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Ciclopirox Olamine inhibits mTORC1 signaling by activation of AMPK

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Abstract

Ciclopirox olamine (CPX), an off-patent antifungal agent, has recently been identified as a potential anticancer agent. The mammalian target of rapamycin (mTOR) is a central controller of cell growth, proliferation and survival. Little is known about whether and how CPX executes its anticancer action by inhibiting mTOR. Here we show that CPX inhibited the phosphorylation of p70 S6 kinase 1 (S6K1) and eukaryotic initiation factor 4E binding protein 1 (4E-BP1), two downstream effector molecules of mTOR complex 1 (mTORC1), in a spectrum of human tumor cells, indicating that CPX inhibits mTORC1 signaling. Using rhabdomyosarcoma cells as an experimental model, we found that expression of constitutively active mTOR (E2419K) conferred resistance to CPX inhibition of cell proliferation, suggesting that CPX inhibition of mTORC1 contributed to its anticancer effect. In line with this, treatment with CPX inhibited tumor growth and concurrently suppressed mTORC1 signaling in RD xenografts. Mechanistically, CPX inhibition of mTORC1 was neither via inhibition of IGF-I receptor or phosphoinositide 3-kinase (PI3K), nor by activation of phosphatase and tensin homolog (PTEN). Instead, CPX inhibition of mTORC1 was attributed to activation of AMP-activated protein kinase (AMPK)-tuberous sclerosis complexes (TSC)/raptor pathways. This is supported by the findings that CPX activated AMPK; inhibition of AMPK with Compound C or ectopic expression of dominant negative AMPK α .

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Conflict of interest

The authors declare no conflict of interest.

partially prevented CPX from inhibiting mTORC1; silencing TSC2 attenuated CPX inhibition of mTORC1; and CPX also increased AMPK-mediated phosphorylation of raptor (S792). Therefore, the results indicate that CPX exerts the anticancer effect by activating AMPK, resulting in inhibition of mTORC1 signaling.

Graphical Abstract

Keywords

Ciclopirox; mTOR; AMPK; TSC2; Raptor

1. Introduction

Ciclopirox olamine (CPX) is a broad-spectrum fungicide against dermatophytes, yeast, filamentous fungi and bacteria [1, 2]. Although CPX has been used to treat mycoses of the skin and nails for over two decades, the mechanism of its antifungal action is poorly understood. CPX may disrupt membrane function in fungi or target different metabolic (respiratory) and energy producing processes in bacteria [1, 2]. In the yeast *Saccharomyces cerevisiae*, CPX may also exert its effect by disrupting DNA repair, cell division signals and structures (mitotic spindles) as well as some elements of intracellular transport [3]. Also, CPX can chelate iron and inhibit the functions of certain iron-containing enzymes (e.g. catalase and peroxidase), interfering with cellular metabolism [4].

Recent preclinical and clinical studies have demonstrated that CPX is a potential anticancer agent [5–7]. To facilitate repurposing this off-patent fungicide for cancer therapy, it is of great importance to elucidate the molecular mechanism of anticancer action of CPX. It has been shown that CPX induces cell death in murine and human myeloma and lymphoma cells by inhibiting the iron-containing enzyme ribonucleotide reductase [5], and Wnt/ β -catenin pathway [8]. Also, CPX inhibits cell proliferation by downregulation of cyclin D1 and cyclin dependent kinases (CDK2 and CDK4), induces apoptosis by downregulation of Bcl-xL and survivin [7], and induces autophagy by activating c-Jun N-terminal kinase (JNK) in solid tumor cells [9]. CPX inhibits angiogenesis by inhibiting deoxyhypusine hydroxylase activity and vascular endothelial cell growth [10], although this is controversial [11]. CPX inhibits lymphangiogenesis by inhibiting vascular endothelial growth factor receptor-3 (VEGFR-3) expression in lymphatic endothelial cells [12]. Of interest, a recent study has reported that CPX enhances parthenolide-induced cell death in leukemic cells, by inhibiting the phosphorylation of ribosomal p70 S6 kinase 1 (S6K1) on T389, implying inhibition of the mammalian target of rapamycin (mTOR) complex 1 (mTORC1) [13]. mTOR, a serine/ threonine (S/T) kinase, is a central controller of cell growth, proliferation, survival, motility,

angiogenesis and lymphangiogenesis, which plays an important role in the tumorigenesis and metastasis [14–16]. However, the role of CPX inhibition of mTORC1 in its anticancer action remains to be determined. Particularly, how CPX inhibits mTORC1 is largely unknown.

mTOR forms two structurally and functionally distinct complexes, mTOR complex 1 (mTORC1) and mTORC2 [14]. In response to environmental cues, such as growth factors, energy, amino acids, stress and redox levels, mTORC1 regulates cell growth and proliferation by controlling protein synthesis and lipid synthesis, by mediating S6K1 and eukaryotic initiation factor 4E (eIF4E) binding protein 1 (4E-BP1) [14, 17]. In response to growth factors, mTORC2 regulates the phosphorylation or activity of Akt [18], glucocorticoid-inducible kinase 1 (SGK1) [19], focal adhesion proteins and small GTPases, and controls cell survival and actin cytoskeleton [20–23].

Dysregulation of mTOR signaling is associated with human diseases including cancer [14, 24]. Because many tumors are addictive to activated mTOR signaling, mTOR has become an attractive target for cancer therapy [15, 16]. Two rapamycin analogs (rapalogs), temsirolimus and everolimus, which are allosteric inhibitors of mTORC1, have been approved by the US Food and Drug Administration (FDA) for treatment of advanced renal cell carcinoma, metastatic pancreatic neuroendocrine tumors, and certain breast cancer [15]. However, only modest efficacy has been achieved in many other solid tumors when treated with rapalogs alone, probably due to the fact that rapalogs inhibit S6K1, which results in a feedback activation of insulin receptor substrate 1 (IRS1)-Akt pathway [15]. Recently a new generation of ATP-competitive mTOR kinase inhibitors (e.g. INK128, AZD8055 and OSI-027) has emerged, which compete with ATP in the catalytic site of mTOR and inhibit both mTORC1 and mTORC2 [16]. However, it has been observed that mTOR kinase inhibitors may relieve feedback inhibition of receptor tyrosine kinases, leading to re-activation of phosphatidylinositol 3' kinase (PI3K)-Akt as well [25]. Furthermore, none of mTOR kinase inhibitors alone has clinically proven effective against any type of cancer yet, despite promising results from preclinical studies [26]. Therefore, it is still of great value to identify new class of mTOR inhibitors.

mTORC1 can be positively regulated by the type I insulin-like growth factor (IGF-I) receptor (IGFR)-PI3K pathway, which is antagonized by the phosphatase and tensin homolog (PTEN) [14]. In addition, mTORC1 responds to cellular energy signals through adenosine monophosphate (AMP)-activated protein kinase (AMPK) [27]. Under conditions of low cellular energy (high AMP/ATP ratio), LKB1 phosphorylates AMPK (T172) [28, 29]. Once activated, AMPK phosphorylates a number of targets, most of which are directly involved in controlling cellular energy metabolism, such as acetyl-CoA carboxylase (ACC) and glycogen synthase [29]. Activated AMPK also phosphorylates tuberous sclerosis complex 2 (TSC2) at T1227 and S1345, promoting the formation and activation of TSC1/2 complex [27, 30], which catalyzes the conversion of Rheb-GTP to Rheb-GDP and thus inhibits mTORC1 activity [31–33]. In addition, activated AMPK can phosphorylate raptor (S792), resulting in inhibition of mTORC1 as well [34]. Therefore, in response to energy stress, mTORC1 can be inhibited by activation of AMPK-TSC and AMPK-raptor pathways.

In the present study, we present evidence that CPX inhibits mTORC1 signaling in a number of tumor cells. Using rhabdomyosarcoma cells (Rh30 and RD) as an experimental model, we demonstrated that CPX inhibited RMS tumor cell growth *in vitro* and *in vivo* by inhibiting mTORC1 signaling. CPX inhibition of mTORC1 was not by inhibition of IGFR/PI3K or activation of PTEN, but was associated with activation of AMPK-TSC and AMPK-raptor pathways. Therefore, our results indicate that CPX exerts the anticancer effect through AMPK-mediated inhibition of mTORC1 signaling.

2. Material and methods

2.1. Materials

Ciclopirox olamine (CPX) was purchased from Sigma (St. Louis, MO, USA). RPMI 1640, Dulbecco's Modified Eagle Medium (DMEM), DMEM/F12, and 0.05% Trypsin-EDTA were purchased from Mediatech (Herndon, VA, USA). Fetal bovine serum (FBS) was from Hyclone (Logan, UT, USA). Type I insulin-like growth factor (IGF-1) (PeproTech, Rocky Hill, NJ, USA) was rehydrated in 0.1 M acetic acid to prepare a stock solution (10 ng/ml), aliquoted and stored at -80°C . Compound C (EMD Millipore, MA, USA) was dissolved in DMSO to prepare a 10 mM stock solution and was stored at -20°C . The antibodies to β -tubulin, β -actin, S6K1, S6, Akt, and c-myc were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA), while the antibodies to p-S6K1 (Thr389), p-mTOR (Ser2448), mTOR, p-AMPK α (Thr172), AMPK, p-ACC (Ser79), ACC, TSC2, p-S6 (Ser235/236), 4E-BP1, p-4E-BP1 (Thr37/46), p-4E-BP1 (Thr70), p-4E-BP1 (Ser65), p-Akt (S473), p-Akt (T308), p-raptor (Ser792) were purchased from Cell Signaling Technology (Beverly, MA, USA). Lipofectamine LTX with PLUS Reagent was from Invitrogen (Carlsbad, CA, USA). pcDNA3-AU1-mTOR-E2419K [35] was a gift from Dr. Fuyuhiko Tamanoi (University of California, Los Angeles, CA, USA). All other chemicals were obtained from Sigma (St. Louis, MO, USA). Fibroblast basal medium (Cat# PCS-201-030) and fibroblast growth kit-low serum (Cat# PCS-201-041) were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA).

2.2. Cell culture

Human rhabdomyosarcoma Rh30 (gift from Dr. Peter J. Houghton, St. Jude Children's Research Hospital, Memphis TN) and RD (Cell Bank of the Chinese Academy of Sciences, Shanghai, China), human head and neck squamous cell carcinoma PCI-13 cells, and lung adenocarcinoma A427 cells (ATCC) were grown in RMPI 1640 supplemented with 10% FBS. Human breast carcinoma (MDA-MB-231 and MDA-MB-435) and prostate carcinoma (PC-3) cells (ATCC) were grown in DMEM supplemented with 10% FBS. Human skin squamous cell carcinoma SRB1-M7 cells [36] (gift from Dr. John Clifford, Louisiana State University Health Sciences Center, Shreveport, LA) were grown in DMEM/F12 supplemented with 10% FBS. Primary human normal dermal fibroblasts PCS-201-012 cells (ATCC) were grown in fibroblast basal medium (Cat.# PCS-201-030, ATCC) supplemented with fibroblast growth kit-low serum (Cat.# PCS-201-041, ATCC). All cell lines were cultured in a humidified atmosphere at 37°C with 5% CO_2 .

2.3 Cell proliferation assay

Rh30, RD and PCS-201-012 cells were seeded in 6-well plates (1×10^5) under standard culture conditions and kept overnight. The next day, cells were treated with 0–20 μ M of CPX for 72 h. Cell proliferation was assessed by counting the trypsinized cells with a Beckman Coulter Counter (Beckman Coulter, Fullerton, CA, USA).

2.4. Cell transfection

For expression of constitutively active mTOR, RD and Rh30 cells (4×10^5) were seeded in 6-well plates. The next day, 2 μ g of pcDNA3.0 (empty vector) or pcDNA3-AU1-mTOR-E2419K plasmid DNA was transfected into the cells by using Lipofectamine LTX with PLUS Reagent. After transfection for 24 h, the cell lysates were prepared for further analysis. Expression of AU1-tagged constitutively active mTOR was confirmed by Western blotting with indicated antibodies.

2.5. Cell viability assay

Briefly, 100 μ l of cell suspension, which was respectively transfected with pcDNA3.0 vector or pcDNA3-AU1-mTOR-E2419K plasmid DNA, was seeded into 96-well plates (8×10^3 cells/well) and incubated overnight. RD cells were then exposed to different concentrations of CPX in triplicates for 48 h, followed by MTS assay, as described previously [7]. Rh30 cells were treated with or without 2.5 μ M of CPX and MTS assay was performed at 0, 24, 48, 72 and 96 h.

2.6. Recombinant adenoviral infection of cells

Recombinant adenovirus encoding c-myc-tagged dominant-negative AMPK α (Ad-AMPK α -DN) [37] was generously provided by Dr. Joohun Ha (Kyung Hee University, Seoul, Korea). The control virus encoding the green fluorescence protein (GFP) alone (Ad-GFP) was described previously [38]. For experiments, the cells were infected with the individual adenovirus for 24 h at 1 of multiplicity of infection (MOI = 1). Cells infected with Ad-GFP served as control. Expression of c-myc-tagged Ad-AMPK α -DN was determined by Western blot with antibodies to c-myc.

2.7. Determination of intracellular ATP levels

Intracellular ATP levels were measured using an ATP Determination Kit (Invitrogen) according to the manufacturer's instruction. Briefly, cells were treated with indicated concentrations of CPX for 8 h. To assess the intracellular ATP contents, cells were washed and lysed with 0.1% Triton X-100. The resulting cell lysates were added to a reaction mixture containing 0.5 mM D-luciferin, 1 mM DTT, and 1.25 μ g/ml of firefly luciferase and incubated for 5 min at room temperature. Luminescence was measured with a luminometer (Thermo Scientific, Waltham, MA USA). ATP solutions with concentrations ranging from 1 nM to 50 nM were used as standards. Reactions were carried out in triplicates and intracellular ATP levels were expressed in nmol ATP/ 10^6 cells.

2.8. Lentiviral shRNA cloning, production, and infection

To generate lentiviral shRNAs to TSC2 (sh-TSC2), oligonucleotides containing the target sequences were synthesized, annealed and inserted into FSIPPW lentiviral vector via the EcoRI/BamHI restriction enzyme site, as described [38]. Oligonucleotides used were: TSC2 sense: 5'-AATTCCTCCGGCTTCTCCAGA ACTGACTTGCAAGAGAAGTCAGTTCTGGAGAAGCCTTTTGTG-3'; anti-sense: 5'-GATCCAAAAAGGCTTCTCCAGAACTGACTTCTCTTGCAAGTCAGTTCTG GAGAAGCCGGG-3'. To produce lentiviral shRNAs, above constructs were co-transfected with plasmids pMD2.G and psPAX2 (Addgene, Cambridge, MA, USA) to 293TD cells as described [38]. After transfection for 48 h and 72 h, the supernatants containing lentiviral particles were collected twice. The lentivirus expressing GFP-target shRNA (sh-GFP) was described previously [38], and used as a control. When grown to about 70% confluence, monolayer Rh30 cells were respectively infected with sh-GFP or sh-TSC2 lentivirus-containing supernatant in the presence of 8 µg/ml of polybrene for 12 h twice at an interval of 6 h. After the second infection for 24 h, the cells were exposed to 2 µg/ml of puromycin for 48 h. In 5 days, the cells were used for experiments.

2.9. Study in RD xenografts

All procedures used in this study were approved by the Animal Care Committee of Kunming Institute of Botany (Kunming, China), and were carried out in accordance with the Guidelines for Experimental Animals of the Ministry of Science and Technology (China). Female BALB/c nude mice (Vital River, Beijing, China) were housed and maintained under specific-pathogen-free condition. Human rhabdomyosarcoma RD cells (6×10^6 , resuspended in PBS) were injected orthotopically into the gastrocnemius muscle in the left hind leg of the mice, as described [39]. Three weeks later, to study the anticancer activity of CPX, the mice were randomized into 3 groups (8 mice/group). The experimental groups were intraperitoneally (i.p.) injected daily with 20 and 60 mg/kg of CPX prepared in a solution (4% ethanol, 5.2% Tween 80, and 5.2% PEG 400), respectively. The control group received equal volume of the vehicle. Tumor volume $[(\text{length} \times \text{width}^2) / 2]$ was determined with a digital caliper. Tumor growth and mice body weight were monitored every three days and analyzed. At the end of experiments, animals were sacrificed, and the tumors were collected, photographed, and analyzed.

2.10. Immunohistochemistry and TUNEL assay

At the end of experiments, tumor tissues were excised and fixed with 10% neutral buffered formalin, embedded in paraffin and sectioned using a standard histological procedure. The tissue sections were respectively stained with hematoxylin and eosin (H&E) and Ki-67 as described previously [7]. The TUNEL assay on tumor section was carried out using DeadEnd Colorimetric TUNEL System (Promega) as described [7].

2.11. Western blot analysis

For Western blot analysis in cultured cells, cells were treated with indicated concentrations of CPX for 24 h, or with 20 µM of CPX for indicated time. For experiments where cells were deprived of serum, cells were incubated in the serum-free medium for 24 h and then

treated with CPX for 24 h, followed by stimulation with 10 ng/ml of IGF-I for 1 h. Western blot analysis was performed as described [7]. All bands were semi-quantified using NIH ImageJ software.

At the end of RD xenograft study, tumor samples were collected, flash frozen in liquid nitrogen, and stored at -80°C until processed. Tumor lysates were prepared using the following lysis buffer: 50 mM Tris (pH 7.4) containing 1% Triton X-100, 0.1% SDS, 150 mM NaCl, 1 mM EDTA, 1% sodium deoxycholate, 0.1 mM Na_3VO_4 , and protease inhibitor cocktail (1:1,000, Sigma). The protein concentration was determined by BCA assay (Thermo Scientific). Immunoblotting was performed as described [7]. All bands were semi-quantified using NIH ImageJ software.

2.12. Statistical analysis

The results were expressed as mean values $\pm$ standard deviation (mean $\pm$ SD). Group variability and interaction were compared using one-way ANOVA followed by Bonferroni's post-tests to compare replicate means. Significance was accepted at $p < 0.05$.

3. Results

3.1. CPX inhibits mTORC1 signaling in tumor cells

A recent study has shown that CPX inhibits mTORC1 signaling in leukemia cells [13]. To determine whether CPX inhibition of mTORC1 signaling is a general mechanism, a spectrum of cell lines derived from human rhabdomyosarcoma (Rh30 and RD), breast adenocarcinoma (MDA-MB-231 and MDA-MB-435), prostate adenocarcinoma (PC-3), head and neck squamous cell carcinoma (PCI-13), and human skin squamous cell carcinoma (SRB1-M7) were employed in this study. As shown in Fig. 1A (I, II), treatment with CPX (0–20 μM) for 24 h inhibited the phosphorylation of S6K1 and 4E-BP1, two best known downstream effector molecules of mTORC1, in Rh30 cells in a concentration-dependent manner. Besides, treatment with 20 μM of CPX also decreased the phosphorylation of S6K1 (T389) in the cells in a time-dependent manner. After 4-h exposure, p-S6K1 was obviously inhibited; and following 24-h exposure, p-S6K1 was almost completely blocked. In addition, 20 μM of CPX also decreased the overall phosphorylation of 4E-BP1 in a time-dependent manner, as visualized by changes in mobility—the reduction of the intensity of the uppermost band γ and the increase in the lower band α , which corresponds to less phosphorylated forms of 4E-BP1 (Fig. 1A (I, II)). Similarly, CPX also inhibited the phosphorylation of S6K1 and 4E-BP1 in RD, MDA-MB-231, PC-3, PCI-13, MDA-MB-435, and SRB1-M7 cells in a concentration-dependent manner (Fig. 1B and C (I, II)). As a positive control, rapamycin, a known mTORC1 inhibitor, significantly inhibited the phosphorylation of S6K1 and 4E-BP1 in Rh30 cells (Fig. 1C (I, II)). Therefore, the results strongly support the notion that CPX inhibition of mTORC1 signaling is a general mechanism, which is independent of cell lines.

As mTOR lies downstream of IGFR, and dysregulation of IGFR-mTOR pathway occurs frequently in a variety of tumors, including rhabdomyosarcoma [14, 24], we also investigated whether CPX inhibits IGF-I-activated mTORC1 signaling. For this, Rh30

rhabdomyosarcoma cells were used, as this cell line is very responsive to IGF-I stimulation [38]. As predicted, treatment with IGF-I (10 ng/ml) for 1 h stimulated mTORC1-mediated phosphorylation of S6K1 (T389) and 4E-BP1 (S65 and T70) in serum-starved Rh30 cells (Fig. 1D (I, II)). Noticeably, pretreatment with CPX for 24 h inhibited IGF-I-induced phosphorylation of S6K1 and 4E-BP1 in a concentration-dependent manner (Fig. 1D (I, II)). Taken together, the above results indicate that CPX inhibits mTORC1 signaling in tumor cells.

mTORC2 phosphorylates Akt on S473 [18]. As CPX has been found to activate the phosphorylation of Akt (S473) in leukemia cells [13], we also examined whether this is the case in the solid tumor cells. For this, four cell lines (Rh30 and MDA-MB-231, PCI-13 and A427) were used. Unexpectedly, treatment with CPX induced the phosphorylation of Akt (S473) in Rh30 and PCI-13 cells (Fig. 2A and C (I, II)), but inhibited the phosphorylation of Akt (S473) in MDA-MB-231 and A427 cells under serum starvation or normal growth condition (Fig. 2B and D (I, II)). Therefore, our results suggest that the effect of CPX on mTORC2-mediated Akt phosphorylation was cell line-dependent.

3.2. Antiproliferative activity of CPX

Cumulative evidence demonstrates that dysregulation of mTOR signaling pathway often occurs in a variety of human tumors, and these tumor cells have shown higher susceptibility to mTOR inhibitors than normal cells [14, 24]. Therefore, the antiproliferative activity of CPX was evaluated in human rhabdomyosarcoma cells and human normal dermal fibroblasts PCS-201-012 cells. As shown in Fig. 3A, CPX exhibited stronger antiproliferative effect on rhabdomyosarcoma cells (RD and Rh30) than normal fibroblasts PCS-201-012 cells. Treatment with 20 μ M of CPX for 72 h reduced the proliferation of RD and Rh30 cells by 84%, but only inhibited the proliferation of PCS-201-012 cells by 22%. These data suggests that tumor cells are more sensitive to CPX than normal cells, which supports the further investigation of CPX as a potential antitumor agent.

3.3 Expression of constitutively active mTOR confers resistance to CPX inhibition of cell proliferation

Given the critical role of mTOR in cell proliferation [14], next, we determined whether inhibition of mTORC1 signaling contributes to CPX inhibition of cell proliferation. For this, rhabdomyosarcoma RD and Rh30 cells were used as an experimental model, and transfected with the empty vector pcDNA3 and pcDNA3-mTOR (E2419K) [35]. The results showed that transfection with pcDNA3-mTOR (E2419K) increased the level of mTOR expression by approximately 3 fold, compared with the transfection with the empty vector control, as detected by antibodies against AU1 and mTOR (Fig. 3B (I, II)). Both RD and Rh30 cells expressing the constitutively active mTOR also exhibited higher phosphorylation of S6K1, S6 and 4E-BP1 than the control cells (Fig. 3B (I, II)), suggesting that the constitutively active mTOR was functional in the cells. In line with this, expression of constitutively active mTOR also significantly enhanced the cell proliferation, compared with the vector control (Fig. 3C (I, II)). Of interest, expression of the constitutively active mTOR rendered a significant resistance to CPX inhibition of cell proliferation (Fig. 3C (I, II)), indicating that inhibition of mTORC1 signaling is crucial for the anticancer effect of CPX.

3.4. CPX inhibits tumor growth and mTORC1 signaling in RD xenografts

Given the potent inhibitory effect of CPX on RD cell proliferation *in vitro* (Fig. 3A), we further evaluated the antitumor effect of CPX on RD xenografts in nude mice. The results showed that treatment with CPX (0–60 mg/kg, i.p.) daily for 21 days dose-dependently inhibited RD tumor growth in volume or size, compared with the vehicle control (Fig. 4A and 4B). Of note, at the end of the experiment, the average weight of the tumors was reduced by 21.7% and 50.8% in the animals treated with 20 and 60 mg/kg of CPX, respectively, compared with the vehicle control (Fig. 4B).

To understand how CPX inhibits the tumor growth, the level of proliferation and apoptosis in tumor tissues was assessed by Ki-67 and TUNEL staining, respectively. As shown in Fig. 4C and D, CPX decreased the percentage of Ki-67-positive cells and increased TUNEL-positive cells in the tumor tissues in xenografts significantly and dose-dependently. The results indicate that CPX inhibits the xenografted tumor growth by inhibiting cell proliferation and inducing apoptosis of RD cells in the tumors. In line with the above *in vitro* data (Fig. 1), the *in vivo* results revealed that CPX also remarkably inhibited mTORC1 signaling in the RD xenografted tumors, as detected by Western blotting for the phosphorylation of S6 and 4E-BP1 (Fig. 4E and F). Thus, our *in vivo* studies support that CPX inhibits tumor growth by suppressing mTORC1 signaling.

3.5. CPX does not suppress mTORC1 by inhibiting IGFR/PI3K or by activating PTEN

As the effect of CPX on mTORC2 is cell line dependent, this study focused on investigating how CPX inhibits mTORC1. Since mTORC1 can be regulated by the upstream kinases IGFR and PI3K positively and by the upstream phosphatase PTEN negatively [14], we reasoned that CPX might inhibit mTORC1 by inhibiting IGFR/PI3K and/or by activating PTEN. To this end, serum-starved Rh30 and MDA-MB231 cells were pretreated with CPX (0–20 μ M) for 24 h and then stimulated with IGF-1 (10 ng/ml) for 1 h, followed by Western blotting. As shown in Fig. 5A (I, II), CPX altered neither the total cellular protein level nor the phosphorylation level of IGFR, PI3K or PTEN. As PDK1 is regulated positively by PI3K and negatively by PTEN [14], to verify the above finding, we also determined whether CPX affects the activity of PDK1 by Western blot analysis of PDK phosphorylation. In agreement with the above data, CPX did not influence the phosphorylation of PDK1 either (Fig. 5A (I, II)). Collectively, these results imply that CPX inhibition of mTORC1 is not through inhibiting IGFR/PI3K or by activating PTEN.

3.6. CPX inhibits mTORC1 signaling via activation of AMPK related to reduced ATP level

Accumulating evidence indicates that AMPK is one of the major negative regulators of mTORC1 signaling [14]. Next, we asked whether CPX inhibits mTORC1 signaling via activation of AMPK. For this, serum-starved Rh30 cells were pretreated without or with 10 μ M of compound C for 30 min, and then exposed to CPX (0–20 μ M) for 24 h, followed by stimulation with IGF-I for 1 h. Cell lysates were subjected to Western blot analysis. Consistent with the above findings (Fig. 1D), CPX inhibited IGF-I stimulated phosphorylation of S6K1, S6 and 4E-BP1 (Fig. 5B (I, II)). Concurrently, CPX also induced the phosphorylation of AMPK α on T172 and its substrate ACC on S79 in a concentration-dependent manner (Fig. 5B (I, II)). Pretreatment with Compound C, a selective inhibitor of

AMPK, profoundly blocked CPX-induced p-AMPK α and p-ACC (Fig. 5B (I, II)). Importantly, Compound C substantially prevented CPX from inhibiting phosphorylation of S6K1, S6 and 4E-BP1 (Fig. 5B (I, II)). The data suggest that CPX-induced activation of AMPK is linked to inhibition of mTORC1. In MDA-MB231 cells, pretreatment with Compound C also reversed the inhibitory effect of CPX on phosphorylation of S6K1, S6 and 4E-BP1 (Fig. 5C (I, II)). This result further demonstrates that AMPK activation is involved in CPX -induced mTORC1 inhibition.

To confirm the above finding, Rh30 cells were infected with recombinant adenovirus expressing a c-myc-tagged dominant negative (DN) AMPK α (Ad-AMPK α -DN) [37], or GFP (Ad-GFP, control) for 24 h, followed by exposure to CPX (0–20 μ M) for 24 h. We found that infection with Ad-AMPK α -DN resulted in high expression of myc-tagged AMPK α -DN (Fig. 5D (I, II)). Ectopic expression of AMPK α -DN markedly reduced the basal or CPX-induced p-ACC (Fig. 5D (I, II)), indicating that AMPK α -DN functioned in the cells, as expected. Of interest, the cells infected with Ad-AMPK α -DN, but not Ad-GFP, were highly resistant to the inhibitory effect of CPX on mTORC1 signaling (Fig. 5D (I, II)). Therefore, our results further demonstrate that CPX inhibits mTORC1 by activating AMPK.

AMPK, an energy sensor, can be activated in response to decreased intracellular ATP level [29]. Likely, CPX activates AMPK by reducing intracellular ATP level. For this, Rh30 and RD cells were treated with various concentrations of CPX for 8 h, followed by the assay for intracellular ATP level. As shown in Fig. 5E, CPX did reduce intracellular ATP levels in both RD and Rh30 cells. Therefore, the results suggest that CPX may activate AMPK by reducing intracellular ATP level.

3.7. CPX inhibits mTORC1 signaling via activation of AMPK-TSC and AMPK-raptor pathways

Since activated AMPK inhibits mTORC1 by activating TSC1/2 complex [27, 30], we further investigated whether CPX inhibits mTORC1 involving the participation of TSC1/2 complex. To address this question, RNA interference was employed to silence the expression of TSC2. As shown in Fig. 6A (I, II), lentiviral shRNA to TSC2 downregulated the expression of TSC2 by approximately 70% in Rh30 cells, compared with lentiviral shRNA to GFP (control). Interestingly, downregulation of TSC2 conferred high resistance to CPX inhibition of p-S6K1 and p-S6, but failed to prevent CPX from inhibiting the phosphorylation of 4E-BP1 (Fig. 6A (I, II)). The results suggest that CPX inhibits mTORC1 signaling in part by activation of AMPK-TSC axis.

As AMPK inhibits mTORC1 also by phosphorylating raptor (S792) [34], we checked whether CPX inhibits mTORC1 by phosphorylating raptor. As expected, treatment with CPX for 16 h indeed induced the phosphorylation of raptor (S792) in a concentration dependent manner (Fig. 6B (I, II)). The results suggest that CPX inhibits mTORC1 signaling also in part by activation of AMPK-raptor pathway. Collectively, our data reveal that the inhibition of mTORC1 by CPX involves activation of both AMPK-TSC and AMPK-raptor pathways.

4. Discussion

CPX has been widely used for the treatment of topical fungal infection for decades, although the mechanism of its antifungal action remains poorly understood [2]. Recently, preclinical studies have found that this off-patent fungicide possess anticancer effect, by inhibiting cell proliferation, inducing cell death, as well as inhibiting angiogenesis and lymphangiogenesis [5, 7–10, 12]. Most recently, a phase I clinical trial has demonstrated that oral administration of CPX at a dose of 40 mg/m² once daily for 5 days is well tolerated in all patients, and induces disease stabilization and/or hematologic improvement in 2/3 patients with advanced hematologic malignancies [6]. These findings strongly support that systemic administration of CPX has a great potential to be repositioned for cancer therapy. To facilitate repurposing CPX for cancer therapy, many groups have investigated the molecular mechanisms underlying its anticancer action [5, 7–10, 12]. Of interest, a recent study has reported that CPX enhances parthenolide-induced cell death in acute myeloid leukemia cells, by inhibiting the phosphorylation of S6K1 (T389), implying inhibition of mTORC1 [13]. mTOR is a master kinase regulating cell growth, proliferation, survival, motility, angiogenesis and lymphangiogenesis, and is crucial for tumorigenesis and metastasis [14–16]. However, whether CPX inhibits mTORC1 is a general mechanism of its anticancer action remains to be determined. Here, for the first time, we show that CPX inhibited the phosphorylation of S6K1 and 4E-BP1, two downstream effector molecules of mTORC1, in a spectrum of human tumor cells derived from rhabdomyosarcoma (Rh30 and RD), breast adenocarcinoma (MDA-MB-231 and MDA-MB-435), prostate adenocarcinoma (PC-3), head and neck squamous cell carcinoma (PCI-13), and human skin squamous cell carcinoma (SRB1-M7) (Fig.1). Therefore, our results strongly support the notion that CPX inhibits mTORC1 signaling in tumor cells, which is a general mechanism and independent of cell lines.

In this study, using rhabdomyosarcoma cells as an experimental model, we found that ectopic expression of constitutively active mTOR (E2419K) conferred a significant resistance to CPX inhibition of proliferation in RD and Rh30 cells (Fig. 3). In agreement with this, treatment with CPX also inhibited the growth of RD xenografts in nude mice, and coincidentally suppressed mTORC1 signaling in the RD tumors (Fig. 4). Thus, our *in vitro* and *in vivo* data reveal that CPX inhibition of mTORC1 plays a critical role in the anticancer action of CPX. It is known that mTOR positively regulates the expression or activity of a number of proteins, such as cyclin D1 [40], CDKs [41], Bcl-xL [42], survivin [43], β -catenin [44], and VEGFR3 [45], and negatively regulates the activity of JNK [46]. Interestingly, CPX has been found to inhibit the expression or activity of cyclin D1, CDKs, Bcl-xL, survivin [7], β -catenin [8], and VEGFR3 [12], and activates JNK [9]. Likely, the effects of CPX on the expression or activity of these proteins are the consequence of mTORC1 inhibition. Further research on this aspect would help understand whether CPX exerts the anticancer action by primarily targeting mTORC1 signaling.

To our knowledge, so far it is largely unknown how CPX inhibits mTORC1 signaling. In this study, CPX was found to activate Akt phosphorylation in Rh30 (Fig. 2) and RD cells (data not shown), suggesting that CPX should not bind and inhibit mTOR as the mTOR kinase inhibitors (e.g. INK128, AZD8055 and OSI-027) do, which compete with ATP and bind to

the catalytic site of mTOR, inhibiting both mTORC1 and mTORC2 [15, 16]. This was supported by our protein-ligand docking analysis. Hence, we mainly focused on determining whether CPX inhibits mTORC1 through mediating the known positive and negative regulators of mTORC1. Our results showed that CPX inhibition of mTORC1 was independent of IGFR/PI3K inhibition or PTEN activation, but was attributed to AMPK activation. This is evidenced by the findings that 1) CPX did not alter the level of either the cellular protein or the phosphorylation of IGFR, PI3K or PTEN (Fig. 5A); 2) CPX induced the phosphorylation of AMPK (T172) (Fig. 5B); 3) Inhibition of AMPK with Compound C or ectopic expression of dominant negative-AMPK α partially prevented CPX from inhibiting mTORC1 (Fig. 5B, C and D). Taken together, the data presented here support that CPX exerts the anticancer effect at least in part by activating AMPK, resulting in inhibition of mTORC1 signaling.

AMPK is a physiological cellular energy sensor by sensing the cellular AMP/ATP ratio [29]. It has been shown that prolonged hypoxia can induce an energy-depleting response evidenced by decreased cellular ATP level and AMPK activation [47]. The heterodimeric hypoxia-inducible factor (HIF) is a key regulator of oxygen homeostasis [29]. Previous studies have shown that CPX is a hypoxia-mimicking agent, which stabilizes HIF-1 α or induce HIF-1 α mRNA production under normoxic conditions [11, 48]. In the present study, we observed that treatment with CPX reduced the intracellular ATP level in a concentration-dependent manner (Fig. 5D). Collectively, these findings suggest that CPX might mimic hypoxia condition, reducing intracellular ATP level, which results in activation of AMPK.

It has been described that activated AMPK inhibits mTORC1 by inducing formation of TSC1/2 complex [27, 30] and phosphorylation of raptor (S792) [34]. In this study, we found that downregulation of TSC2 conferred high resistance to CPX inhibition of S6K1 phosphorylation, but failed to prevent CPX from inhibiting 4E-BP1 phosphorylation (Fig. 6A). Currently we do not have a good explanation of this unexpected observation. In the meantime, we also found that CPX induced the phosphorylation of raptor (S792) (Fig. 6B), which is mediated by AMPK and directly inhibits mTORC1 activity [34]. Therefore, these findings suggest that CPX inhibits mTORC1 through AMPK-TSC and AMPK-raptor pathways.

Since CPX can activate the phosphorylation of Akt (S473) in leukemia cells [13], here we also investigated whether this is the case in solid tumor cells. Our results indicate that the effect of CPX on mTORC2 was cell line dependent. Consistent with the finding in leukemia cells [13], the phosphorylation of Akt (S473) was indeed induced by 24 h-treatment with CPX in rhabdomyosarcoma (Rh30) and head and neck cancer (PCI-13) cells (Fig. 2A and C). In contrast, the phosphorylation of Akt (S473) was inhibited by 24 h-treatment with CPX in breast (MDA-MB-231) and lung cancer (A427) cells (Fig. 2B and D). It has been noticed that the effect of the mTORC1 inhibitor rapamycin on Akt phosphorylation is also cell line dependent. For instance, rapamycin induced the phosphorylation of Akt (S473) in rhabdomyosarcoma (Rh30) cells [38] and lung cancer (H157 and A549) cells [49], but prolonged treatment (24 h) with rapamycin can inhibit the phosphorylation of Akt (S473) in prostate cancer (PC-3) and lymphoma (U937) cells [50]. Rapamycin inhibits Akt phosphorylation by reducing mTORC2 assembly [50], whereas rapamycin activates Akt

phosphorylation through multiple mechanisms, such as S6K1-insulin receptor substrate-1 (IRS-1) feedback regulation [51–53], Grb10 inactivation [54, 55], and protein phosphatase 2A (PP2A)-dependent DNA protein kinase (DNA-PK) activation [49]. More studies are needed to address whether CPX inhibits or activates Akt as rapamycin does.

In summary, here, for the first time, we show that CPX inhibits mTORC1 signaling in tumor cells *in vitro* and *in vivo*, but may inhibit or activate mTORC2-mediated phosphorylation of Akt in a cell line dependent manner. CPX inhibition of mTORC1 was not through inhibition of IGFR/PI3K or activation of PTEN, but via activation of AMPK-TSC/raptor pathways. Our results support that CPX exerts the anticancer effect at least by AMPK-mediated inhibition of mTORC1 signaling.

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References

1. Abrams BB, Hanel H, Hoehler T. Ciclopirox olamine: a hydroxypyridone antifungal agent. *Clin. Dermatol.* 1991; 9:471–477. [PubMed: 1822407]
2. Gupta AK. Ciclopirox: an overview. *Int J Dermatol.* 2001; 40:305–310. [PubMed: 11554991]
3. Leem SH, Park JE, Kim IS, Chae JY, Sugino A, Sunwoo Y. The possible mechanism of action of ciclopirox olamine in the yeast *Saccharomyces cerevisiae*. *Mol. Cells.* 2003; 15:55–61. [PubMed: 12661761]
4. Niewerth M, Kunze D, Seibold M, Schaller M, Korting HC, Hube B. Ciclopirox olamine treatment affects the expression pattern of *Candida albicans* genes encoding virulence factors, iron metabolism proteins, and drug resistance factors. *Antimicrob. Agents Chemother.* 2003; 47:1805–1817. [PubMed: 12760852]
5. Eberhard Y, McDermott SP, Wang X, Gronda M, Venugopal A, Wood TE, et al. Chelation of intracellular iron with the antifungal agent ciclopirox olamine induces cell death in leukemia and myeloma cells. *Blood.* 2009; 114:3064–3073. [PubMed: 19589922]
6. Minden MD, Hogge DE, Weir SJ, Kasper J, Webster DA, Patton L, et al. Oral ciclopirox olamine displays biological activity in a phase I study in patients with advanced hematologic malignancies. *Am. J. Hematol.* 2014; 89:363–368. [PubMed: 24273151]
7. Zhou H, Shen T, Luo Y, Liu L, Chen W, Xu B, et al. The antitumor activity of the fungicide ciclopirox. *Int. J. Cancer.* 2010; 127:2467–2477. [PubMed: 20225320]
8. Kim Y, Schmidt M, Endo T, Lu D, Carson D, Schmidt-Wolf IG. Targeting the Wnt/beta-catenin pathway with the antifungal agent ciclopirox olamine in a murine myeloma model. *In Vivo.* 2011; 25:887–893. [PubMed: 22021681]
9. Zhou H, Shen T, Shang C, Luo Y, Liu L, Yan J, et al. Ciclopirox induces autophagy through reactive oxygen species-mediated activation of JNK signaling pathway. *Oncotarget.* 2014; 5:10140–10150. [PubMed: 25294812]
10. Clement PM, Hanauske-Abel HM, Wolff EC, Kleinman HK, Park MH. The antifungal drug ciclopirox inhibits deoxyhypusine and proline hydroxylation, endothelial cell growth and angiogenesis *in vitro*. *Int. J. Cancer.* 2002; 100:491–498. [PubMed: 12115536]
11. Linden T, Katschinski DM, Eckhardt K, Scheid A, Pagel H, Wenger RH. The antimycotic ciclopirox olamine induces HIF-1alpha stability, VEGF expression, and angiogenesis. *FASEB J.* 2003; 17:761–763. [PubMed: 12594177]

12. Luo Y, Zhou H, Liu L, Shen T, Chen W, Xu B, et al. The fungicide ciclopirox inhibits lymphatic endothelial cell tube formation by suppressing VEGFR-3-mediated ERK signaling pathway. *Oncogene*. 2011; 30:2098–2107. [PubMed: 21217783]
13. Sen S, Hassane DC, Corbett C, Becker MW, Jordan CT, Guzman ML. Novel mTOR inhibitory activity of ciclopirox enhances parthenolide antileukemia activity. *Exp. Hematol*. 2013; 41:799–807. e794. [PubMed: 23660068]
14. Laplante M, Sabatini DM. mTOR signaling in growth control and disease. *Cell*. 2012; 149:274–293. [PubMed: 22500797]
15. Sun SY. mTOR kinase inhibitors as potential cancer therapeutic drugs. *Cancer Lett*. 2013; 340:1–8. [PubMed: 23792225]
16. Zhang YJ, Duan Y, Zheng XF. Targeting the mTOR kinase domain: the second generation of mTOR inhibitors. *Drug Discov. Today*. 2011; 16:325–331. [PubMed: 21333749]
17. Inoki K, Kim J, Guan KL. AMPK and mTOR in cellular energy homeostasis and drug targets. *Annu. Rev. Pharmacol. Toxicol*. 2012; 52:381–400. [PubMed: 22017684]
18. Sarbassov DD, Guertin DA, Ali SM, Sabatini DM. Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. *Science*. 2005; 307:1098–1101. [PubMed: 15718470]
19. Garcia-Martinez JM, Alessi DR. mTOR complex 2 (mTORC2) controls hydrophobic motif phosphorylation and activation of serum- and glucocorticoid-induced protein kinase 1 (SGK1). *Biochem. J*. 2008; 416:375–385. [PubMed: 18925875]
20. Gulhati P, Bowen KA, Liu J, Stevens PD, Rychahou PG, Chen M, et al. mTORC1 and mTORC2 regulate EMT, motility, and metastasis of colorectal cancer via RhoA and Rac1 signaling pathways. *Cancer Res*. 2011; 71:3246–3256. [PubMed: 21430067]
21. Jacinto E, Loewith R, Schmidt A, Lin S, Ruegg MA, Hall A, et al. Mammalian TOR complex 2 controls the actin cytoskeleton and is rapamycin insensitive. *Nat. Cell Biol*. 2004; 6:1122–1128. [PubMed: 15467718]
22. Liu L, Chen L, Chung J, Huang S. Rapamycin inhibits F-actin reorganization and phosphorylation of focal adhesion proteins. *Oncogene*. 2008; 27:4998–5010. [PubMed: 18504440]
23. Sarbassov DD, Ali SM, Kim DH, Guertin DA, Latek RR, Erdjument-Bromage H, et al. Rictor, a novel binding partner of mTOR, defines a rapamycin-insensitive and raptor-independent pathway that regulates the cytoskeleton. *Curr. Biol*. 2004; 14:1296–1302. [PubMed: 15268862]
24. Huang S, Houghton PJ. Targeting mTOR signaling for cancer therapy. *Curr Opin. Pharmacol*. 2003; 3:371–377. [PubMed: 12901945]
25. Rodrik-Outmezguine VS, Chandarlapaty S, Pagano NC, Poulikakos PI, Scaltriti M, Moskatel E, et al. mTOR kinase inhibition causes feedback-dependent biphasic regulation of AKT signaling. *Cancer Discov*. 2011; 1:248–259. [PubMed: 22140653]
26. Huang Z, Wu Y, Zhou X, Qian J, Zhu W, Shu Y, et al. Clinical efficacy of mTOR inhibitors in solid tumors: a systematic review. *Future Oncol*. 2015; 11:1687–1699. [PubMed: 26043220]
27. Inoki K, Zhu T, Guan KL. TSC2 mediates cellular energy response to control cell growth and survival. *Cell*. 2003; 115:577–590. [PubMed: 14651849]
28. Woods A, Johnstone SR, Dickerson K, Leiper FC, Fryer LG, Neumann D, et al. LKB1 is the upstream kinase in the AMP-activated protein kinase cascade. *Curr. Biol*. 2003; 13:2004–2008. [PubMed: 14614828]
29. Hardie DG. AMPK--sensing energy while talking to other signaling pathways. *Cell Metab*. 2014; 20:939–952. [PubMed: 25448702]
30. Tee AR, Manning BD, Roux PP, Cantley LC, Blenis J. Tuberous sclerosis complex gene products, Tuberlin and Hamartin, control mTOR signaling by acting as a GTPase-activating protein complex toward Rheb. *Curr. Biol*. 2003; 13:1259–1268. [PubMed: 12906785]
31. Garami A, Zwartkruis FJ, Nobukuni T, Joaquin M, Rocco M, Stocker H, et al. Insulin activation of Rheb, a mediator of mTOR/S6K/4E-BP signaling, is inhibited by TSC1 and 2. *Mol. Cell*. 2003; 11:1457–1466. [PubMed: 12820960]
32. Inoki K, Li Y, Xu T, Guan KL. Rheb GTPase is a direct target of TSC2 GAP activity and regulates mTOR signaling. *Genes Dev*. 2003; 17:1829–1834. [PubMed: 12869586]
33. Zhang Y, Gao X, Saucedo LJ, Ru B, Edgar BA, Pan D. Rheb is a direct target of the tuberous sclerosis tumour suppressor proteins. *Nat. Cell Biol*. 2003; 5:578–581. [PubMed: 12771962]

34. Gwinn DM, Shackelford DB, Egan DF, Mihaylova MM, Mery A, Vasquez DS, et al. AMPK phosphorylation of raptor mediates a metabolic checkpoint. *Mol. Cell.* 2008; 30:214–226. [PubMed: 18439900]
35. Urano J, Sato T, Matsuo T, Otsubo Y, Yamamoto M, Tamanoi F. Point mutations in TOR confer Rheb-independent growth in fission yeast and nutrient-independent mammalian TOR signaling in mammalian cells. *Proc. Natl. Acad. Sci. U. S. A.* 2007; 104:3514–3519. [PubMed: 17360675]
36. Yin W, Cheepala S, Roberts JN, Syson-Chan K, DiGiovanni J, Clifford JL. Active Stat3 is required for survival of human squamous cell carcinoma cells in serum-free conditions. *Mol. Cancer.* 2006; 5:15. [PubMed: 16603078]
37. Kim YD, Park KG, Lee YS, Park YY, Kim DK, Nedumaran B, et al. Metformin inhibits hepatic gluconeogenesis through AMP-activated protein kinase-dependent regulation of the orphan nuclear receptor SHP. *Diabetes.* 2008; 57:306–314. [PubMed: 17909097]
38. Liu L, Li F, Cardelli JA, Martin KA, Blenis J, Huang S. Rapamycin inhibits cell motility by suppression of mTOR-mediated S6K1 and 4E-BP1 pathways. *Oncogene.* 2006; 25:7029–7040. [PubMed: 16715128]
39. Wan X, Shen N, Mendoza A, Khanna C, Helman LJ. CCI-779 inhibits rhabdomyosarcoma xenograft growth by an antiangiogenic mechanism linked to the targeting of mTOR/Hif-1 α /VEGF signaling. *Neoplasia.* 2006; 8:394–401. [PubMed: 16790088]
40. Hashemolhosseini S, Nagamine Y, Morley SJ, Desrivieres S, Mercep L, Ferrari S. Rapamycin inhibition of the G1 to S transition is mediated by effects on cyclin D1 mRNA and protein stability. *J. Biol. Chem.* 1998; 273:14424–14429. [PubMed: 9603954]
41. Gao N, Flynn DC, Zhang Z, Zhong XS, Walker V, Liu KJ, et al. G1 cell cycle progression and the expression of G1 cyclins are regulated by PI3K/AKT/mTOR/p70S6K1 signaling in human ovarian cancer cells. *Am. J. Physiol. Cell Physiol.* 2004; 287:C281–C291. [PubMed: 15028555]
42. Tirado OM, Mateo-Lozano S, Notario V. Rapamycin induces apoptosis of JN-DSRCT-1 cells by increasing the Bax : Bcl-xL ratio through concurrent mechanisms dependent and independent of its mTOR inhibitory activity. *Oncogene.* 2005; 24:3348–3357. [PubMed: 15782132]
43. Stromberg T, Dimberg A, Hammarberg A, Carlson K, Osterborg A, Nilsson K, et al. Rapamycin sensitizes multiple myeloma cells to apoptosis induced by dexamethasone. *Blood.* 2004; 103:3138–3147. [PubMed: 15070696]
44. Feng Z, Fan X, Jiao Y, Ban K. Mammalian target of rapamycin regulates expression of beta-catenin in hepatocellular carcinoma. *Hum. Pathol.* 2011; 42:659–668. [PubMed: 21239045]
45. Luo Y, Liu L, Rogers D, Su W, Odaka Y, Zhou H, et al. Rapamycin inhibits lymphatic endothelial cell tube formation by downregulating vascular endothelial growth factor receptor 3 protein expression. *Neoplasia.* 2012; 14:228–237. [PubMed: 22496622]
46. Huang S, Shu L, Easton J, Harwood FC, Germain GS, Ichijo H, et al. Inhibition of mammalian target of rapamycin activates apoptosis signal-regulating kinase 1 signaling by suppressing protein phosphatase 5 activity. *J. Biol. Chem.* 2004; 279:36490–36496. [PubMed: 15218033]
47. Schneider A, Younis RH, Gutkind JS. Hypoxia-induced energy stress inhibits the mTOR pathway by activating an AMPK/REDD1 signaling axis in head and neck squamous cell carcinoma. *Neoplasia.* 2008; 10:1295–1302. [PubMed: 18953439]
48. Roy A, Volgin DV, Baby SM, Mokashi A, Kubin L, Lahiri S. Activation of HIF-1 α mRNA by hypoxia and iron chelator in isolated rat carotid body. *Neurosci. Lett.* 2004; 363:229–232. [PubMed: 15182949]
49. Li Y, Wang X, Yue P, Tao H, Ramalingam SS, Owonikoko TK, et al. Protein phosphatase 2A and DNA-dependent protein kinase are involved in mediating rapamycin-induced Akt phosphorylation. *J. Biol. Chem.* 2013; 288:13215–13224. [PubMed: 23536185]
50. Sarbassov DD, Ali SM, Sengupta S, Sheen JH, Hsu PP, Bagley AF, et al. Prolonged rapamycin treatment inhibits mTORC2 assembly and Akt/PKB. *Mol. Cell.* 2006; 22:159–168. [PubMed: 16603397]
51. Shi Y, Yan H, Frost P, Gera J, Lichtenstein A. Mammalian target of rapamycin inhibitors activate the AKT kinase in multiple myeloma cells by up-regulating the insulin-like growth factor receptor/insulin receptor substrate-1/phosphatidylinositol 3-kinase cascade. *Mol. Cancer. Ther.* 2005; 4:1533–1540. [PubMed: 16227402]

52. Sun SY, Rosenberg LM, Wang X, Zhou Z, Yue P, Fu H, et al. Activation of Akt and eIF4E survival pathways by rapamycin-mediated mammalian target of rapamycin inhibition. *Cancer Res.* 2005; 65:7052–7058. [PubMed: 16103051]
53. O'Reilly KE, Rojo F, She QB, Solit D, Mills GB, Smith D, et al. mTOR inhibition induces upstream receptor tyrosine kinase signaling and activates Akt. *Cancer Res.* 2006; 66:1500–1508. [PubMed: 16452206]
54. Hsu PP, Kang SA, Rameseder J, Zhang Y, Ottina KA, Lim D, et al. The mTOR-regulated phosphoproteome reveals a mechanism of mTORC1-mediated inhibition of growth factor signaling. *Science.* 2011; 332:1317–1322. [PubMed: 21659604]
55. Yu Y, Yoon SO, Poulogiannis G, Yang Q, Ma XM, Villen J, et al. Phosphoproteomic analysis identifies Grb10 as an mTORC1 substrate that negatively regulates insulin signaling. *Science.* 2011; 332:1322–1326. [PubMed: 21659605]

Fig. 1.

CPX inhibits mTORC1 signaling in tumor cells. (A–I) Rh30 cells were treated with indicated concentrations of CPX for 24 h, or with CPX (20 μ M) for indicated time. (B–I) RD, MDA-MB-231, PC-3 and (C–I) PCI-13, MDA-MB-435, SRB1-M7 cells were treated with indicated concentrations of CPX for 24 h. Rh30 cells were treated with 0–100 ng/ml of rapamycin for 24 h. (D–I) Serum-starved Rh30 cells were treated with CPX (0–20 μ M) for 24 h, followed by stimulation with 10 ng/ml of IGF-I for 1 h. Whole cell lysates were subjected to Western blot analysis using indicated antibodies (A–D (I)). (A–II, B–II, C–II,

D–II) Blots for indicated protein expressions were semi-quantified using NIH ImageJ software. Results are presented as mean $\pm$ SD (n = 3). ^a $p < 0.001$, difference versus untreated control.

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Fig.2.

The effect of CPX on Akt phosphorylation is cell line dependent. (A-I, B-I) Serum-starved Rh30 and MDA-MB-231 cells were treated with CPX (0–20 μM) for 24 h, followed by stimulation with 10 ng/ml of IGF-I for 1 h. Whole cell lysates were subjected to Western blot analysis using indicated antibodies. (A-II, B-II) Blots for indicated protein expressions were semi-quantified using NIH ImageJ software. Results are presented as mean ± SD (n = 3). ^a*p* < 0.001, difference versus IGF-I-treated group. (C-I, D-I) PCI-13 and A427 cells, grown under normal growth condition, were treated with CPX (0–10 μM) for 24 h. Whole

cell lysates were subjected to Western blot analysis using indicated antibodies. (C–II, D–II) Blots for indicated protein expressions were semi-quantified using NIH ImageJ software. Results are presented as mean $\pm$ SD (n = 3). ^a $p < 0.01$, ^b $p < 0.001$, difference versus untreated control.

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Fig.3.

Expression of constitutively active mTOR confers resistance to CPX inhibition of cell proliferation. (A–I) Rh30, RD and PCS-201-012 cells were seeded in 6-well plates (1×10^5) under standard culture conditions and kept overnight. Cells were treated with 0–20 μ M of CPX for 72 h and cell proliferation was assessed by counting the trypsinized cells. ^a $p < 0.01$, ^b $p < 0.001$, difference versus untreated control. (B–I) RD and Rh30 cells were transfected with the empty vector (pcDNA3) or pcDNA3-AU1-mTOR-E2419K for 16 h by using Lipofectamine LTX with PLUS Reagent. Whole cell lysates were subjected to Western blot analysis using indicated antibodies. (B–II) Blots for indicated protein expressions were

semi-quantified using NIH ImageJ software. Results are presented as mean $\pm$ SD (n = 3). ^a $p < 0.001$, difference versus vector transfected control. (C–I) RD cells, transfected with the empty vector or pcDNA3-AU1-mTOR-E2419K, were treated with indicated concentrations of CPX for 48 h. (C–II) Rh30 cells, transfected with the empty vector or pcDNA3-AU1-mTOR-E2419K, were treated with or without 2.5 μ M of CPX for 0, 24, 48, 72 and 96 h. Cell proliferation was determined by one solution assay. Results are presented as mean $\pm$ SD (n = 3). ^a $p < 0.01$, ^b $p < 0.001$, difference versus 0 μ M CPX treatment group. ^c $p < 0.05$, ^d $p < 0.01$, ^e $p < 0.001$, difference versus vector transfected group.

Fig.4.

CPX inhibits RD tumor growth in nude mice. (A, B) Nude mice bearing RD xenografts were treated daily by i.p. injection of CPX (20, 60 mg/kg) or vehicle. Tumor volume (A) was measured at indicated time. Results are presented as mean $\pm$ SD (n = 8). ^a $p < 0.05$, ^b $p < 0.01$, ^c $p < 0.001$, difference versus vehicle control group. (B) At the end of the experiment, mice were sacrificed, and the tumors were weighed. Tumor weights are presented as mean $\pm$ SD (n = 8). $p < 0.01$ and $p < 0.001$, difference versus vehicle control group. (C) Representative images of H&E, Ki-67 and TUNEL stainings for RD xenografts treated with the vehicle or

CPX (20 and 60 mg/kg). (D) Quantitative data (mean $\pm$ SD, n=3) show Ki-67 staining and TUNEL staining. Results are presented as mean $\pm$ SD (n = 8). ^c $p < 0.001$, difference versus vehicle-treated control group. (E) The inhibitory effect of CPX on mTORC1 in tumor tissues was determined by Western blot analysis. β -actin was used as a loading control. (F) Quantitative data (mean $\pm$ SD, n=4) of relative protein level of p-S6, p-4E-BP1 (T37/46) and 4E-BP1 (α) in (E). ^a $p < 0.05$, ^b $p < 0.01$, difference versus vehicle-treated control group.

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Fig.5.

CPX inhibits mTORC1 signaling by activation of AMPK pathway. (A–I) CPX does not suppress mTORC1 by inhibiting IGFIR/PI3K or by activating PTEN. Serum-starved Rh30 and MDA-MB231 cells were treated with CPX (0–10 μ M) for 24 h, and then stimulated with 10 ng/ml of IGF-I for 1 h, followed by Western blotting with indicated antibodies. (A–II) Blots for indicated protein expressions were semi-quantified using NIH ImageJ software. Results are presented as mean $\pm$ SD (n = 3). (B–I) Serum-starved Rh30 cells were pretreated without or with 10 μ M of compound C for 30 min, and then exposed to indicated

concentrations of CPX for 24 h, followed by stimulation with IGF-I for 1 h. Whole cell lysates were subjected to Western blot analysis with indicated antibodies. (C–I) MDA-MB231 cells were pretreated without or with 10 μ M of compound C for 30 min, and then exposed to indicated concentrations of CPX for 24 h. Whole cell lysates were subjected to Western blot analysis with indicated antibodies. (B–II, C–II) Blots for indicated protein expressions were semi-quantified using NIH ImageJ software. Results are presented as mean $\pm$ SD (n = 3). ^a p <0.05, ^b p <0.01, ^c p <0.001, difference versus compound C untreated group. (D–I) Rh30 cells, infected with Ad-GFP (as control) and Ad-AMPK α -DN, were treated with indicated concentrations of CPX for 24 h. Whole cell lysates were subjected to SDS-PAGE followed by Western blot analysis using indicated antibodies. β -tubulin served as a loading control. (D–II) Blots for indicated protein expressions were semi-quantified using ImageJ software. Results are presented as mean $\pm$ SD (n = 3). ^a p <0.05, ^b p <0.01, ^c p <0.001, difference versus Ad-GFP-transfected group. (E) Cells were treated with indicated concentrations of CPX for 8 h. Intracellular ATP levels were measured using an ATP Determination Kit (Invitrogen) according to the manufacturer's instruction. ^a p <0.05, ^b p <0.01, difference versus control group.

Fig.6.

CPX inhibits mTORC1 signaling via activation of AMPK-TSC and AMPK-raptor pathways. (A-I) Serum-starved Rh30 cells, infected with lentiviral shRNA to GFP (as control) or TSC2, were exposed to indicated concentrations of CPX for 16 h, and then stimulated with IGF-I for 1 h. Whole cell lysates were subjected to SDS-PAGE followed by Western blot analysis using indicated antibodies. β-tubulin served as a loading control. (A-II) Blots for indicated protein expressions were semi-quantified using NIH ImageJ software. Results are presented as mean ± SD (n = 3). ^a*p* < 0.05, ^b*p* < 0.01, difference versus sh-GFP-transfected group. (B-I) Rh30 and RD cells were exposed to indicated concentrations of CPX for 16 h, followed by Western blotting with indicated antibodies. (B-II) Blots for p-Raptor (S792) expression were semi-quantified using NIH ImageJ software. Results are presented as mean ± SD (n = 3). ^a*p* < 0.05, ^b*p* < 0.01, difference versus untreated control.