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A Novel Semisynthetic Inhibitor of the FRB Domain of Mammalian Target of Rapamycin Blocks Proliferation and Triggers Apoptosis in Chemoresistant Prostate Cancer Cells

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ABBREVIATIONS: FRB, FKBP12-rapamycin-binding domain; mTOR, mammalian target of rapamycin; C-KβBA, cinnamoyl-11-keto-β-boswellic acid; PTEN, phosphatase and tensin homolog deleted on chromosome ten; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labelling; S6K1, p70 ribosomal S6 kinase; elF4E, eukaryotic translation initiation factor 4E; 4E-BP, 4E-binding protein; mTORC1, mTOR complex 1; mTORC2, mTOR complex 2; ERK, extracellular signal regulated kinase; KβBA, 11-keto-β-boswellic acid; LKB1, liver kinase B1; AMPK, AMP-activated protein kinase; TSC, tuberous sclerosis complex; PI3K, phosphatidylinositol 3-kinase; AKβBA, acetyl-11-keto-β-boswellic acid; NF-κB, nuclear factor κB; FITC, fluorescein isothiocyanate; FOXO1, forkhead box protein O1; PDK1, 3-phosphoinositide-dependent protein kinase 1;

ABSTRACT

The mammalian target of rapamycin (mTOR) is a key regulator of cell growth and its uncontrolled activation is a hallmark of cancer. Moreover, mTOR activation has been implicated in the resistance of cancer cells to many anticancer drugs rendering this pathway a promising pharmacotherapeutic target. Here we explored the capability of a semisynthetic compound to intercept mTOR signaling. We synthesized and chemically characterized a novel, semisynthetic triterpenoid derivative, 3-cinnamoyl-11-keto-β-boswellic acid (C-KβBA). Its pharmacodynamic effects on mTOR and several other signaling pathways were assessed in a number of prostate and breast cancer cell lines as well as in normal prostate epithelial cells. C-KβBA exhibits specific antiproliferative and proapoptotic effects in cancer cell lines *in vitro* as well as in PC-3 prostate cancer xenografts *in vivo*. Mechanistically, the compound significantly inhibits the cap-dependent transition machinery, decreases expression of eIF4E, cyclin D1, and induces G_1 cell cycle arrest. In contrast to conventional mTOR inhibitors, C-KβBA downregulates the phosphorylation of S6K1, the major downstream target of mTORC1, without concomitant activation of mTORC2/Akt and ERK pathways, and independently of protein phosphatase 2A, LKB1/AMPK/TSC, and F12-protein binding. At the molecular level, the compound binds to the FRB domain of mTOR with high affinity thereby competing with the endogenous mTOR activator phosphatidic acid. C-KβBA represents a new type of proapoptotic mTOR inhibitor that due to its special mechanistic profile might overcome the therapeutic drawbacks of conventional mTOR inhibitors.

Introduction

The mammalian target of rapamycin (mTOR) pathway is a central regulator of ribosome biogenesis and protein synthesis. Increasing evidence suggests that its exacerbated activation is associated with diseases involving deregulated growth, such as cancer, but also with metabolic diseases, such as type 2 diabetes and aging (Zoncu et al., 2011). Epidemiological data indicate that deregulation of the PI3K/Akt/mTOR pathway, either via PTEN mutations or through Ras-mediated activation of PI3K, are, together with p53, among the most prevalent alterations in human cancer (Antonarakis et al., 2010; Hay and Sonenberg, 2004). In addition, the mTOR signaling pathway has been implicated in the resistance of cancer cells to many anticancer drugs such as vincristine, doxorubicin, retinoic acid, cisplatin, trastuzumab, and others (Hay and Sonenberg, 2004; Jiang and Liu, 2008). Indeed, activated mTOR and elevated levels of its downstream targets, p70 ribosomal S6 kinase 1 (S6K1), eukaryotic translation initiation factor 4E (eIF4E) and its phosphorylated inhibitory binding protein (4E-BP), have been detected in high grade human malignancies including prostate cancer (Antonarakis et al., 2010; Zoncu et al., 2011). Moreover, activation of the mTOR pathway correlates with poor prognosis and reduced patient survival (Brown et al., 2008; Graff et al., 2009; Hollander et al., 2011). Therefore, mTOR has been considered as a promising target for the treatment of human malignancies (Hay and Sonenberg, 2004) including chemoresistant, androgen-independent prostate cancer (Antonarakis et al., 2010). To date, however, in clinical trials, the mTOR inhibitor rapamycin showed only limited success as an anticancer drug (Zoncu et al., 2011). Activation of prosurvival and proliferative signals through Akt and ERK cascades is held responsible for the cytostatic rather than cytotoxic effects of rapamycin and its analogues in the majority of human cancers (Efeyan and Sabatini, 2010). The disappointing therapeutic efficacy motivated the search for new mTOR inhibitors with different mechanisms of action (Zoncu et al., 2011).

mTOR is a highly conserved Ser/Thr kinase belonging to the PI3K pathway activated by growth factors, nutrients, and stress. It serves as a catalytic subunit of two structurally and functionally distinct complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) containing, besides mTOR, RAPTOR and RICTOR, respectively. The two complexes signal via different effector pathways, thereby allowing control of distinct cellular processes. mTORC1 controls the translation machinery and regulates growth and metabolism via phosphorylation and activation of S6K1 and the inhibitory phosphorylation of the repressor of mRNA translation, 4E-BP1 which, in turn, releases and activates eIF4E. eIF4E

binds to the cap structure at the 5'-end of mRNAs, promotes recruitment of ribosome, and the initiation of translation (Hay and Sonenberg, 2004; Zoncu et al., 2011). Important functions of mTORC2 are actin organization and cell polarization. mTORC2 phosphorylates and activates the pro-survival kinase Akt as well as a number of other kinases supporting cell survival, cell-cycle progression, and anabolism (Hay and Sonenberg, 2004; Zoncu et al., 2011).

Growth factors and increased ATP concentration positively regulate the mTORC1 activity (Hay and Sonenberg, 2004; Zoncu et al., 2011). Also small ligands, such as amino acids and phosphatidic acid elicit mTOR activation. Increased activity of phospholipase leading to enhanced production of phosphatidic acid in breast cancer cells is held responsible for the resistance of these cells to rapamycin (Chen et al., 2003).

Recently, we have identified distinct tetracyclic triterpenoids as potent Akt inhibitors (Estrada et al., 2010). Furthermore, the pentacyclic triterpenoid acetyl-11-keto-ß-boswellic acid (AKβBA) was found to induce apoptosis *in vitro* and *in vivo* in human prostate cancer (Syrovets et al., 2005b), colorectal cancer (Yadav et al., 2012), and in human pancreatic tumor xenografts (Park et al., 2011). AKβBA inhibits IκB kinase activity and subsequently nuclear factor-κB (NF-κB) both *in vitro* and in murine models *in vivo* (Cuaz-Perolin et al., 2008; Syrovets et al., 2005a; Wang et al., 2009). Cinnamic acid is a plant-derived organic acid frequently used in flavors because of its special scent; natural derivatives of cinnamic acid may also exhibit antitumor properties (De et al., 2011). Here we introduce a novel esterified KβBA derivative, cinnamoyl-KβBA (C-KβBA), which inhibits mTOR and exhibits antiproliferative and proapoptotic activity on prostate cancer cells *in vitro* and *in vivo* in the chick embryo chorioallantoic membrane model.

Materials and Methods

Reagents: Calyculin A was from Santa Cruz Biotechnology (Santa Cruz, CA), protease inhibitor cocktail III, Akt inhibitor VIII (1,3-dihydro-1-(1-((4-(6-phenyl-1H-imidazo[4,5-g]quinoxalin-7-yl)phenyl)methyl)-4-piperidinyl)-2H-benzimidazol-2-one, Akti-1/2), compound C (6-[4-(2-piperidin-1-yl-ethoxy)-phenyl)]-3-pyridin-4-yl-pyrrazolo[1,5-a]-pyrimidine, dorsomorphin, BMP inhibitor I) were from Calbiochem (San Diego, CA). CCI-779 (temsirolimus) was from LC Laboratories (Woburn, MA). Annexin V-FITC and the TUNEL Kit were from Roche Diagnostics (Mannheim, Germany). Cinnamic acid, rapamycin, proteinase K, RNase A, propidium iodide, phosphatidic acid were purchased from Sigma (St. Louis, MO). Protein phosphatase peptide substrate H-Arg-Arg-Ala-pThr-Val-Ala-OH (RRApTVA) was from Enzo Life Sciences (Farmingdale, NY). Antibodies against the following proteins were used: actin (Chemicon, Billerica, MA), eIF4E, S6K1, p-eIF4E^{Ser209}, topoisomerase I, cyclin D1 (all from Santa Cruz), Akt1, ERK1/2, IκB-α, p-IκBα, p-ERK1/2, p-p38, p-PDK^{Ser241} (Cell Signaling Technology, Danvers, MA), NF-κB p65, p-STAT3, and p-S6K1 (Epitomics, Burlingame, CA), Ki-67 (DAKO, Glostrup, Denmark), p-Akt^{Ser473} (Upstate, Billerica, MA), α-tubulin (Oncogene Research Products, La Jolla, CA). *L*-α-phosphatidic acid sodium salt from egg yolk, *L*-α-phosphatidylcholine type XVI-E and LPS (*Escherichia coli* serotype 055:B5) were from Sigma. Human mTOR, (also known as mechanistic target of rapamycin, mammalian target of rapamycin, FRAP, RAPT1, EC=2.7.11.1) a.a. 1362-end, expressed in a baculovirus-infected Sf9 cell expression system was from Biomol (Hamburg, Germany). HPLC solvents and reagents were obtained from Merck (Darmstadt, Germany). African frankincense, i.e. oleogum resin from *Boswellia carterii*, was from Caesar & Lorentz GmbH (Hilden, Germany) (batch number 72598287).

Synthesis of C-KβBA from KβBA. AKβBA was isolated from the oleogum resin of African frankincense (*Boswellia carterii*) and purified to chemical homogeneity (>99.5% purity) by reversed phase high performance liquid chromatography (Belsner et al., 2003; Büchele and Simmet, 2003). For the chemical characterization of the starting compound AKβBA, see Supplemental Material. KβBA was generated from AKβBA by deacetylation. Derivatization of KβBA by Steglich esterification (Neises and Steglich, 1978) at position 3 of the ring A with cinnamic acid yielded C-KβBA, which was further purified by reversed-phase high performance liquid chromatography (HPLC) to chemical homogeneity (Fig. 1A); its

structure was confirmed by mass spectrometry and one and two dimensional nuclear magnetic resonance spectroscopy (Supplemental Fig. 1 and 2) and compared to that of the parent compound, KβBA (Supplemental Fig. 3). The purity of the synthesized C-KβBA was more than 99.9% as analyzed by thin layer chromatography, mass spectrometry, and high performance liquid chromatography (Supplemental Fig. 4). UV λ_{max} = 266 nm (methanol); CI-MS: *m/z* = 601 M^+-H (calculated for M^+-H Peak $C_{39}H_{53}O_5$: 601.38). C-KβBA was stable in human plasma for at least 4 h (Supplemental Fig. 5).

Cell lines. PC-3, LNCaP, DU 145 prostate cancer cells, RWPE-1 normal prostate cells, MDA-MB-231 breast cancer cells and THP-1 monocytic leukemia cells were all from ATCC (Manassas, VA). The PC-3 cell line overexpressing PTEN (PC-3-PTEN) (Zhao et al., 2004) was a generous gift from Dr. Derek LeRoith (Mount Sinai School of Medicine, New York). The $TSC2^{-/-}$ and $TSC2^{+/+}$ mouse embryonic fibroblast cell lines (MEFs) (Zhang et al., 2007) were generous gifts from Dr. David J. Kwiatkowski (Brigham and Women's Hospital, Harvard Medical School, Boston).

Cytotoxicity and apoptosis parameters. Cytotoxicity was quantified by the mitochondrial reduction of XTT (Roche Diagnostics) and by flow cytometric analyses of the phosphatidylserine expression on the outer membrane leaflet by binding of FITC-labeled annexin V and of the subG_0/G_1 peak of PI-stained cells. DNA fragmentation was analyzed by agarose gel electrophoresis (Estrada et al., 2010; Lunov et al., 2010). For clonogenic cell survival, PC-3 (300 cells/cm^2) were treated with the compounds for 24 h followed by 6 days in medium, and assayed spectrophotometrically after crystal violet staining. Caspase-3 activation was analyzed in cell lysates with a fluorogenic caspase substrate Z-DEVD–R110 (Molecular Probes, Eugene, OR).

Molecular pharmacological analyses. For Western blotting analysis, cells were starved in serum-free medium overnight, were treated with compounds as indicated, and stimulated with 1 % FCS for the indicated time. Nuclear extracts were isolated and Western immunoblotting was performed as described (Syrovets et al., 2005b). Activation of FOXO1 transcription factor was analyzed by TransAM™ (Active Motif, Carlsbad, CA). For the analysis of Ser/Thr protein phosphatase activity, PC-3 cells were pretreated with calyculin A (100 nM) for 20 min then treated with C-KβBA for 30 min, followed by stimulation with 1% FCS for 1 h. Clarified cell lysates were assayed spectrophotometrically using the phosphopeptide substrate RRApTVA.

mTOR kinase assay was performed using recombinant S6K1-GST and either the mTOR standard supplied in the K-LISA mTOR activity kit (Calbiochem) or mTOR precipitated with anti-mTOR/FRAP antibody (Calbiochem) from PC-3 cells (Syrovets et al., 2005b). mTOR was pretreated with KβBA, C-KβBA, rapamycin, or wortmannin for 15 min before addition of the substrate for additional 30 min. The phosphorylated S6K1 was resolved by gel electrophoresis and visualized by phosphorimaging (Syrovets et al., 2005b). Akt1 kinase was assayed as described (Estrada et al., 2010).

The cell cycle was analyzed in cells stained with propidium iodide in the presence of RNase A by flow cytometry using ModFit (BD) (Syrovets et al., 2005b).

The interaction of mTOR with phosphatidic acid was analyzed by photon correlation spectroscopy using a Zetasizer Nano ZS90 (Malvern, Worcestershire, UK) (Hanlon et al., 2010; Röcker et al., 2009). Liposomes containing phosphatidic acid were prepared as described using a Liposo Fast lipid extruder (polycarbonate filters, 0.2 μm) from Avestin (Mannheim, Germany) (Guo et al., 2011). The dissociation constant (K_D) of the mTOR/phosphatidic acid complex was calculated by fitting the experimental data to a mathematical binding model (Röcker et al., 2009).

Molecular modeling. Computational docking and scoring studies of the ligand-protein interactions based on binding free energy (ΔG) were performed using Molegro Virtual Docker 5 (Estrada et al., 2010; Thomsen and Christensen, 2006). The NMR structure of the FKBP12-rapamycin binding (FRB) domain of mTOR has been published (RCSB accession number 2GAQ) (Leone et al., 2006). To confirm the specificity of the analysis, we have also performed molecular docking of C-KβBA with two other proteins, an mTORC1 downstream target, p70S6 kinase 1 (RCSB accession number 3A60) and protein phosphatase 2 (PP2A), RCSB accession number 3DW8.

Tumor xenografts. PC-3 cells ($0.7x10^6$) were seeded in medium/Matrigel ((1:1 (v/v); BD Biosciences, San Jose, CA) onto the chorioallantoic membrane of chicken eggs 6 days after fertilization. Starting 2 days after seeding, the cells were treated topically once daily for 4 days; tissue sections were finally analyzed immunohistochemically with an Axiophot microscope (Carl Zeiss, Göttingen, Germany) (Syrovets et al., 2005b).

Statistical analysis. Multiple group comparisons were conducted using the Newman-Keuls test (StatSoft, Statistica).

Results

C-KβBA is cytotoxic for prostate cancer cell lines. Searching for novel compounds with therapeutic efficacy against chemoresistant, androgen-independent prostate cancer, we have previously identified a series of plant-derived pentacyclic triterpenoids, which inhibit IκB kinase (IKK) that is essential for prosurvival signaling in many cancer cells (Syrovets et al., 2005a; Syrovets et al., 2005b). In addition, we classified several tetracyclic triterpenoids as inhibitors of Akt kinase (Estrada et al., 2010). The presence of an acetyl group at position 3 of the ring A was consistently associated with an increased antiproliferative and antitumor activity of terpenoids. On the other hand, derivatives of cinnamic acid exhibit antitumor activity in various lymphomas, leukemias, and solid tumors (De et al., 2011). Therefore, we synthesized a number of pentacyclic triterpenoids on the basis of the terpenoid KβBA. Derivatization by Steglich esterification (Neises and Steglich, 1978) at position 3 of ring A of the parent compound, KβBA, with cinnamic acid led to C-KβBA (Supplemental Fig. S1-S5).

C-KβBA induces time- and concentration-dependent inhibition of the PC-3 cell proliferation with IC_{50}'s of about 4.2 and 3.6 μM after 24 and 48 h of exposure, respectively (Fig. 1A). By contrast, neither cinnamic acid nor its analog cinnamic acid methyl ester, which features better cell membrane permeability, elicited any significant cytotoxicity. C-KβBA was more potent than the structurally related, triterpenoid AKβBA, which inhibits IKK. C-KβBA exhibited comparable cytotoxic efficacy also on other prostate cancer cell lines such as the androgen-dependent LNCaP and the androgen-independent DU 145 cells (Fig. 1B). Compared to normal prostate epithelial cells, the toxicity of C-KβBA was preferentially directed against prostate cancer cells (Fig. 1C).

C-KβBA triggers apoptosis in PC-3 cells *in vitro*. Treatment of PC-3 cells with C-KβBA triggered a concentration-dependent expression of phosphatidylserine on the outer membrane leaflet, an early sign of apoptosis (Fig. 2A and 2B) and induced a concentration-dependent caspase-3 activation (Fig. 2C). Likewise, treatment of PC-3 cells with C-KβBA caused the typical DNA laddering pattern, a biochemical hallmark of irreversible apoptosis (Fig. 2D).

C-KβBA inhibits neither Akt nor NF-κB signaling. Epidemiological data indicate that sporadic mutation or deregulation of p53, PI3K, Akt, and PTEN are the most prevalent alterations in human cancer (Zoncu et al. 2011). C-KβBA is equally toxic for two prostate cell lines bearing p53 mutations (PC-3 and DU-145) and for LNCaP expressing wt p53 (Carroll et al. 1993) precluding the involvement of p53. PC-3 is a PTEN-null cell line. Loss of PTEN phosphatase, a negative regulator of Akt, results in constitutive activation of Akt, a

phenomenon frequently observed in cancer (Hay and Sonenberg, 2004) and correlated with poor prognosis (Hollander et al., 2011). We found that overexpression of active PTEN in PC-3 cells renders them less sensitive to C-KβBA (Fig. 3A) suggesting that C-KβBA might target Akt or key downstream kinases, such as IKK or mTOR.

Akt phosphorylates FOXO1 leading to their cytoplasmic retention thereby rendering them transcriptionally inactive. Consistently, Akt inhibitor VIII increased significantly FOXO1 activation. However, C-KβBA had no such effect (Fig. 3B). Similarly, C-KβBA did not affect phosphorylation of Akt, neither on Ser473 nor on Thr308, both of which are indispensable for Akt kinase activation. Moreover, C-KβBA did not inhibit phosphorylation of the major Akt substrate, GSK-3β (Fig. 3D). C-KβBA had also no effect on the constitutively active PDK1, a kinase upstream of Akt, and did not influence the activity of ERK1/2 (Fig. 3C,D). Likewise, C-KβBA does not inhibit phosphorylation and activation of the MAPK ERK1/2 and p38, or NF-κB and STAT3 in monocytic leukemia cells (Supplemental Fig. 6); those cells were chosen, because they respond with all the aforementioned signaling pathways to the standard stimulus LPS. These data indicate that, although an intact Akt signaling pathway is indispensable for the anticancer activity of C-KβBA, this compound has no direct inhibitory effect on Akt, PDK1, or ERK1/2. Interestingly, at variance to C-KβBA, CCI-779 and rapamycin caused a marked increase in phosphorylation of GSK-3β, the downstream target of Akt (Fig. 3D). This is believed to be a consequence of a concomitant activation of mTORC2 known to induce Akt activation when mTORC1 is inhibited by rapamycin or rapamycin analogues (Zoncu et al., 2011).

We have previously shown that the acetylated form of KβBA, AKβBA, induces apoptosis by downregulation of the NF-κB signaling pathway (Syrovets et al., 2005b). However, C-KβBA did not affect NF-κB (Fig. 3E). Akin to C-KβBA, the rapamycin analog CCI-779 is a less potent inhibitor of the proliferation of PC-3-PTEN cells exhibiting low Akt activity (Fig. 3F). These data suggest that C-KβBA might target mTOR.

C-KβBA inhibits the mTOR signaling pathway *in vitro* and *in vivo.* Within 30 min, hence far ahead of any detectable cytotoxicity, C-KβBA induced a concentration-dependent downregulation of the phosphorylation of S6K1 kinase in PC-3 cells (Fig. 4A) and enhanced the phosphorylation of eIF4E, though to a lesser extent than CCI-779 or rapamycin (Fig. 4B). The phosphorylation of eIF4E was, however, absent in DU 145 prostate cancer cells treated with C-KβBA (Supplemental Fig. S6). These cells differ from PC-3 by having a low basal Akt/mTOR activity due to PTEN expression. C-KβBA, CCI-779 and rapamycin also inhibited

the expression of eIF4E (Fig. 4B), which might reduce the cap-dependent translation of preferentially pro-tumorigenic genes (Graff et al., 2009), including cell cycle regulators such as cyclin D1 (Zoncu et al., 2011) as shown in Fig. 4B.

The proto-oncogene cyclin D1 is a key regulator of the transition from the G_1 to the S phase (Alao et al., 2006). Consistently, treatment of PC-3 cells with C-KβBA elicited arrest in the G_1 phase of the cell cycle (Fig. 4C,D). Notably, a single treatment with C-KβBA for 24 h inhibited the clonogenic survival of PC-3 cells grown for the next 6 days in medium without the inhibitor (Fig. 4E,F).

Next, C-KβBA concentration-dependently inhibited proliferation of PC-3 prostate cancers grafted on chick chorioallantoic membranes as assessed by immunohistochemical analysis of proliferation marker Ki-67 and impeded phosphorylation of the mTOR substrate S6K1, thereby confirming inhibition of mTOR signaling *in vivo*. Concomitantly, C-KβBA, but not rapamycin, increased the rate of apoptosis in the tumor xenografts (Fig. 4G,H).

C-KβBA inhibits the mTOR signaling pathway independently of LKB1-AMPK, TSC complex, and protein phosphatases. The AMPK-TSC network is a negative upstream regulator of mTORC1. In response to low energy level or high AMP level, AMPK is activated by phosphorylation by Liver Kinase B1 (LKB1) leading to phosphorylation of TSC2 and activation of the TSC complex and inhibition of the mTORC1 signaling (Zoncu et al., 2011). As expected, inhibition of AMPK by compound C enhanced the mTORC1-induced phosphorylation of S6K1. However, inhibition of AMPK did not revert the inhibitory effect of C-KβBA on the mTORC1-induced phosphorylation of S6K1 (Fig. 5A). Moreover, C-KβBA proved to be cytotoxic to MDA-MB-231 cells, which do not express LKB1 (Shen et al., 2002) (Fig. 5B). Thus, C-KβBA inhibits mTOR independently of AMPK and LKB1 signaling.

Ser/Thr protein phosphatases such as PP2A are essential components of mTOR signaling in mammalian cells, where they control the activity of the mTORC1 substrates (Rohde et al., 2001). The activity of the Ser/Thr protein phosphatases in cells treated with C-KβBA remained, however, unaffected when compared to the phosphatase inhibitor calyculin A-treated cells (Fig. 5C).

In addition to Akt and AMPK, the TSC2 subunit can be phosphorylated by ERK1/2, and RSK1, a kinase downstream of ERK. Inhibition of any of these kinases would also result in the inhibition of mTORC1 signaling (Bai and Jiang, 2010). C-KβBA, though, did not inhibit the phosphorylation and activation of Akt, whereas the ERK kinases were not activated in the prostate cancer cells (Fig. 3B-D). However, C-KβBA might target the TSC complex directly,

thereby inhibiting mTOR signaling. Two mouse embryonic fibroblast cell lines, one expressing TSC2, the other one not, were treated with C-KβBA. C-KβBA is equally cytotoxic to either cell line, suggesting independence from the expression of TSC2 (Fig. 5D).

These data suggest that C-KβBA might target mTOR directly. However, the mechanism of C-KβBA seems to differ from that of rapamycin and CCI-779, which are cytostatic rather than cytotoxic (Fig. 5E). Indeed, comparison of KβBA, C-KβBA, CCI-779, and rapamycin revealed that in terms of the subG_0/G_1 peak, C-KβBA is a much better inducer of apoptosis than rapamycin, the rapamycin analog, or the parent compound KβBA (Fig. 5F).

Evidence for competition of C-KβBA with phosphatidic acid binding to the FRB domain of mTOR. The activity of mTOR is modulated by a number of small ligands including phosphatidic acid and rapamycin, both of which bind to the FRB domain of mTOR pointing to a special role of this domain in controling the catalytic activity of mTOR (Veverka et al., 2008; Yoon et al., 2011; Zoncu et al., 2011). *In silico* molecular docking did not reveal any significant binding affinity of C-KβBA to S6K1 or PP2A. In contrast, it predicted high affinity binding of C-KβBA to the FRB domain of mTOR (Fig. 6A and 6B) with low energy interaction indicative for a stable binding system (Thomsen and Christensen, 2006). Recently, binding of phosphatidic acid has been characterized by NMR (Veverka et al., 2008; Yoon et al., 2011). Our molecular modeling approach confirmed the earlier identified binding mode of phosphatidic acid and indicated that C-KβBA could interact with the same amino acid residues of mTOR that are involved in the interaction with phosphatidic acid but with higher affinity. The calculated K_D of C-KβBA binding to the FRB domain in mTOR (Fig. 6C) correlated well with the inhibitory values obtained in cell-based assays (Fig. 1) and was superior to the structurally related KβBA and AKβBA. Consistent with the biochemical data, the calculated binding efficacy of C-KβBA for Akt1, ERK2, IKKα, and IKKβ was negligible (Supplemental Table 1).

Phosphatidic acid is produced by hydrolysis of phosphatidylcholine by phospholipase D. Cells which exhibit high phospholipase D activity and high levels of phosphatidic acid are highly resistant to rapamycin, such as, for example, MDA-MB-231 breast cancer cells (Chen et al., 2003). However, C-KβBA is cytotoxic to MDA-MB-231 cells indicating that it successfully competes with phosphatidic acid for binding to mTOR within the cells (Fig. 5B). Addition of phosphatidic acid to prostate cancer cells increased the mTOR activity and S6K1 phosphorylation as expected. However, C-KβBA effectively inhibited the phosphatidic acid-induced mTOR activation (Fig. 6D). Moreover, in the presence of C-KβBA, the affinity of

mTOR to phosphatidic acid decreased almost 10-fold (Fig. 6E). Detailed analysis of the specific binding showed a parallel shift of the binding curve to the right indicative for a competitive mode of inhibition (Supplemental Fig. S7). These data are in agreement with our molecular modeling showing higher affinity of mTOR for C-KβBA than for phosphatidic acid. Finally, a kinase assay using either commercial active mTOR or mTOR immunoprecipitated from PC-3 cells and devoid of FKB12 demonstrated that C-KβBA indeed inhibited mTOR activity and the S6K1 phosphorylation, whereas rapamycin was inactive in the absence of FKBP12 cofactor protein (Fig. 6F).

Discussion

Due to the crucial role of mTOR signaling for the proliferation of malignant cells and the fact that even full inhibition of mTORC1 and mTORC2 could be well tolerated in adult tissues (Zoncu et al., 2011), mTOR inhibitors have been tested in a number of clinical trials. The immunosuppressive macrolide rapamycin is considered the classical mTOR inhibitor. Since rapamycin and its derivatives CCI-779 (temsirolimus) and RAD-001 (everolimus) exhibited potent antiproliferative activity against various cancer cell lines and favorable toxicity profiles, they have been clinically explored in several trials for the treatment of neoplastic diseases (Antonarakis et al., 2010). Indeed, CCI-779 and RAD-001 are currently approved for the treatment of advanced metastatic renal cell carcinoma. AP23573 (deforolimus) is presently being evaluated in clinical studies against docetaxel-refractory, advanced, castration-resistant prostate cancer. It is, however, expected that classical mTOR inhibitors such as rapamycin and its analogues will have little impact as single agents, and are likely to be more promising in combination therapy with other chemotherapeutics, where they increase cancer cell sensitivity to these drugs (Antonarakis et al., 2010).

To achieve an effective mTOR-based cancer therapy, some limitations of the rapamycin therapy have to be overcome. The most important one is a severe upregulation of MEK-ERK and PI3K/Akt signaling due to the S6K1-mediated feedback loop and TORC2-mediated activation of Akt. These mechanisms upregulate important pro-survival and proliferative signals, which dampen the inhibitory effects of rapamycin in preclinical models and in cancer patients (Efeyan and Sabatini, 2010; Zoncu et al., 2011). Activation of these anti-apoptotic feedback loops might also explain why rapamycin and its analogues emerge as cytostatic rather than cytotoxic drugs (Efeyan and Sabatini, 2010; Zoncu et al., 2011). On this

background, development of catalytic inhibitors of mTOR with mechanisms of action different from that of rapamycin may prove especially useful (Zoncu et al., 2011).

The mechanism of action of C-KβBA identified in this study differs from that of rapamycin and its analogues. Notably, C-KβBA does not induce a feedback activation of Akt observed as hyperphosphorylation of GSK-3β, when cells had been treated with rapamycin or CCI-779. However, similar to rapamycin and CCI-779, C-KβBA induces a modest increase in phosphorylation of eIF4E. The physiological consequences of eIF4E phosphorylation are not well established. Thus, fluorescence spectroscopy and surface plasmon resonance analyses demonstrated that phosphorylation of eIF4E markedly reduces its affinity for capped RNA, primarily due to an increased rate of dissociation (Scheper et al., 2002). In other studies, the oncogenic activity of eIF4E correlates with its phosphorylation on Ser209 (Bianchini et al., 2008; Furic et al., 2010). elF4E phosphorylation may result from residual mTORC2 activity, known to induce phosphorylation of eIF4E via MNK (Wang et al., 2007). Although C-KβBA upregulates the eIF4E phosphorylation, it markedly reduces the eIF4E expression in prostate cancer cells. In this regard, the overall expression levels of eIF4E might be a better indicator of the prostate cancer malignancy. The elevated levels of eIF4E are associated with prostate cancer progression (Kremer et al., 2006), and its reduction in prostate cancer cells elicits apoptosis (Graff et al., 2009). Accordingly, eIF4E is hardly expressed in normal prostate tissues, whereas in prostate cancer, it is highly expressed and phosphorylated (Graff et al., 2009). Cyclin D1 is considered the prime downstream target protein of eIF4E-mediated protein translation, and expression of eIF4E significantly correlates with the level of cyclin D1 protein expression because eIF4E enhances the nuclear export of cyclin D1 mRNA (Hay and Sonenberg, 2004). Cyclin D1, on the other hand, is a key regulator of the G_1 phase of the cell cycle, and drives cells through the G_1/S phase transition (Stacey, 2003). Indeed, we found that C-KβBA reduced the expression of cyclin D1 and arrested cells in the G_1 phase.

To elucidate the molecular mechanisms of C-KβBA, we used different approaches. C-KβBA was cytotoxic to MDA-MB-231, a cell line, which does not express LKB1 (Shen et al., 2002), and for $TSC^{-/-}$ MEFs ruling out an effect of C-KβBA on the TSC2 signaling network. These data implicated that C-KβBA affects mTOR directly. A number of new catalytic inhibitors of mTORC1 and mTORC2 competing with binding of ATP have been synthesized. Such catalytic inhibitors of mTOR are well tolerated in the clinical setting in adults (Zoncu et al., 2011). However, under conditions of prolonged treatment with catalytic mTOR inhibitors, mTOR-independent activation of Akt was observed, which led to activation of some Akt

substrates, such as FOXO1 (Zoncu et al., 2011). In contrast, a single treatment with C-KβBA effectively inhibited the clonogenic growth of prostate cells, in the absence of FOXO1 activation in C-KβBA-treated cells. Hence, the compound shows a greatly improved pharmacodynamic profile.

The lipid second messenger phosphatidic acid along with amino acids is a strong activator of mTOR. It is required for the association of mTOR with Raptor within mTORC1 and that of mTOR with Rictor within mTORC2 (Toschi et al., 2009). It displaces FKBP38 from mTOR and, in addition, allosterically stimulates the catalytic activity of mTORC1 (Veverka et al., 2008; Yoon et al., 2011). C-KβBA, which competes with phosphatidic acid for binding to mTOR, might interfere with this dual activation, which results in inhibition of the mTORC1 catalytic activity. We did not observe inhibition of mTORC2-mediated activation of Akt by C-KβBA. On the other hand, whereas in rapamycin- and CCI-779-treated cells GSK-3β was hyperphosphorylated, C-KβBA-treated cells apparently did not exhibit activated Akt signaling. This might be a result of partial inhibition of the mTORC2 complex by C-KβBA, or of direct inhibitory effects on Akt. The latter is, however, less probable, because the concentrations of C-KβBA used, did not detectably affect Akt. Thus, effects of C-KβBA on mTORC2 cannot be excluded. In fact, this might be an advantage of the compound, because mTORC2, though neglected for some time, appears to be of special importance for tumorigenesis under conditions of PTEN deficiency (Guertin et al., 2009), particularly in metastatic prostate cancer (Antonarakis et al., 2010).

Taken together, the novel semisynthetic terpenoid C-KβBA inhibits cell proliferation and induces apoptosis *in vitro* and *vivo* in the chick embryo chorioallantoic membrane model even at low concentrations and after a single, rather short exposure. These effects of C-KβBA are triggered by its inhibition of the mTOR signaling pathway that occurs independent from the upstream kinases Akt and PDK1, and independent from the negative regulators of mTOR signaling, TSC2 and PP2A. C-KβBA exhibits a preferential efficacy against prostate cancer cells, i.e. the PC-3 cell line harboring defects in the apoptosis pathway, compared to normal prostate epithelial cells.

The capability of C-KβBA to inhibit the mTOR signaling pathway renders it an interesting lead compound for the development of novel pharmacotherapeutic approaches for the treatment of cancer and as a tool for dissecting signaling pathways on the molecular level.

Disclosure of Potential Conflicts of Interest

None.

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Authorship Contributions

Participated in research design: Morad, Syrovets, Simmet

Conducted experiments: Morad, Schmid, El Gafaary, Lunov

Contributed new reagents or analytical tools: Schmid, Lunov, Büchele

Performed data analysis: Morad, Schmid, Siehl, Lunov, Syrovets, Simmet

Wrote or contributed to the writing of the manuscript: Morad, Siehl, Syrovets, Simmet

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Footnotes

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Figure Legends

Fig. 1. Effects of C-KβBA and the parent compounds on prostate cancer cell viability. A, PC-3 cells were treated with the compounds for 24 and 48 h and analyzed by XTT assay. B, The prostate cancer cell lines treated with C-KβBA were analyzed as in (A). C, C-KβBA exhibits selectivity against PC-3 prostate cancer cells compared to normal RWPE-1 prostate epithelial cells. Cells were treated for 48 h and analyzed as in (A). Half maximal inhibitory concentration (IC_{50}) values were calculated using the Hill three-parameter mathematical model. All data are mean ± SEM, $n = 3$, $^{*}P < 0.05$.

Fig. 2. C-KβBA triggers apoptosis in prostate cancer cells *in vitro*. A, C-KβBA induces phosphatidylserine expression on the outer cell membrane. PC-3 cells treated for 24 h were double-stained with annexin V/propidium iodide and analyzed by flow cytometry. B, Representative graphs show expression of phosphatidylserine by PC-3 cells treated for 24 h. C, C-KβBA induces caspase-3 activation as analyzed by the proteolytic cleavage of the fluorogenic substrate Z-DEVD-R110 (24 h). D, C-KβBA (5 μM) induces DNA laddering (96 h). All data are mean ± SEM, $n = 3$, $^{*}P < 0.05$, $^{**}P < 0.01$.

Fig. 3. Downregulation of the PI3K/Akt pathway renders PC-3 cells less sensitive to C-KβBA. A, C-KβBA shows selective antiproliferative efficacy against the PC-3 (PTEN null) cell line. Cells were treated with C-KβBA for 48 h and analyzed by XTT assay. B, C-KβBA does not inhibit the downstream target of Akt, FOXO1. PC-3 cells were treated with C-KβBA, KβBA (3 μM), or Akt inhibitor VIII (1 μM) for 3 h and FOXO1 activity in nuclear extracts was analyzed by Trans^{AM} ELISA. Data are mean ± SEM, $n = 3$, $^{*}P < 0.05$. C, C-KβBA does not inhibit Akt signaling. PC-3 cells treated with C-KβBA, KβBA (3 μM), or the mTOR inhibitors CCI-779 (3 μM) and rapamycin (10 nM) for 30 min and stimulated for 30 min with FCS were analyzed by Western blotting. D, C-KβBA does not affect prolonged Akt activation. PC-3 cells treated as in (C) and stimulated for 8 h with FCS were analyzed by Western blotting. E, C-KβBA does not inhibit NF-κB signaling. Whole cell lysates, cytosolic, and nuclear fractions of PC-3 cells treated as in (C) were analyzed. F, Similarly to C-KβBA, CCI-779 is more cytotoxic to PC-3 (PTEN "null"cells) compared to PC-3-PTEN cells. All data are representative of three independent experiments.

Fig. 4. C-KβBA inhibits mTOR signaling *in vitro* and *in vivo*. A and B, C-KβBA inhibits phosphorylation of S6K1 and induces phosphorylation and degradation of eIF4E. PC-3 cells treated with C-KβBA, KβBA (3 μM), or the mTOR inhibitors CCI-779 (3 μM) and rapamycin (10 nM) for 30 min and stimulated for 30 min (A) or 8 h (B) with FCS were analyzed. C and D, C-KβBA induces G_1 cell-cycle arrest in prostate cancer cells. PC-3 cells treated for 24 h with C-KβBA were analyzed by flow cytometry (C) and quantified using ModFit (D). E and F, C-KβBA inhibits clonogenic survival of PC-3 cells. PC-3 cells (300 cells/cm^2) were treated with as in (A) for 24 h. After recovering for 6 days in medium free of compounds, cell survival was assayed spectrophotometrically. G and H, C-KβBA inhibits proliferation of PC-3 tumor xenografts on the chick chorioallantoic membrane. PC-3 cells were grafted onto the chorioallantoic membrane of fertilized chicken eggs. After 2 days, the tumors were treated with C-KβBA, rapamycin, or solvent (control) for 4 days. The tumor sections were stained against Ki-67 (proliferation marker, dark violet), TUNEL (TdT, apoptosis marker, dark brown), and p-S6K1 (pink) (G). Quantification of the proliferating cells in tumors (H). Where applicable, data are mean ± SEM, $n = 3$, $^{**}P < 0.01$.

Fig. 5. C-KβBA inhibits the mTORC1 signaling pathway independently of the AMPK signaling, TSC network, and phosphatase activation. A, PC-3 cells, treated with C-KβBA (3 μM) or compound C (1 μM), an AMPK inhibitor, alone or in combination for 30 min, followed by stimulation with FCS for 30 min were analyzed by Western blotting. B, Rapamycin-resistant MDA-MB-231 expressing high levels of phosphatidic acid or PC-3 were treated with C-KβBA for 48 h and analyzed by XTT assay. C, C-KβBA does not activate Ser/Thr protein phosphatases. PC-3 cells were pretreated with calyculin A (100 nM), then treated with C-KβBA for 30 min, followed by stimulation with FCS for 1 h. Cleared cell lysates were assayed using phosphopeptide substrate. D, Antiproliferative effect of C-KβBA on MEF cells either expressing TSC2 ($TSC2^{+/+}$) or TSC2-null ($TSC2^{-/-}$) was measured by the XTT assay after exposure for 48 h. E, C-KβBA is cytotoxic to PC-3 cells, which are resistant to rapamycin and the rapamycin analogue CCI-779. Cell viability was measured by XTT assay (24 h). E, C-KβBA is cytotoxic to PC-3 cells, which are resistant to rapamycin and the rapamycin analogue CCI-779.

Fig. 6. C-KβBA competes with phosphatidic acid for binding to mTOR and inhibits mTOR kinase activity. A, Molecular modelling, C-KβBA and phosphatidic acid are shown as green

carbons. Left panel: surface charge distribution of mTOR FRB is shown in red (negative charge) and blue (positive charge). Right panel: mapping of mTOR residues involved in the interactions with C-KβBA and phosphatidic acid. Red sticks – oxygen, grey sticks – carbons, purple sticks – nitrogen. B, Predicted interaction energy and calculated K_D for C-KβBA binding. A low (negative) energy of interaction is an indicator of a stable system and favourable binding interaction. C, Comparison of the predicted interaction energy and the calculated K_D for C-KβBA and phosphatidic acid binding to the mTOR FRB domain. D, C-KβBA inhibits mTOR activity induced by phosphatidic acid. mTOR activity in PC-3 cells treated with C-KβBA or phosphatidic acid alone or in combination for 30 min followed by stimulation with FCS for 30 min was analyzed by Western blotting. E, C-KβBA inhibits binding of phosphatidic acid to mTOR. Increase in the average hydrodynamic radii of mTOR/phosphatidic acid complexes (Δr) was measured by photon correlation spectroscopy. mTOR (1-300 pM) preincubated for 15 min with 10 μM C-KβBA or vehicle, was mixed with phosphatidic acid-containing liposomes for 15 min, and analyzed. F, C-KβBA inhibits the mTORC1 activity in an *in vitro* kinase assay. The mTOR standard (20 μL) or immunoprecipitated mTOR in the absence of FKBP12 were pretreated for 15 min with KβBA (10 μM), C-KβBA, wortmannin (each 10 μM), or rapamycin (10 nM) prior to addition of recombinant S6K1 for 30 min. Phosphorylated substrate was visualized by phosphor imaging. G, Scheme of the mTORC1 signaling pathway.

Figure 1

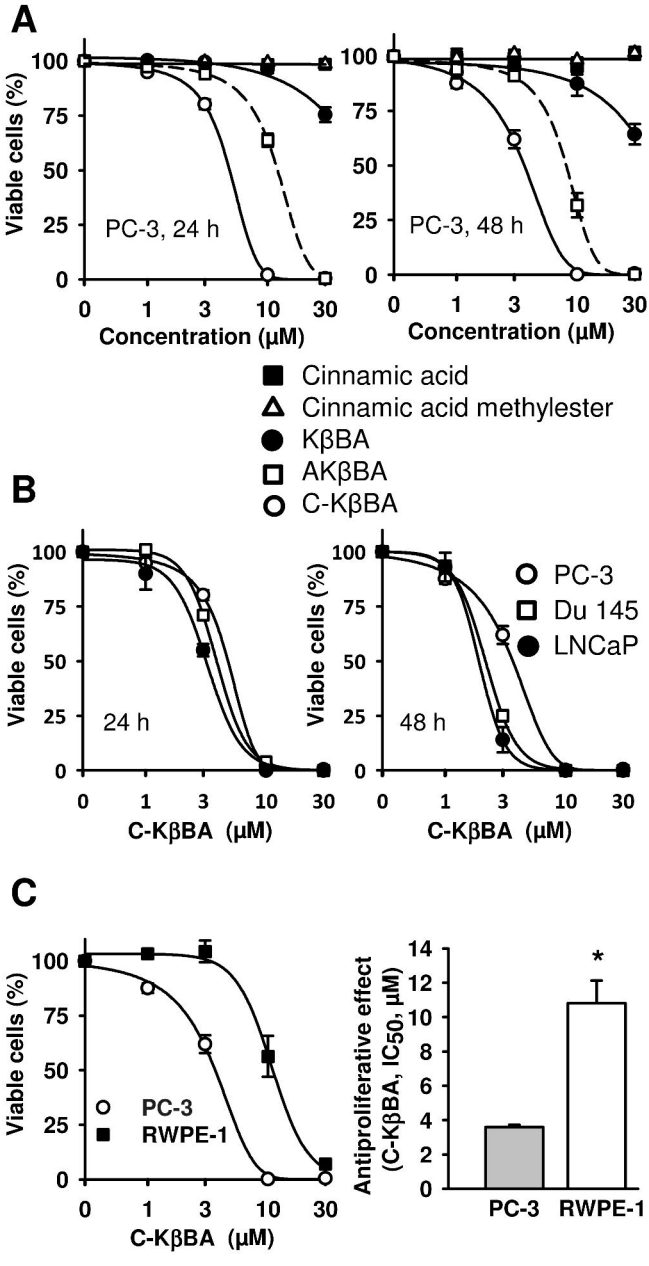
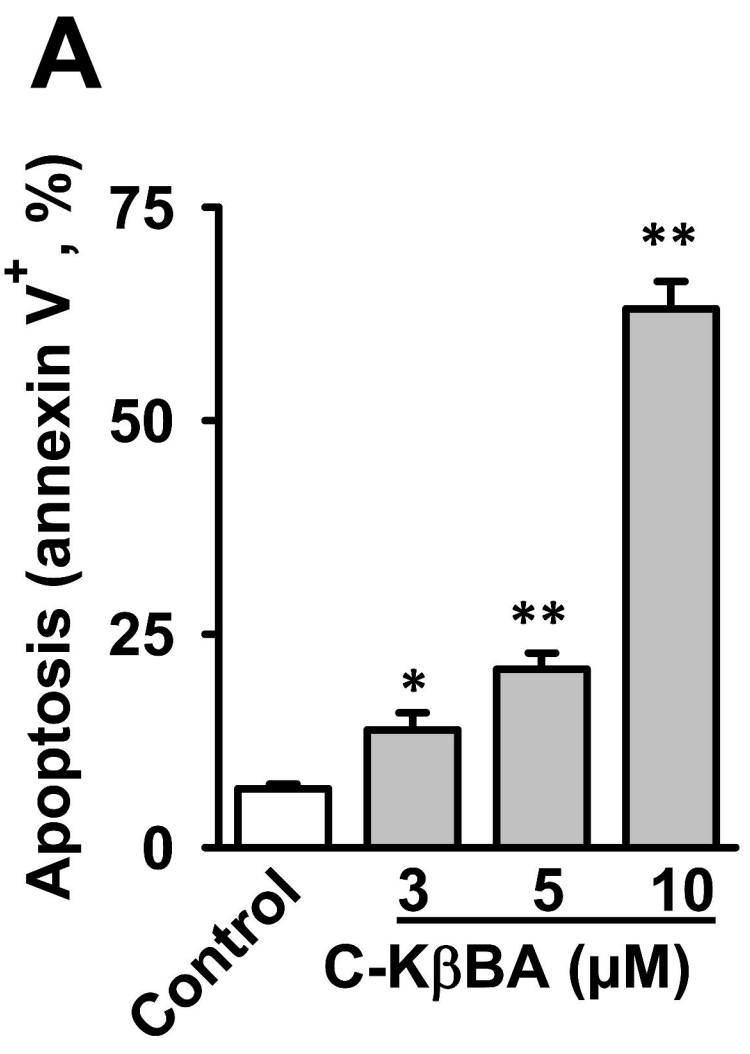
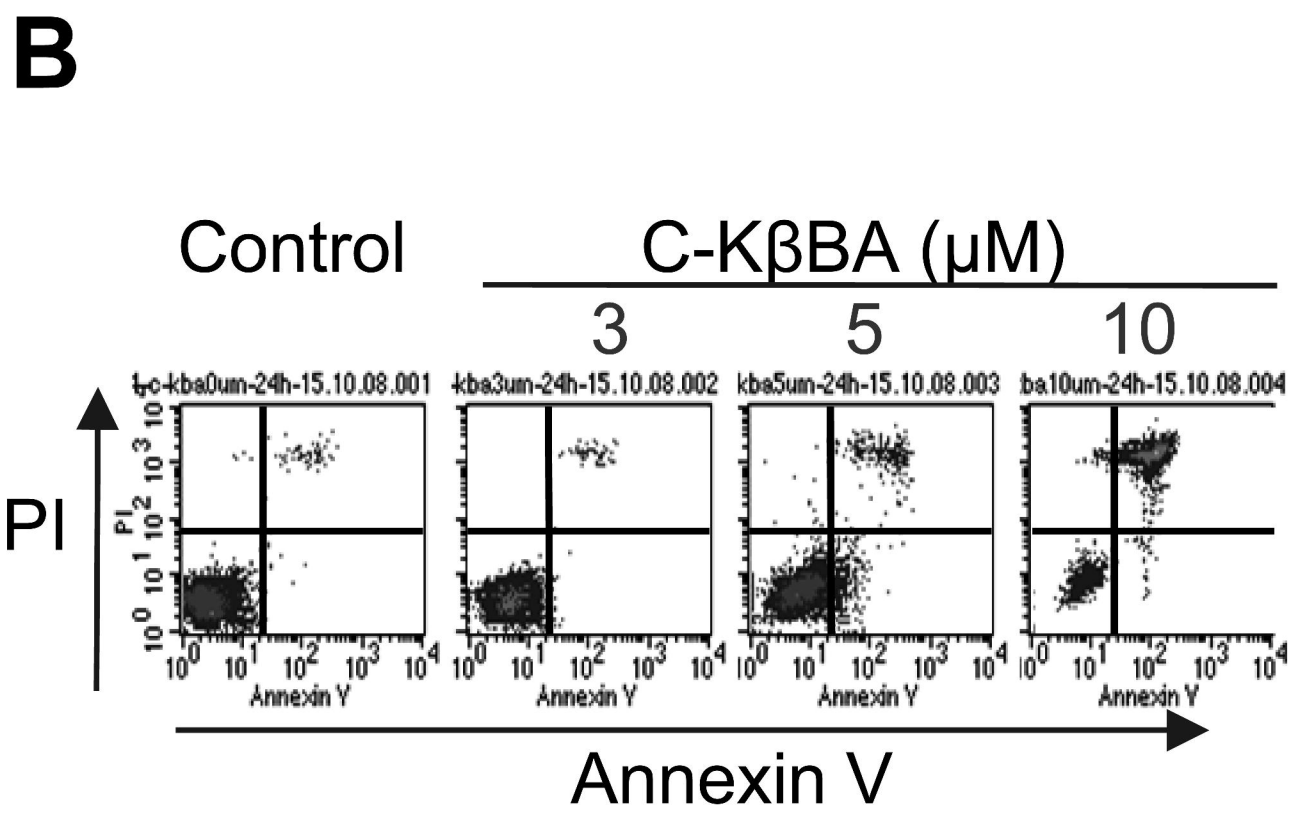
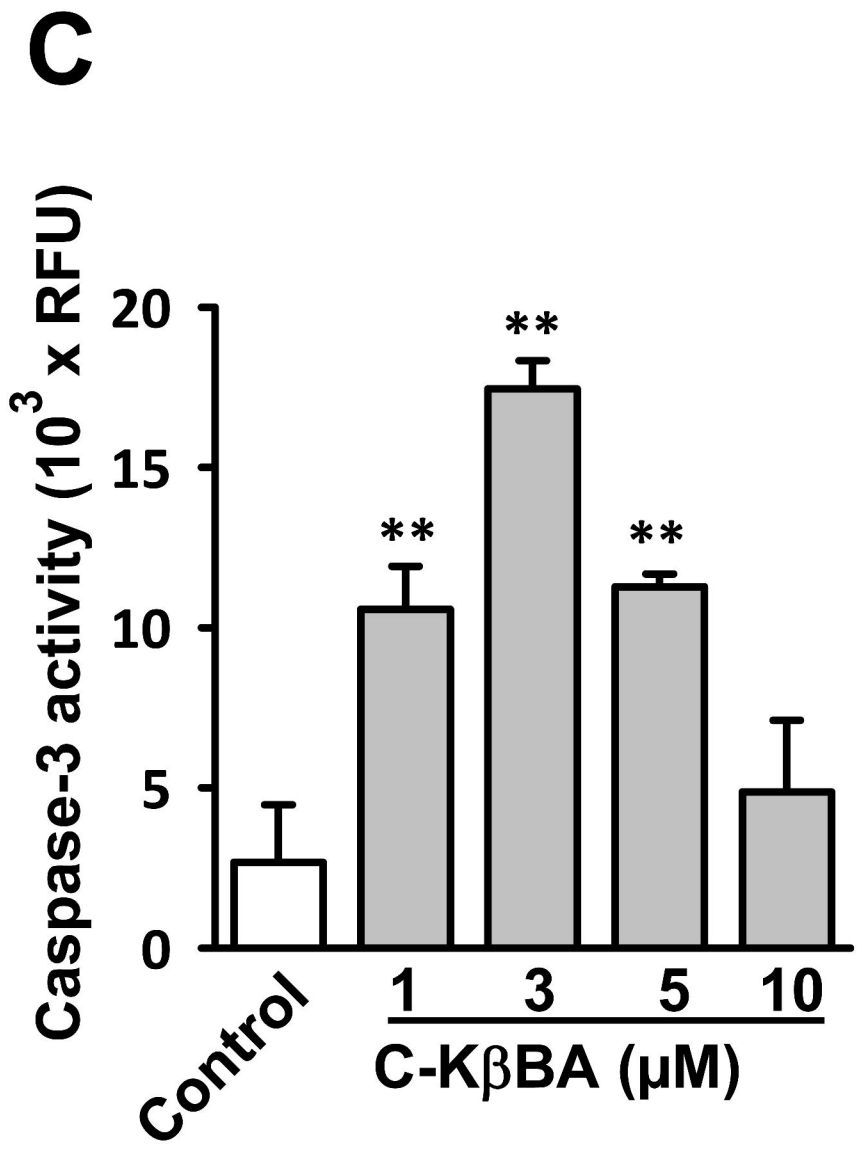

A
Viable cells (%)
PC-3, 24 h
PC-3, 48 h
Concentration (μM)
Cinnamic acid
Cinnamic acid methylester
KβBA
AKβBA
C-KβBA
B
24 h
48 h
C-KβBA (μM)
PC-3
Du 145
LNCaP
C
PC-3
RWPE-1
Antiproliferative effect
(C-KβBA, IC50, μM)
*

Figure 2


A
Apoptosis (annexin V+, %)
75
50
25
0
*
**
**
Control
3
5
10
C-KβBA (μM)


B
Control
C-KβBA (μM)
3
5
10
PI
Annexin V
Annexin V


C
Caspase-3 activity (10^3 x RFU)
20
15
10
5
0
**
**
**
Control
1
3
5
10
C-KβBA (μM)


D
Control
C-KβBA

Figure 3

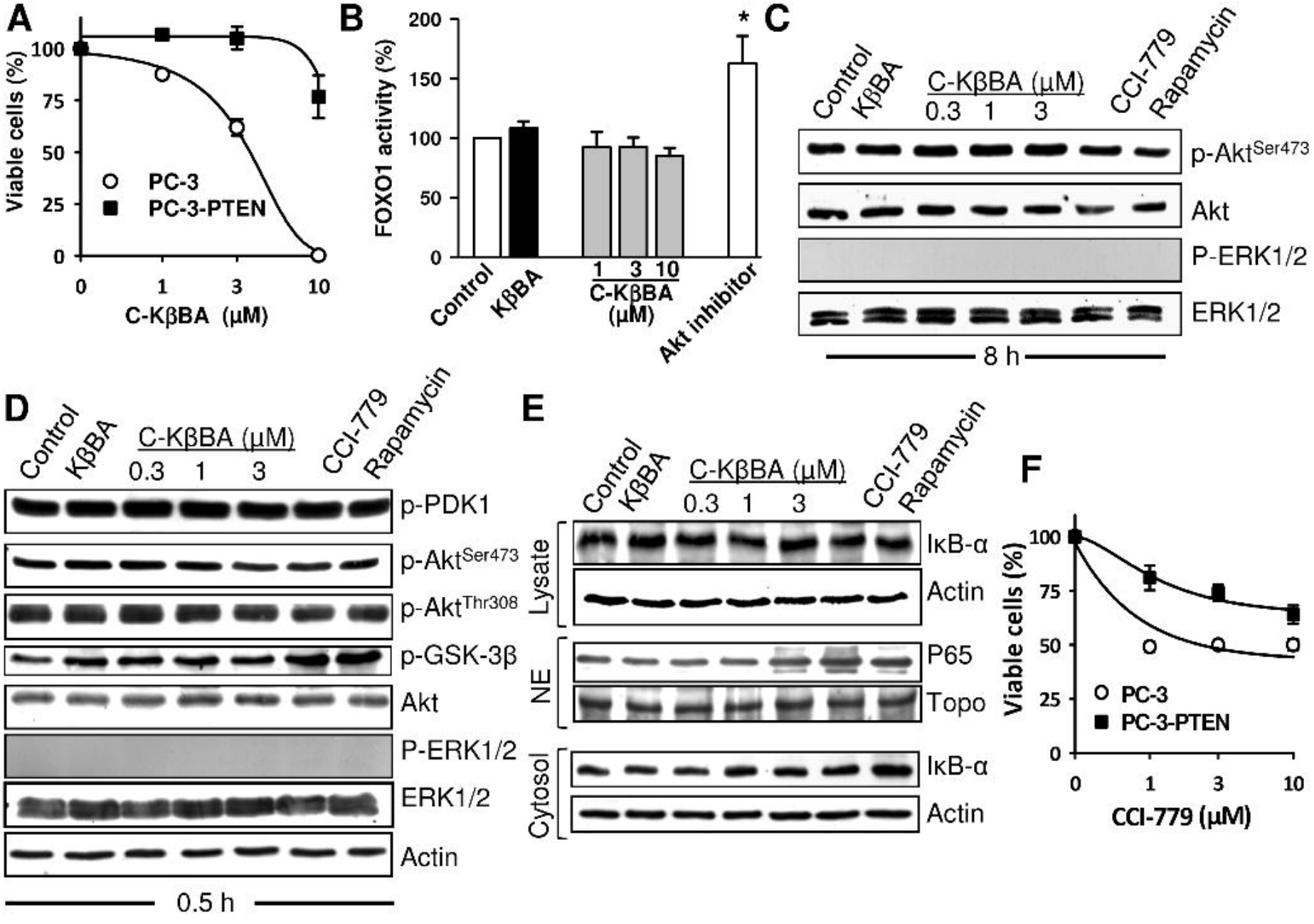

A
Viable cells (%)
100
75
50
25
0
PC-3
PC-3-PTEN
0
1
3
10
C-KβBA (μM)
B
FOXO1 activity (%)
200
150
100
50
0
*
Control
KβBA
1
3
10
C-KβBA (μM)
Akt inhibitor
C
Control
KβBA
C-KβBA (μM)
0.3
1
3
CCI-779
Rapamycin
p-Akt^Ser473
Akt
P-ERK1/2
ERK1/2
8 h
D
Control
KβBA
C-KβBA (μM)
0.3
1
3
CCI-779
Rapamycin
p-PDK1
p-Akt^Ser473
p-Akt^Thr308
p-GSK-3β
Akt
P-ERK1/2
ERK1/2
Actin
0.5 h
E
Control
KβBA
C-KβBA (μM)
0.3
1
3
CCI-779
Rapamycin
Lysate
IκB-α
Actin
NE
P65
Topo
Cytosol
IκB-α
Actin
F
Viable cells (%)
100
75
50
25
0
PC-3
PC-3-PTEN
0
1
3
10
CCI-779 (μM)

Figure 4

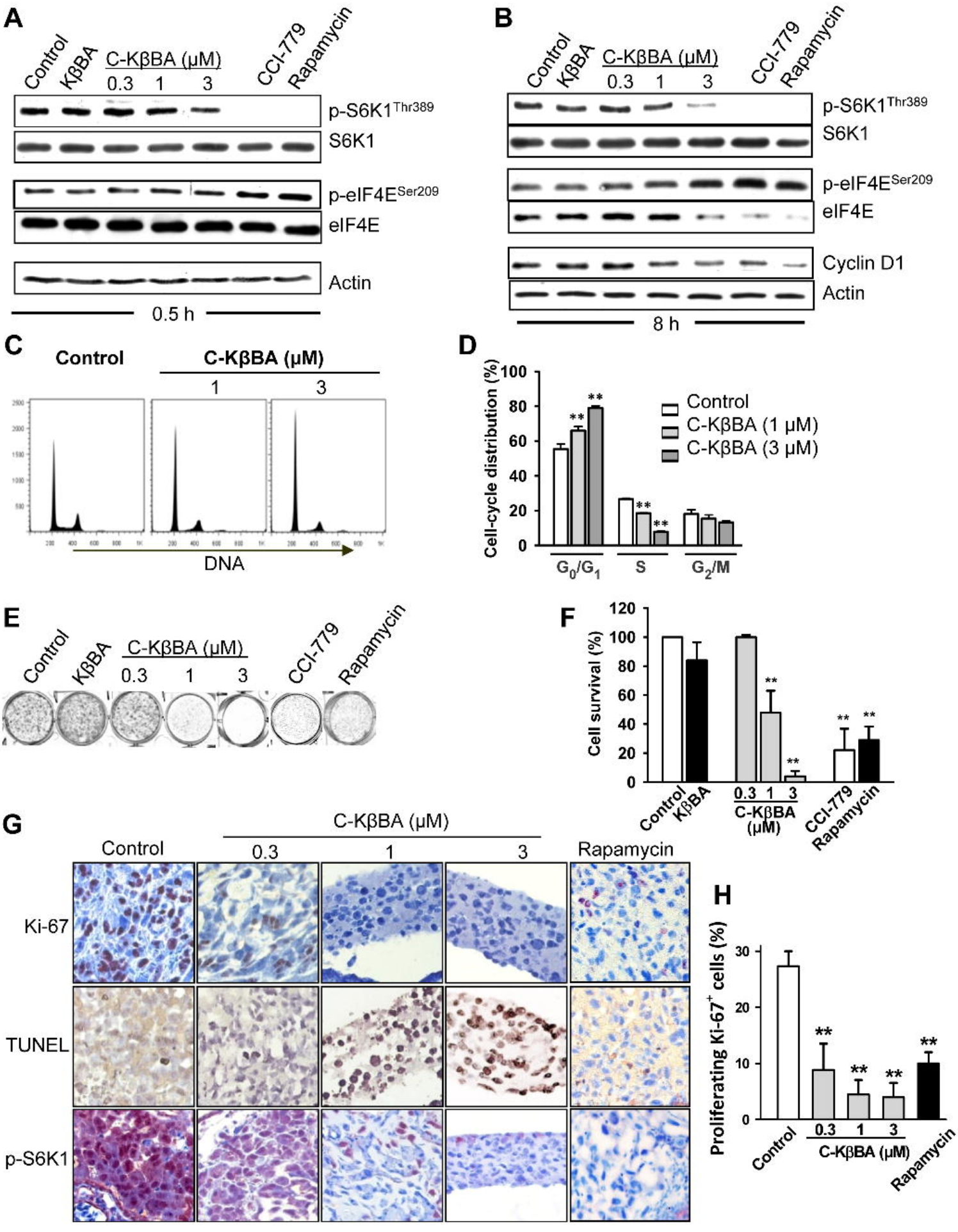

A
Control
KβBA
C-KβBA (μM)
0.3
1
3
CCI-779
Rapamycin
p-S6K1Thr389
S6K1
p-eIF4ESer209
eIF4E
Actin
0.5 h
B
Control
KβBA
C-KβBA (μM)
0.3
1
3
CCI-779
Rapamycin
p-S6K1Thr389
S6K1
p-eIF4ESer209
eIF4E
Cyclin D1
Actin
8 h
C
Control
C-KβBA (μM)
1
3
DNA
D
Cell-cycle distribution (%)
Control
C-KβBA (1 μM)
C-KβBA (3 μM)
G0/G1
S
G2/M
E
Control
KβBA
C-KβBA (μM)
0.3
1
3
CCI-779
Rapamycin
F
Cell survival (%)
Control
KβBA
0.3
1
3
C-KβBA (μM)
CCI-779
Rapamycin
G
Control
C-KβBA (μM)
0.3
1
3
Rapamycin
Ki-67
TUNEL
p-S6K1
H
Proliferating Ki-67+ cells (%)
Control
0.3
1
3
C-KβBA (μM)
Rapamycin

Figure 5

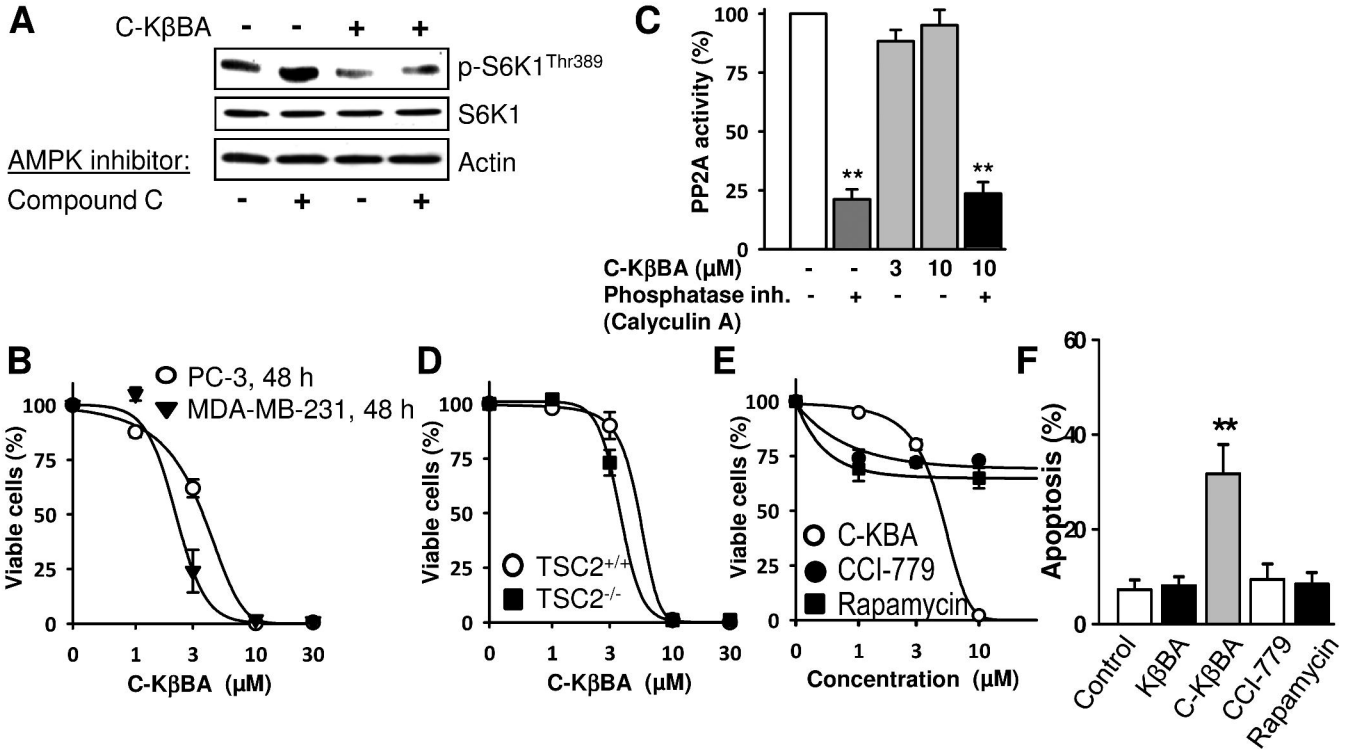
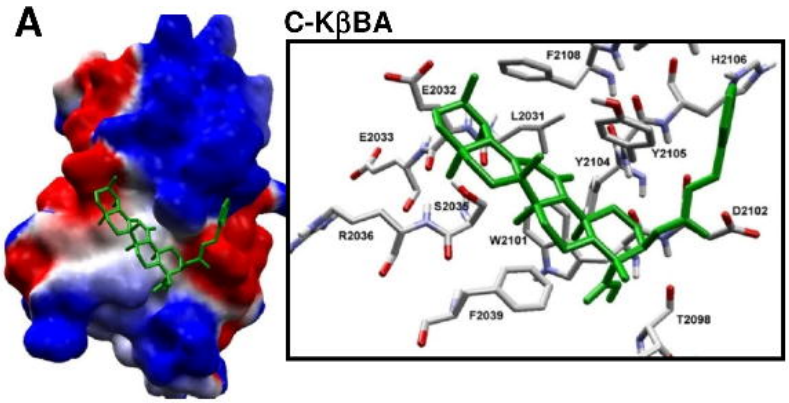
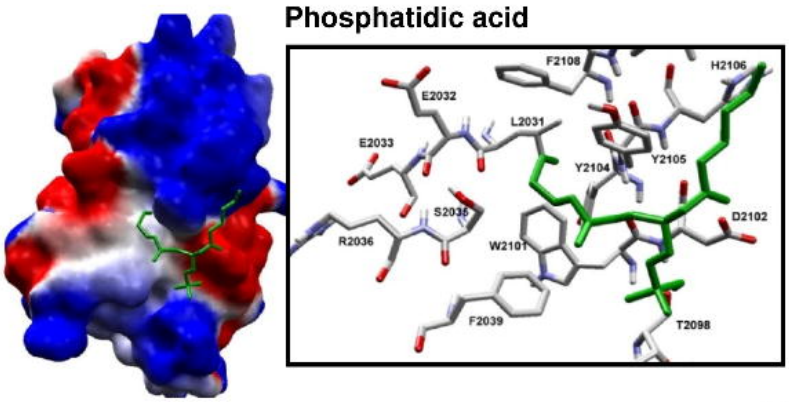

A
C-KβBA - - + +
p-S6K1Thr389
S6K1
AMPK inhibitor:
Actin
Compound C - + - +
C
PP2A activity (%)
100
75
50
25
0
**
**
C-KβBA (μM) - - 3 10 10
Phosphatase inh. - + - - +
(Calyculin A)
B
PC-3, 48 h
MDA-MB-231, 48 h
Viable cells (%)
C-KβBA (μM)
D
TSC2+/+
TSC2-/-
E
C-KBA
CCI-779
Rapamycin
Concentration (μM)
F
Apoptosis (%)
Control
KβBA
C-KβBA
CCI-779
Rapamycin

Figure 6



B

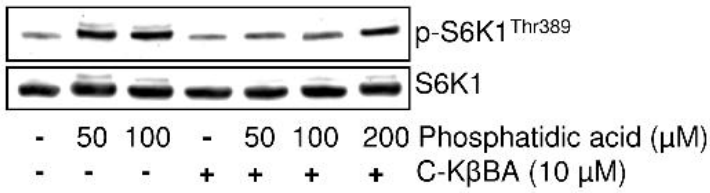
Protein	PDB entry	Interaction energy, kJ/mol/L	Calculated K_D, μM
mTOR	2GAQ	-189.6 ± 20.5	2.1 ± 0.6
S6K1	3A60	-74.6 ± 15.6	381.8 ± 25.4
PP2A	3DW8	-40.4± 9.2	6439.8 ± 36.4



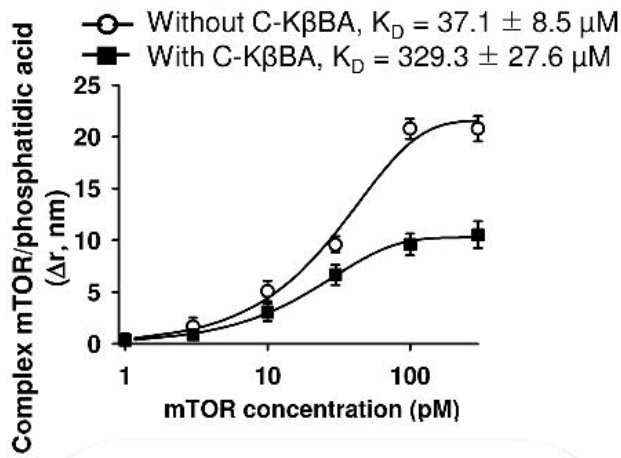
C

Protein	Ligand	Interaction energy, kJ/mol/L	Calculated K_D, μM
mTOR	C-KβBA	-189.6 ± 20.5	2.1 ± 0.6
	Phospha-tidic acid	-125.1 ± 19.9	33.9 ± 17.7

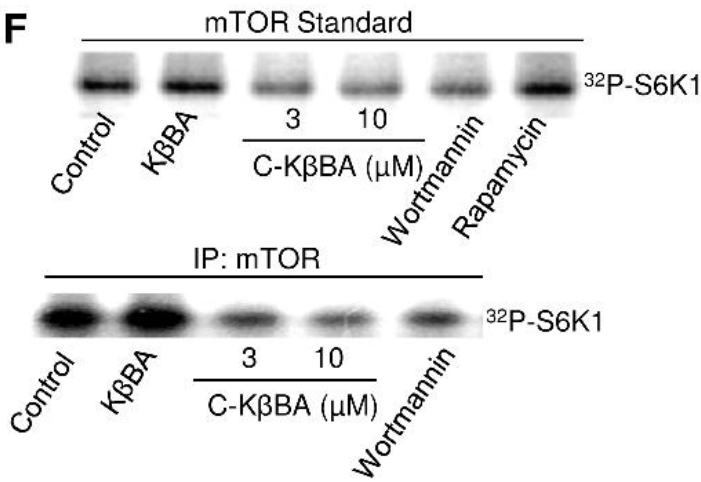
D



E



F



G