alone. Primary AML samples or healthy cord blood samples were treated for 24 hours with PTL and ciclopirox, alone or in combination (Fig. 1). Treatment with PTL or ciclopirox alone was moderately toxic to the AML cells; however, the combination of the two compounds demonstrated a much greater toxicity with more than a sevenfold decrease compared to treatment with each individual compound alone (Fig. 1A and B; $p < 0.05$). Notably, these effects extended to phenotypically defined CD34⁺CD38⁻ LSCs (Fig. 1C). Next, we determined the effects of the combination using functional assays. First, we tested the ability of PTL and ciclopirox to inhibit AML colony formation in methylcellulose, alone or in combination. When used as single agents, PTL and ciclopirox each had only a modest effect in impairing AML colony formation. However, the combination of PTL and ciclopirox resulted in a 10-fold decrease in the number of AML colonies relative to the untreated controls, demonstrating a significant ability of the PTL-ciclopirox combination to impair colonyforming capacity of AML stem and progenitor cells. Importantly, the PTL-ciclopirox combination did not significantly impair the formation of normal erythroid and myeloid colonies (Fig. 1D). To determine the ability of the combination of PTL and ciclopirox to impair the LSCs, three different primary AML samples were treated with the drug combination for 24 hours, and then transplanted into sublethally irradiated NOD/SCID mice. We found that the PTL-ciclopirox combination significantly decreased levels of AML engraftment compared with the untreated controls (Fig. 1E), indicating that the combination of PTL and ciclopirox was effectively impairing LSC function.

To determine whether the combination of drugs was additive or synergistic, we tested different concentrations of both agents to determine combination indices (CIs). We found the combination of PTL and ciclopirox was synergistic with CI <1 (Supplementary Figure E1, online only, available at www.exphem.org).

Ciclopirox inhibits the PTL-induced activation of mTOR signaling pathway

To elucidate the mechanism by which ciclopirox augments the activity of PTL, we determined the effect of the PTL-ciclopirox combination on molecular pathways modulated by PTL [13,17]. PTL is known to inhibit NF- κ B and also has been shown to activate the mTOR pathway [17]. As described previously, we found that treatment with PTL resulted in the inhibition of NF- κ B, as shown by the loss of phosphorylation on the NF- κ B p65 subunit. As reported, PTL exposure resulted in the activation of the mTOR pathway, indicated by an increase in the phosphorylation of p70 S6 kinase (p70S6K) (Fig. 2A and Supplementary Figure E2, online only, available at www.exphem.org). Importantly, we noted that treatment with ciclopirox could abrogate the PTL-induced phosphorylation of p70S6K. The levels of total p70S6K were unaltered. These results were further validated by flow cytometric analyses in AML cell lines with detectable CD34⁺CD38⁻ population [28]. As shown in Fig. 2B, treatment with PTL resulted in almost a threefold increase of phospho-S6 ribosomal protein, a direct substrate of p70S6K. Indeed, treatment with ciclopirox prevented the PTL-induced elevation of phospho-S6 ribosomal protein. Abrogation of phospho-S6 ribosomal protein phosphorylation was also evident in CD34⁺ and the CD34⁺CD38⁻ subpopulations. In addition to the PTL-induced activation of the PI3K/mTOR pathway, PTL also resulted in the activation of a cytoprotective response characterized by the activation of HMOX1 and NQO1. Inhibition of either PI3K or mTOR abrogated the ability of PTL to increase expression of Nrf2-regulated genes [17]. As observed with PI3K and mTOR inhibition, ciclopirox also decreased the ability of PTL to induce HMOX1 and NQO1 (Supplementary

Figure E3, online only, available at www.exphem.org). These findings indicate that ciclopirox impairs PTL-induced activation of cytoprotective responses.

Ciclopirox inhibits mTORC1 signaling

The aforementioned data suggest that ciclopirox can inhibit mTOR signaling. mTOR functions in mammalian cells through two major complexes: mTOR complex 1 (mTORC1) and complex 2 (mTORC2) [29]. mTORC1 is rapamycin sensitive and regulates phosphorylation of P70 S6 kinase and eukaryotic initiation factor 4E (eIF4E) binding protein 1 (4E-BP1). The function of mTORC1 is primarily in the control of translation initiation and nutrient sensing [30]. In contrast, studies indicate that mTORC2 is rapamycin insensitive and acts via phosphorylation of Akt at Ser473, which mediates its effects in the regulation of actin cytoskeleton [31,32]. To further dissect the effect of ciclopirox on mTOR signaling, primary AML cells were treated with increasing concentrations of ciclopirox for 6 hours, followed by immunoblotting. Treatment with PTL and temsirolimus were used as control groups for the activation and inhibition of mTOR signaling, respectively [17]. We examined the status of phospho-p70S6K at Thr-421/Ser424 [33] or T389 [34,35], or both as an indicator of mTORC1 activity, because these sites represent its immediate downstream targets of mTORC1. Figure 3 shows that treatment with increasing concentrations of ciclopirox resulted in a dose-dependent decrease in the phosphorylation of P70 S6 kinase. Another important response controlled by mTORC1 is the initiation of cap-dependent translation, which occurs mainly through 4EBP1 phosphorylation [36]. Phosphorylation of 4EBP-1 is essential for its deactivation and dissociation from the initiation factor eIF4E, so cap-dependent translation can proceed. We found that treatment with ciclopirox also resulted in a decreased phosphorylation of 4EBP1. The decrease in the phosphorylation of p70S6K coincided with an increase in phosphorylation of Akt at serine 473, a marker of mTORC2 activity [31]. The levels of total p70S6K and total Akt were unaffected. Furthermore, we observed no change in Akt (Thr308; Supplementary Figure E4, online only, available at www.exphem.org). These results suggest that ciclopirox acts as an inhibitor of mTORC1, whereby an increase in Akt serine 473 phosphorylation arises as a consequence of a negative-feedback loop activation of mTORC2 [37,38].

Ciclopirox is known to inhibit the enzyme deoxyhypusine hydroxylase, which is responsible for the hypusination of the translation initiation factor eIF5A [39]. Hypusination is a unique posttranslational modification that has been implicated in the activation of eIF5A [40,41]. To determine whether the inhibition of mTORC1 signaling was unique to ciclopirox or can also occur with other drugs that inhibit eIF5, we treated primary AML cells with increasing doses of N1-guanyl-1,7-diaminoheptane (GC7), a selective inhibitor of deoxyhypusine synthase [42]. We found that GC7 treatment did not significantly alter phosphorylation of Akt at serine 473, even at doses as high as 50–100 $\mu\text{mol/L}$ (Fig. 4; lanes 10–12), unlike that observed after treatment with ciclopirox (Fig. 4, lane 6). Moreover, GC7 treatment did not enhance the ability of PTL to induce cell death (Supplementary Figure E5A, online only, available at www.exphem.org) and did not decrease the PTL-induced phosphorylation of p70S6K (Supplementary Figure E5C, online only, available at www.exphem.org).

We also investigated whether other reported functions for ciclopirox can affect the mTOR pathway, such as iron chelation. To determine this, primary AML cells were treated with deferoxamine. Unlike ciclopirox, exposure of AML cells to deferoxamine did not show a decrease in the phosphorylation of p70S6K (induced by PTL; Supplementary Figure E5C, online only, available at www.exphem.org), and deferoxamine had no significant effect on Akt serine 473 phosphorylation (Fig. 4, lanes 7–9). Furthermore, the combination of PTL with deferoxamine did not result in a significant increase in cell death (Supplementary Figure E5B, online only, available at www.exphem.org). In addition, cells were also pretreated with ferric ammonium citrate to determine whether iron supplementation could

prevent the effects of ciclopirox. The addition of iron had no appreciable effect on the PTL or ciclopirox-mediated cytotoxicity, and it was unable to inhibit the PTL-induced p70S6K phosphorylation (Supplementary Figures E5D and 5E, online only, available at www.exphem.org). Taken together, these results confirm that ciclopirox inhibits signaling via the mTOR pathway, and mTORC1 inhibition is likely to be independent of the iron chelator or eIF5 inhibitor activities of ciclopirox.

Ciclopirox inhibits mTORC1 kinase activity

To further understand the effect of mTOR inhibition on the enhancement of PTL-induced cell death, we investigated how mTORC1 and mTORC2 knockdown affect the sensitivity of leukemic cells to PTL. We used an siRNA-based approach to selectively knockdown Raptor and Rictor to disrupt mTORC1 and mTORC2, respectively (Fig. 5A). As shown in Fig. 5B, we found that knockdown of Raptor resulted in a significant increase in sensitivity of AML cells to PTL treatment compared with Rictor knockdown; however, Rictor knockdown also had an effect in PTL-induced cell death. Importantly, knockdown of Raptor (but not Rictor) was able to abrogate the PTL-induced increase of phospho-S6 ribosomal protein (Fig. 5C), similar to the effect seen with ciclopirox. In addition, treatment with PTL also resulted in an increased phosphorylation of Akt serine 473 in the Raptor knockdown cells (Supplementary Figure E6, online only, available at www.exphem.org), likely because of feedback mediated by Rictor [38,43]. Indeed, we observed that PTL treatment did not result in increased phosphorylation of Akt-ser473 in the Rictor siRNA-transfected cells (Supplemental Figure E6, online only, available at www.exphem.org). Together, these results further indicate that downregulation of mTORC1 is important for enhancing the activity of PTL.

To determine whether ciclopirox inhibited the kinase activity of mTOR, we performed an in vitro enzymelinked immunosorbent assay–based activity assay using re-combinant mTOR, FKBP12, and p70S6K-GST fusion protein as a specific substrate for mTOR. The mechanism of action of rapamycin is such that it first binds FKBP12, and the rapamycin-FKBP12 complex then binds and inhibits mTOR [44]. We observed that, similar to rapamycin-FKBP12, the combination of FKBP12 and ciclopirox showed a dose-dependent decrease in the activity of mTOR (Fig. 6). On the other hand, in the absence of FKBP12, ciclopirox was unable to inhibit mTOR kinase activity in vitro. This finding reveals that ciclopirox can directly inhibit the kinase activity of mTOR and that it functions specifically as an mTOR inhibitor and not as a PI3K inhibitor such as wortmannin.

Discussion

Ciclopirox is an antifungal agent that is commonly used in the treatment of mycoses of skin and nails [45,46]. It is an off-patent compound and has been shown recently to have antitumor properties. It was identified in a screen for agents potentially cytotoxic to leukemia cells by searching for compounds inhibiting the growth of TEX and M9-ENL1 cell lines [24]. It is a known chelator of iron [47] and an inhibitor of eIF5A hypusination [39]. Both the aforementioned properties of ciclopirox have been implicated in its cytotoxicity; however, its antitumor mechanism is complex and not well understood [48,49].

In this study, we describe that ciclopirox possesses mTOR inhibitory activity that results in a synergistic antileukemia effect when combined with PTL. These observations were confirmed with phosphoflow analyses as well as mTOR-specific kinase assays in vitro. Immunoblot analyses of primary AML cells treated with increasing doses of ciclopirox showed that a decrease in the phosphorylation of p70S6K was associated with a concomitant increase in Akt phosphorylation at Ser 473. This result is likely due to the triggering of a negative feedback loop, causing activation of Akt signaling and usually seen as a result of mTORC1 inhibition by rapamycin and rapalogs [37,38].

The importance of Raptor (mTORC1) in enhancing the activity of PTL was also confirmed with siRNA transfections. It was observed that similar to ciclopirox treatment, Raptor (or mTORC1) knockdown could abrogate the PTL-induced activation of phospho-S6 ribosomal protein, and it resulted in significant enhancement of cell death. Furthermore, in vitro kinase assay demonstrated that similar to rapamycin-FKBP12, ciclopirox-FKBP12 inhibited mTOR activity, whereas ciclopirox alone did not. These results suggest that ciclopirox functions like an allosteric inhibitor of mTORC1 similar to rapamycin.

Interestingly, although we observed an increase in Akt (Ser473) after treatment with ciclopirox and following PTL treatment on the Raptor siRNA-transfected cells, there is higher cell death in the presence of PTL. This finding is likely the result of the cooperation with other antileukemia activities of PTL (e.g., inhibition of NF- κ B and induction of ROS) [13].

We previously reported that treatment of AML cells with PTL activated signaling via the mTOR pathway, and PI3K/mTOR inhibitors, such as rapamycin, synergized with PTL to ablate human LSCs. In the current study, we similarly observed that the combination of ciclopirox with PTL was more effective in targeting AML cells compared with each drug alone. Furthermore, functional stem and progenitor assays demonstrated that the effects of the PTL-ciclopirox combination extended to AML progenitors and LSCs. In this study, we demonstrate that the antifungal agent ciclopirox can function through a FKBP12-dependent mechanism of mTOR inhibition to augment the antileukemia activity of PTL. The ability of ciclopirox to inhibit recombinant mTOR is demonstrated; we also show its ability to inhibit this pathway in situ. It has been reported that ciclopirox-induced cell death is dependent on iron chelation [24]. We did not observe an iron-dependent mechanism of cell death in our study. Iron homeostasis in leukemia is complex; the relative amounts of iron can vary depending on the particular cell lines and patient samples, and the potential mechanisms underlying the antitumor effects of iron-chelating agents has been understood only recently [50,51]. Our results also suggest that mTOR inhibition is unique to ciclopirox and is not shared by other hypusine inhibitors, such as GC7. The observed increase of PTL-induced cytotoxicity, therefore, is likely due to the ability of ciclopirox to modulate PTL-induced mTOR activation, although it is possible that a combination of more than one mechanism is involved in ciclopirox-enhanced cytotoxicity. Ciclopirox has been reformulated for oral use and is being tested in clinical trials for hematologic malignancies (ClinicalTrials.gov Identifier: NCT00990587). Our current findings demonstrate a novel mTOR inhibitory activity for ciclopirox that could provide a rationale for additional combinations with agents that activate the mTOR pathway in other malignancies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Cyclopirox synergizes with parthenolide eliminating bulk, progenitor, and leukemia stem cells. (A) Representative example of flow cytometric analysis of CD34⁺CD38⁻CD123⁺ population in a primary AML. Cells are shown in the presence or absence of 2.5 μ mol/L PTL, 20 μ mol/L cyclopirox, or both. Dot plots show 7-aminoactinomycin D (y axis) versus annexin V (x axis). (B) Graphical representation for the percent viability in primary patient AML samples (n = 4) and normal samples (n = 3) treated with PTL and cyclopirox alone, or in combination for 18–24 hours. (C) Percent viability of CD34⁺CD38⁻ subpopulation in primary patient AML samples (n = 4) and normal samples (n = 3) treated with PTL and cyclopirox alone or in combination at 18–24 hours. Solid closed bars represent normal cells, and open bars represent AML cells. (D) Colony-forming assays for primary AML (n = 4) versus normal cells (n = 2) treated with PTL and cyclopirox in combination for 18 hours. The percentage of colony-forming units (CFUs) was normalized to an untreated control group. (E) Percentage of human AML engraftment in NOD/SCID mice for primary AML cells after exposure with PTL-cyclopirox combination for 18 hours (n = 3). Open bars represent untreated samples, and closed bars represent cells treated with PTL-cyclopirox. (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). Error bars represent SEM.

Figure 2.

Ciclopirox inhibits the PTL-induced activation of mTOR signaling pathway. (A) Immunoblot for the following antibodies: phospho-p65 (S536), phospho-p70S6K (T421/S424), phospho-Akt (S473), total p70S6K, total Akt, and -actin. Cell lysates were prepared 6 hours after exposure with the indicated compounds: 10 μ mol/L PTL, 10 μ mol/L ciclopirox (Ciclo), or both. (B) Graphical representation of flow cytometric analyses of Kasumi-1 cells treated with 10 μ mol/L PTL, 10 μ mol/L ciclopirox (Ciclo), or both. The fold change in the mean fluorescence intensities of phospho-S6 ribosomal protein (S235/236) in CD34⁺ and CD34⁺CD38⁻ populations are shown evaluated by phosphoflow. Each dot represents the average mean fluorescence intensity of one experiment performed in triplicate (n = 3; **p* < 0.05; ***p* < 0.01).

Figure 3.

Cyclopirox inhibits the mTOR signaling pathway. Representative immunoblot of a primary AML sample treated with 10 μ mol/L PTL, 2.5 μ g/mL temsirolimus (Tem), and increasing concentrations of cyclopirox (ciclo) for 6 hours. The blot was probed for phospho-p70S6K (T421/S424), phospho-p70S6K (T389), phospho-4EBP1(T37/46), phospho-Akt (S473), total p70S6K, total Akt, and -actin.

Figure 4.

Iron, iron chelators, and a hypusination inhibitor do not affect mTOR signaling.

Representative immunoblot of a primary AML sample treated with 10 μ mol/L PTL, 10 μ mol/L ciclopirox (ciclo), 2.5 μ g/mL temsirolimus (Tem), and increasing concentrations of ferric ammonium citrate (Fe), deferoxamine, or GC-7 for 6 hours. The blot was probed as indicated with antibodies to phospho-p70S6K (T421/S424), phospho-Akt (S473), total p70S6K, total Akt, p-P65 (S536), and -actin.

Figure 5.

Effect of Raptor and Rictor knockdown on the sensitivity of AML cells to parthenolide. (A) Representative immunoblot of Kasumi-1 AML cells transfected with scrambled siRNA and siRNA targeting Raptor or Rictor. (B) Bar chart for the percent viability in Kasumi-1 cells transfected with scrambled siRNA and siRNA targeting Raptor or Rictor following treatment with 5 μ mol/L PTL. (C) Bar chart of flow cytometric analyses of Raptor or Rictor knockdown Kasumi-1 cells treated with 5 μ mol/L PTL. The fold change in the mean fluorescence intensities of phospho-S6 ribosomal protein (S235/236) evaluated by phosphoflow are indicated.

Figure 6.

Ciclopirox inhibits mTOR activity. The activity of recombinant mTOR was determined after incubation with increasing concentrations of ciclopirox and 3 μ g/mL of FKBP12, or increasing concentrations of ciclopirox alone without FKBP12. Parthenolide-FKBP12 was also tested, whereas rapamycin-FKBP12 and wortmannin were used for control groups. The activity was determined by measuring the absorbance at 450 nm, shown on the y axis. Mean absorbance relative to the untreated controls are depicted.