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Characterization of pomiferin triacetate as a novel mTOR and translation inhibitor

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Abstract

Deregulation of the phosphatidylinositol 3-kinase (PI3K)-Akt-mammalian target of rapamycin (mTOR)-70 kDa ribosomal protein S6 kinase 1 (p70^{S6K}) pathway is commonly observed in many tumors. This pathway controls proliferation, survival, and translation, and its overactivation is associated with poor prognosis for tumor-associated survival. Current efforts focus on the development of novel inhibitors of this pathway. In a cell-based high-throughput screening assay of 15 272 pure natural compounds, we identified pomiferin triacetate as a potent stabilizer of the tumor suppressor programmed cell death 4 (Pdc4). Mechanistically, pomiferin triacetate appeared as a general inhibitor of the PI3K-Akt-mTOR-p70^{S6K} cascade. Interference with this pathway occurred downstream of Akt but upstream of p70^{S6K}. Specifically, mTOR kinase emerged as the molecular target of pomiferin triacetate, with similar activities against mTOR complexes 1 and 2. In an *in vitro* mTOR kinase assay pomiferin triacetate dose-dependently inhibited mTOR with an IC₅₀ of 6.2 μM. Molecular docking studies supported the interaction of the inhibitor with the catalytic site of mTOR. Importantly, pomiferin triacetate appeared to be highly selective for mTOR compared to a panel of 17 lipid and 50 protein kinases tested. As a consequence of the mTOR inhibition, pomiferin triacetate efficiently attenuated translation. In summary, pomiferin triacetate emerged as a novel and highly specific mTOR inhibitor with strong

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translation inhibitory effects. Thus, it might be an interesting lead structure for the development of mTOR- and translation-targeted anti-tumor therapies.

Keywords

mammalian target of rapamycin; natural product; translation; pomiferin triacetate

1. Introduction

The PI3K-Akt-mTOR-signaling pathway is important in regulating cell growth, survival, and differentiation [1]. Due to its central role it is commonly deregulated, i.e. overactivated, in many tumors [2]. Within this signaling cascade the serine/threonine kinase mTOR, belonging to the superfamily of PI3K-related protein kinases, represents a central node [3]. mTOR can be activated directly by Akt, but also by Akt-mediated inactivation of its suppressor tuberous sclerosis complex 2 [4]. mTOR exists in two forms, where mTOR complex 1 (mTORC1) enhances translation by phosphorylating and inactivating the eukaryotic initiation factor 4E (eIF4E)-binding protein (4E-BP), which otherwise sequesters eIF4E to inhibit cap-dependent translation [5]. Furthermore, mTORC1 activates p70^{S6K} to phosphorylate the ribosomal protein S6 [6], but also to destabilize the tumor suppressor programmed cell death 4 (Pdc4) [7]. In contrast, mTORC2 provides a strong signal amplification function by phosphorylating the upstream kinase Akt at Ser473 to enhance its activity [8]. Therapeutically, the rapalogs (i.e. rapamycin and its analogs) have been introduced into the clinic for treatment of mantle-cell lymphomas and renal cell carcinomas [9, 10]. Inhibitors in this class bind mTOR complexed with the adaptor protein FKBP12 and allosterically inhibit mTORC1 [11]. In contrast rapamycin does not affect mTORC2. Therefore, the remaining mTORC2 activity was suggested to account for treatment failure and the development of resistances in some tumor types [12]. Consequently, current efforts aim to develop therapeutic options that inhibit both mTORC1 and mTORC2, or apply combinatorial approaches to also eliminate PI3K activity [13, 14].

We have previously established the critical role of p70^{S6K} in phosphorylating and degrading the tumor suppressor Pdc4 [15, 16]. Using a cell-based reporter assay to identify stabilizers of Pdc4 [17], we identified a number of Pdc4-stabilizing agents in a high-throughput screen of natural product libraries at the U.S. National Cancer Institute [18–20]. Here we present the novel, natural product-derived small molecule pomiferin triacetate (PT) that stabilizes cellular levels of Pdc4 by inhibiting the PI3K-Akt-mTOR-p70^{S6K} cascade. We provide evidence that PT interferes directly with the activity of both mTOR complexes 1 and 2, and consequently attenuates translation. PT therefore provides an interesting lead structure for the development of a novel class of mTOR inhibitors.

2. Materials and Methods

2.1. Compound

The pure natural compounds pomiferin triacetate (NSC021570; 4-[5-acetyloxy-8,8-dimethyl-6-(3-methylbut-2-en-1-yl)-4-oxo-4,8-dihydropyrano[2, 3-f]chromen-3-yl]-1,2-phenylene-diacetate) and pomiferin (NSC05113) was provided by the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis, U.S. National Cancer Institute (Bethesda, USA) and dissolved in DMSO. The identity and purity of pomiferin triacetate was confirmed by LC-MS and NMR analyses.

2.2. Reagents

All chemicals were purchased from Sigma-Aldrich (Schnelldorf, Germany) if not indicated otherwise. Rapamycin and TPA (12-*O*-tetradecanolyphorbol-13-acetate) were purchased from LC Laboratories (Woburn, MA, USA). Anti-Pdcd4, anti-phospho-S6 (Ser240/244), anti-S6, anti-phospho-S6K (Thr389), anti-S6K, anti-phospho-Akt (Ser473), anti-Akt, anti-phospho-GSK3 β (Ser9), and anti-GSK3 β antibodies were obtained from Cell Signaling Technology (Frankfurt, Germany). Anti-nucleolin antibody came from Santa Cruz Biotechnology (Heidelberg, Germany), anti-HA from Covance (Munich, Germany), and IRDyes 680LT and 800CW secondary antibodies from Li-COR Biosciences GmbH (Bad Homburg, Germany).

2.3. Cell culture

MCF7 and HEK293 cells were purchased from ATCC-LGC Standard GmbH (Wesel, Germany) and maintained in DMEM supplemented with 10% FBS, 100 U/mL penicillin, 100 μ g/mL streptomycin and 2 mM L-glutamine. Stable HEK293 Pdcd4-luc cells were maintained in regular growth medium supplemented with 3 μ g/mL blasticidin as previously described [17]. HEK293 cells stably transduced with either a control or a HA-tag-containing myristylated-Akt (myr-Akt) expressing vector were maintained in regular growth medium supplemented with 110 mg/L sodium pyruvate and 1 mg/mL G418. Cells were cultivated in a humidified atmosphere with 5% CO₂ at 37°C. Medium, supplements, and FBS came from PAA (Linz, Austria).

2.4. Pdcd4 stabilization assay

Pdcd4 stabilization was assessed using a luciferase-based assay as previously described [17]. Briefly, HEK293 cells stably expressing either Pdcd4₍₃₉₋₉₁₎luc or Pdcd4_(mut39-91)luc were seeded in a 96-well plate (1×10^4) and allowed to attach for 18 h before treatment. After appropriate incubations, cells were harvested in luciferase lysis buffer (25 mM Tris, 2 mM DTT, 1% Triton-X-100, 10% glycerol, pH 7.8) and frozen at -20°C for at least 2 h. After lysis at room temperature, luminescence was measured using *firefly* luciferase substrate solution (20 mM tricine, 2.67 mM 4MgCO₃*Mg(OH)₂*5H₂O, 1.07 mM MgSO₄*7H₂O, 100 μ M EDTA, 33.3 mM DTT, 530 μ M ATP, 0.213 mg/mL coenzyme A, 470 mM D-luciferin) on a Mithras LB 940 (Berthold, Bad Wildbad, Germany). Pdcd4₍₃₉₋₉₁₎luc expressing cells were used to determine Pdcd4 stabilization and the stabilizing activity of test samples was calculated using the following formula: Pdcd4₍₃₉₋₉₁₎ stabilization [%] = (RLU_{PT} - RLU_{TPA}) / (RLU_{DMSO} - RLU_{TPA}) $\times$ 100. Pdcd4_(mut39-91)luc expressing cells served as a specificity control and effects were calculated according to the following formula: Pdcd4_(mut39-91) RLU [%] = RLU_{PT} / RLU_{TPA} $\times$ 100.

2.5. Cell viability

HEK293 cells stably expressing Pdcd4₍₃₉₋₉₁₎luc were seeded and cultured with TPA (100 nM) and different doses of pomiferin triacetate (0.3–20 μ M) for 8 h. Afterwards MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) solution (5 mg/mL in PBS) was added and the cells were incubated at 37°C for 2 h. Cells were lysed in MTT lysis buffer (20% SDS, 50% dimethyl formamide, pH 4.7). After cell lysis, the absorbance of the solution was measured at 560 nm on a Mithras LB 940 (Berthold, Bad Wildbad, Germany).

2.6. Western blot analysis

For protein analysis, cells were lysed in lysis buffer (50 mM Tris [pH 7.4], 0.27 M sucrose, 1 mM Na-ortho-vanadate, 1 mM EDTA, 1 mM EGTA, 10 mM Na- β -glycerolphosphate, 50 mM NaF, 5 mM Na-pyrophosphate, 1% Triton-X-100, protease inhibitor with EDTA (Roche, Mannheim, Germany)). 50 μ g of protein was separated via SDS-PAGE and

transferred onto nitrocellulose membranes (Neolab, Heidelberg, Germany). Proteins were detected using specific antibodies with appropriate secondary antibodies and visualized and quantified on the Odyssey infrared imaging system (Li-COR Biosciences GmbH, Heidelberg, Germany).

2.7. mTOR activity assay

mTOR kinase inhibition was determined using the K-LISA™ mTOR (recombinant) activity kit (Calbiochem, San Diego, CA) according to the manufacturer's protocol. Briefly, the mTOR substrate (p70^{S6K}-GST-fusion protein) was bound to glutathione-coated wells. Recombinant mTOR (*S. frugiperda*) was pre-incubated with the test compound in mTOR activity buffer. ATP was supplied before the mixture was added to the substrate-containing wells, and incubations lasted for 30 min at 30°C. After stopping the reaction, substrate phosphorylation was determined using anti-phospho-p70^{S6K} (Thr389) antibody, followed by detection with HRP-antibody conjugate and TMB substrate. Absorbance was measured at 450 nm and the activity was calculated relative to the DMSO control. The IC₅₀ value was determined by fitting the data to a sigmoidal dose-response curve using the Prism software package version 5.04.

2.8. Computational modeling

To model the interaction between mTOR and pomiferin triacetate or pomiferin, we performed an Induced Fit Docking (IFD) program from Schrödinger suite 2012 [21]. The three-dimensional structure of mTOR [22] was obtained from the Protein Data Bank (PDB ID: 4JSX), accommodated for docking analysis under Schrödinger suite 2012, and finally IFD was performed to determine a binding model structure of mTOR-pomiferin triacetate or mTOR-pomiferin, which merges the predictive power of Prime with the docking and scoring capabilities of Glide to accommodate the possible protein conformational changes upon ligand binding. Herein we obtained the best-docked representative structure. Images were generated with the UCSF Chimera program [23].

2.9. Selectivity profiling

Specificity was assessed against a panel of 50 protein kinases using radioactive (³³P-ATP) filter-binding assays, and against a panel of 17 lipid kinases using the ADP-Glo™ Assay (Promega, Southampton, UK) at the International Centre for Kinase Profiling of the Medical Research Council Protein Phosphorylation Unit (Dundee, Scotland, UK).

2.10. Polysome analysis

MCF7 cells were seeded in 15-cm dishes and treated with 20 µM pomiferin triacetate, 100 nM rapamycin, and DMSO for 8 h. Cycloheximide (CHX) (100 µg/mL) was added for the last 10 min of the incubation, before the cells were harvested in PBS/CHX (100 µg/mL) and lysed in 750 µL polysome buffer (140 mM KCl, 20 mM Tris-HCl [pH 8.0], 5 mM MgCl₂, 0.5% NP40, 0.5 mg/mL heparin, 1 mM DTT, 100 U/mL RNasin (Promega, Mannheim, Germany), 100 µg/mL CHX). After pelleting, cytoplasmic lysates were layered onto 11-mL 10–50% continuous sucrose gradients. The gradients were centrifuged at 35 000 rpm for 2 h at 4°C without brake using a SW40 rotor in a Beckman ultracentrifuge. The gradients were fractionated using a Biologic LP system (Biorad, Munich, Germany) and the absorbance was measured continuously at 254 nm.

2.11. Statistical analysis

At least three independent experiments were performed. Data are presented as means ± SEM. Statistical analyses were performed using Student's t-test.

3. Results

3.1. Pomiferin triacetate stabilizes Pdc4 from TPA-induced degradation

We recently showed that PI3K-mTOR-p70^{S6K}-dependent phosphorylation facilitates proteasomal degradation of the translation inhibitor and tumor suppressor Pdc4 [15, 16], and established a luciferase-based assay to identify compounds stabilizing Pdc4 from TPA-induced degradation [17, 18]. In this assay a vector containing the phosphorylation-dependent degradation domain of Pdc4, that includes both the p70^{S6K}-phosphorylation and the E3-ligase β -TrCP-recognition motifs (aa 39–91) fused to luciferase (Pdc4_(39–91)luc), served as a sensitive tool to analyze TPA-induced degradation of Pdc4. Mutations of serines 67, 71, and 76 to alanines within the phospho-degron rendered the construct insensitive to TPA-induced degradation. In a high-throughput screen, using HEK293 cells stably expressing Pdc4_(39–91)luc, 15 272 compounds from an NCI library of pure natural products were tested, and PT was identified as a promising stabilizer of cellular Pdc4 levels. PT (Fig. 1) is a prenylated isoflavone derivative that was originally prepared from the fruits of the deciduous tree *Maclura pomifera*, which is also known as Osage orange or hedge apple tree [24].

For an in-depth characterization of PT-effects, we initially determined the maximally effective concentration for stabilizing Pdc4. To this end, HEK293 cells stably expressing the Pdc4 reporter Pdc4_(39–91)luc were treated with TPA [10 nM] for 8 h which resulted in a marked decrease of the luciferase activity. Co-treatment with PT significantly prevented loss of the luciferase-signal at concentrations as low as 1.25 μ M ($23.9 \pm 2.9\%$), while maximal stabilization of the Pdc4 reporter was achieved at concentrations of 10 μ M ($86.3 \pm 5.2\%$) (Fig. 2A). To prove specificity, a cell line expressing the Pdc4_(mut39–91)luc vector was used which was only marginally affected by TPA. In this case, PT co-treatment did not alter the activity of the control vector at all concentrations tested (Fig. 2B). In line, PT did not affect cell viability at concentrations up to 20 μ M (Fig. 2C).

Next, we determined if PT also affects the endogenous Pdc4 protein amount. In fact, PT [20 μ M] significantly rescued endogenous Pdc4 protein levels from TPA-induced degradation similar to the mTOR inhibitor rapamycin [100 nM] and the PI3K inhibitor LY294002 [10 μ M] (Fig. 2D). Importantly, PT suppressed phosphorylation of the ribosomal protein S6, which serves as a marker for p70^{S6K} activity, to a similar degree as the PI3K and mTOR inhibitors.

These data indicate that PT inhibits the activity of the PI3K-Akt-mTOR-p70^{S6K} axis, thereby also affecting Pdc4 stability.

3.2. Pomiferin triacetate interferes with PI3K signaling downstream of Akt but upstream of p70^{S6K}

To determine the direct molecular target of PT within the PI3K-Akt-mTOR-p70^{S6K} cascade, we serum-starved MCF7 breast tumor cells for 48 h prior to stimulation with insulin-like growth factor-1 (IGF-1) [1 ng/mL] for 10 min. While short-term stimulation with IGF-1 cannot be expected to affect Pdc4 protein stability, this experimental set-up greatly enhances the sensitivity and specificity for the PI3K pathway. Pre-treatment of MCF7 cells for 6 h with rapamycin [100 nM] or PT [20 μ M] efficiently blocked IGF-1-induced S6-phosphorylation (Fig. 3A). Moreover, both compounds inhibited phosphorylation of the upstream p70^{S6K}. To assess if PT affects the cellular levels of phosphorylated p70^{S6K} by inhibiting its phosphorylation or by enhancing its dephosphorylation via protein phosphatase 2A (PP2A), we treated samples with rapamycin [100 nM] or PT [20 μ M] for 6 h and added the PP2A inhibitor okadaic acid [50 nM] for the last 30 min before stimulating with IGF-1

[1 ng/mL; 10 min]. Attenuation of p70^{S6K}- and S6-phosphorylation by rapamycin was not affected by the PP2A inhibitor. Similarly, PT-mediated p70^{S6K} inhibition was insensitive to PP2A inhibition (Fig. 3A).

To narrow down the molecular target of PT within the PI3K-Akt-mTOR-p70^{S6K} cascade, we next used HEK293 cells stably expressing myristylated, i.e. constitutively active, Akt (myr-Akt) [15, 25]. After serum deprivation (48 h), cells overexpressing myr-Akt displayed elevated Akt and p70^{S6K} activity as measured by increased GSK3 β - and S6-phosphorylation, respectively, compared to control cells (Fig. 3B). Treatment with rapamycin [100 nM] or PT [20 μ M] for 6 h markedly decreased S6-phosphorylation. In contrast, GSK3 β -phosphorylation, i.e. Akt activity, appeared unaffected by both inhibitors. Based on these observations, we conclude that PT interferes with PI3K-Akt-mTOR-p70^{S6K} signaling downstream of Akt but upstream of p70^{S6K}.

3.3. Pomiferin triacetate inhibits mTOR kinase directly

As the molecular target of PT within the PI3K-Akt-mTOR-p70^{S6K} cascade appeared to reside between Akt and p70^{S6K}, we next analyzed mTOR kinase. To this end, MCF7 cells were serum-starved for 48 h and treated for 6 h with PT [20 μ M], rapamycin [100 nM], or LY294002 [10 μ M] prior to the addition of IGF-1 [1 ng/mL; 10 min]. All three inhibitors significantly reduced activation of p70^{S6K} (Fig. 4A). In line with previous reports that characterized rapamycin as a specific inhibitor of mTORC1 [8], Akt Ser473-phosphorylation, as an indicator of mTORC2 activity, was not attenuated by rapamycin. In contrast, PT as well as the PI3K inhibitor LY294002 efficiently prevented Akt Ser473-phosphorylation. This observation served as a first indicator that PT might inhibit mTOR.

To validate the mTOR inhibitory potential of PT, we employed an *in vitro* mTOR kinase activity assay. Dose response studies revealed a significant reduction in mTOR activity by PT with remaining mTOR activities of $21 \pm 4.1\%$, $7 \pm 0.9\%$, and $5 \pm 0.5\%$ at concentrations of 10, 20, and 50 μ M, respectively. This corresponds to an IC₅₀ of 6.2 μ M for the inhibition of mTOR by PT (Fig. 4B). Of note, non-esterified pomiferin appeared similarly potent in inhibiting mTOR activity as PT (Fig. 5A).

Since the mTOR activity assay is designed such that mTOR activity is measured in the absence of co-factors that determine complex 1 or 2 formation (such as raptor or rictor), these data indicate that PT could directly interact with mTOR. To verify this option we performed molecular modeling and docking analyses. Specifically, PT was docked into the recently solved structure of mTOR kinase [22] with the Induced Fit Docking (IFD) protocol implemented in Schrödinger Suite 12 [21]. According to the docking analysis, PT forms several hydrogen bonds with the catalytic site of the mTOR protein (Fig. 4C). One hydrogen bond formed with the backbone atoms of residue His2242 and one with the side chain atoms of the adjacent Trp2239. Furthermore, two H-bonds involved the side chain atoms of Arg2348 and two of the phenyl rings of PT formed strong π - π stacking interactions with Trp2239.

The molecular docking analysis thus suggests a direct interaction of PT with the ATP-binding pocket of mTOR. Interestingly, non-acetylated pomiferin, while interacting with different residues of mTOR, appeared to also dock into the ATP-binding site of mTOR (Fig. 5B). As our data supported direct inhibition of mTOR activity by PT, we next evaluated kinase selectivity. When testing PT at concentrations of 5 and 10 μ M, i.e. concentrations around the IC₅₀ for mTOR, against a panel of 17 lipid kinases, it showed marginal activity against most kinases tested (Table 1).

The only lipid kinases affected by PT were two PI3K α mutants, E542K and E545K. Remaining activities for PI3K α E542K were $65.3 \pm 1.6\%$ and $46.1 \pm 0.5\%$, and $52.8 \pm 0.5\%$ and $37.6 \pm 4.9\%$ for PI3K α E545K at 5 and 10 μ M PT, respectively. Interestingly, further testing of PT against a panel of 50 protein kinases did not yield additional targets (Table 2).

These data indicate that PT selectively inhibits mTOR *in vitro* and *in vivo* at low micromolar concentrations.

3.4. Pomiferin triacetate inhibits translation

mTOR plays a key role in the regulation of protein synthesis [26]. As the translation inhibitory function of mTOR inhibitors is critical for their tumor therapeutic properties, we finally determined the impact of PT on protein translation. Translational efficiency was assessed using polysome profiling, i.e. the distribution of mRNAs across a sucrose gradient, where translated mRNAs sediment deeper when they are bound by increasing numbers of ribosomal complexes (polysomes) as compared to the non-translated sub-polysomal fraction. Treatment of MCF7 cells with rapamycin [100 nM] for 8 h decreased the polysome-bound mRNA fraction as compared to controls (Fig. 6A, *lower vs. upper panel*). Inversely, mRNA abundance in the sub-polysomal 80S fraction increased in response to rapamycin. PT [20 μ M] treatment provoked an even stronger shift from the polysomal to the sub-polysomal fraction (Fig. 6A, *middle panel*). The ratio of polysome- vs. 80S-associated mRNAs significantly decreased in response to rapamycin (0.58 ± 0.06) and PT (0.46 ± 0.05), relative to controls (Fig. 6B).

Thus, we conclude that PT efficiently impairs protein translation via its mTOR inhibitory activity.

4. Discussion

In this study, we identify a novel molecular functionality of the natural product-derived small molecule pomiferin triacetate. Specifically, we characterize PT as a highly selective mTOR inhibitor that consequently also impacts translation.

PT is an acetylated derivative of the plant-derived prenylated isoflavonoid pomiferin, which was first isolated from the Osage orange (*Maclura pomifera*) [24]. While no biological activities have been previously reported for PT, non-acetylated pomiferin was shown to possess antioxidant, antimicrobial, and histone deacetylase (HDAC) inhibitory properties [27–29], and to exert anti-proliferative effects in tumor cells [30]. These observations are in line with many studies supporting tumor therapeutic properties for various flavonoids [31, 32]. Historically natural product mixtures have been widely used as preventive, curative, or adjuvant agents, and purified natural product-derived molecules are well-accepted as an important source of novel antitumor therapeutics [33]. In a high-throughput screen of pure natural compounds, we identified PT as a stabilizer of Pdc4. In contrast to a previously reported plant-derived compound that specifically interfered with the ubiquitination of Pdc4 [18], PT inhibited p70^{S6K}-dependent phosphorylation of Pdc4. A detailed analysis of the mechanism-of-action revealed that PT specifically inhibits mTOR. In addition, PT might also exhibit some HDAC inhibitory properties as previously reported for pomiferin [30]. In a cellular context, such properties might contribute to the observed mTOR inhibitory action of PT, as HDAC3 depletion was recently proposed to enhance the expression of the PH Domain and Leucine-rich Repeat Phosphatase 1, resulting in attenuated Akt activity and consequently lower mTOR activity [34]. Nevertheless, while Bradley et al. found that HDAC inhibitor-repressed mTOR activity could be rescued by overexpression of constitutively active Akt, PT effectively inhibited mTOR even in cells expressing constitutively active Akt (Fig. 3B). This observation in combination with the *in vitro* mTOR

kinase assays and the modeling further supports the direct inhibition of mTOR by PT, yet does not exclude a contribution of potential HDAC inhibitory properties.

mTOR integrates extracellular input, such as growth factor signaling, transmitted by the PI3K-Akt cascade [3], but also intracellular signals such as the cellular energy status as detected by 5' adenosine monophosphate-activated protein kinase (AMPK) [35]. Functionally, mTOR activation facilitates protein synthesis by antagonizing the inhibitory function of 4E-BP, thus allowing for binding of eIF4E to the 7-methylguanosine cap of mRNAs to initiate cap-dependent translation. As a consequence, mTOR activation enhances proliferation [36]. Since mTOR appears as a central kinase within the PI3K-Akt-mTOR cascade and this signaling pathway is overactivated in many tumors [2], mTOR has been considered a promising molecular target for novel tumor therapeutics [37]. Rapamycin, a bacterial-derived macrocyclic lactone, was the first mTOR inhibitor to be used therapeutically. In addition to the immunosuppressive effects of rapamycin [38], various rapalogs have been shown to be effective against endometrial cancers, mantle cell lymphoma [39], and advanced stage renal cell carcinoma [40]. Yet, rapamycin was found to efficiently inhibit mTORC1 only when bound to the adaptor FKBP12, and mTORC2 appeared to be insensitive to rapamycin [41]. Specificity for mTORC1 vs. mTORC2 is achieved by specific co-factors (such as raptor and rictor), which bind to the shared catalytic mTOR kinase core [3]. Functionally, mTORC1 phosphorylates downstream targets such as 4E-BP and p70^{S6K} [42], whereas mTORC2 provides a potent feed-forward signal by phosphorylating the upstream kinase Akt at Ser473, thereby enhancing the activity of the latter [8]. Therapeutic approaches selectively inhibiting mTORC1 (such as the rapalogs) showed only limited success as the remaining mTORC2 activity enabled tumor cells to overcome anti-tumorigenic effects by overactivation of Akt [12, 43]. Accordingly, we observed that IGF-induced Akt phosphorylation was even further enhanced after rapamycin treatment (Fig. 4A). Consequently, additional inhibitors which target the activity of both mTOR complexes have been developed [14]. Similar to the recently developed mTORC1/C2 inhibitors Torin1/2 or PP242, PT inhibited both mTOR complexes (Fig. 4A). This observation is further supported by the fact that PT also efficiently inhibited recombinant mTOR in an *in vitro* assay, which does not depend on the presence of complex determining co-factors (Fig. 4B). In accordance with these dual-specific inhibitors, which bind to the catalytic domain of mTOR to inhibit its activity [44], our docking analyses suggest that PT binds to the ATP-binding site of mTOR (Fig. 4C). Similarly, non-esterified pomiferin also inhibited mTOR *in vitro* and was modelled to bind to the ATP-binding pocket of mTOR (Fig. 5). Thus, it will be interesting to see if pomiferin triacetate might be hydrolyzed by intracellular esterases to generate the equally potent mTOR inhibitor pomiferin. Along the same lines, 3HOI-BA-01 was recently reported to bind to the catalytic domain of mTOR and to inhibit mTORC1 and 2 [45]. While binding to the ATP-binding pocket provides a rational explanation for the observation that PT inhibits both mTOR complexes, compounds targeting this part of a kinase usually show low specificity as ATP-binding cassettes of various kinases show structural similarities [46]. Surprisingly, PT exhibited a marked specificity for inhibition of mTOR at concentrations around the IC₅₀ for mTOR (6.2 μM), as it did not significantly affect the activity of 50 protein and 15 lipid kinases tested at these concentrations. The only two kinases which showed some concentration-dependent sensitivity to PT were two oncogenic mutant forms of PI3Kα (Table 1). The observation that only the mutant forms were inhibited by PT, while the parent kinase PI3Kα appeared unresponsive, requires further in-depth analyses. Nevertheless, as PI3Kα E542K and PI3Kα E545K are commonly found in various human tumors [47, 48], inhibition of these oncogenic subtypes of PI3Kα (e.g. by PT) might provide some benefit for the treatment of selected tumor types and/or individual patients.

Inhibition of mTOR evokes a number of functional changes including induction of autophagy [49], yet a major proportion of mTOR-targeted therapies has been attributed to the inhibition of translation [50], as mTOR activation promotes translation by inhibiting 4E-BP and by activating p70^{S6K}. As deregulated protein synthesis is increasingly acknowledged to contribute to tumorigenesis, therapeutic approaches interfering at this level are currently under investigation [51]. Therefore, our finding that PT inhibits mTOR activity (Fig. 4A+B), and as a functional consequence efficiently inhibits translation (Fig. 6) appears of interest. In conclusion, we identified PT as a novel mTORC1/C2 inhibitor which is effective at low micromolar concentrations, and efficiently attenuates translation. Due to its selectivity for mTOR, PT could be a promising candidate for the development of future translation-targeted antitumor therapeutic approaches.

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pomiferin triacetate

$C_{31}H_{30}O_9$

M_r 546.57

Fig. 1.
Molecular structure of PT.

Fig. 2.

PT stabilizes Pdc4 from TPA-induced degradation. (A) HEK293 cells stably expressing Pdc4₍₃₉₋₉₁₎luc were treated for 8 h with TPA [10 nM] in combination with increasing concentrations of PT [0.3125–20 μM]. Pdc4 stabilizing activity was determined relative to $\Delta(\text{RLU}_{\text{DMSO}} - \text{RLU}_{\text{TPA}})$. (B) HEK293 cells stably expressing Pdc4_(mut39-91)luc were treated as in (A). Luciferase activity is given relative to TPA-treated controls. Data are presented as means $\pm$ SEM (n = 3, ** $p < 0.01$, *** $p < 0.001$). (C) HEK293 cells stably expressing Pdc4₍₃₉₋₉₁₎luc were treated for 8 h with TPA [10 nM] and different concentrations of pomiferin triacetate (PT) [0.3125–20 μM]. Afterwards cells were incubated

with MTT solution for 2 h at 37°C. After the cell lysis, the absorbance was measured at 560 nm. Data are presented as means $\pm$ SEM (n = 3). (D) HEK293 cells were treated for 8 h with TPA [10 nM] with or without PT [20 μ M], rapamycin [100 nM], or LY294002 [10 μ M]. Whole-cell extracts were subjected to Western analysis and probed with the indicated antibodies. Blots are representative of at least three independent experiments. Band intensities were quantified relative to DMSO only-treated cells and are given as means $\pm$ SEM (n = 3, * p < 0.05, ** p < 0.01).

A **B** **Fig. 3.**

PT inhibits IGF-1-induced signaling downstream of Akt activation. (A) MCF7 cells were serum-starved (48 h) and then treated for 6 h with PT [20 μM] or rapamycin [100 nM]. The protein phosphatase 2A inhibitor okadaic acid [50 nM] was added for the last 30 min of the incubation (lanes 3, 5, and 7) before IGF-1 [1 ng/ml] was added for 10 min. Whole-cell extracts were subjected to Western analysis and probed with the indicated antibodies. Blots are representative of at least three independent experiments. Band intensities were quantified and statistically analyzed relative to IGF-1/DMSO-treated cells and are given as means ± SEM (n = 3, **p < 0.01, ***p < 0.001). (B) HEK293 cells stably expressing either a control vector or a HA-tag-containing myr-Akt expression vector were serum-starved for 48 h and then treated with PT [20 μM] or rapamycin [100 nM] for 6 h. Whole-cell extracts were subjected to Western analysis and probed with the indicated antibodies. Blots are representative of at least three independent experiments. Band intensities were quantified and statistically analyzed relative to myr-Akt expressing/DMSO-treated cells and are given as means ± SEM (n = 3, **p < 0.01, ***p < 0.001).

Fig. 4. PT inhibits mTOR kinase. (A) MCF7 cells were serum-starved (48 h) and then treated for 6 h with PT [20 μM], LY294002 [10 μM], or rapamycin [100 nM]. Whole-cell extracts were subjected to Western analysis and probed with the indicated antibodies. Blots are representative of at least three independent experiments. Band intensities were quantified and statistically analyzed relative to IGF-1/DMSO-treated cells and are given as means ± SEM (n 3, *p < 0.05, **p < 0.01, ***p < 0.001). (B) mTOR activity was measured using an *in vitro* kinase assay. Remaining activity of recombinant mTOR kinase was determined in the presence of increasing concentrations of PT [0.3125–20 μM] relative to the DMSO

control. Data are presented as means $\pm$ SEM (n = 3, *** $p < 0.001$). (C) Predicted computational model of the mTOR-PT complex. PT is shown in a stick model, protein residues in a ribbon model, and hydrogen-bonds appear as red lines. Here, only the kinase domain (Pro2146 to Trp2546) of mTOR is shown for clarity purposes. *Upper panel*, binding of PT inside the ATP-binding pocket of mTOR. *Lower panel*, interaction between PT and specific amino acids within the ATP-binding pocket.

Fig. 5. Pomiferin inhibits mTOR kinase. (A) mTOR activity was measured using an *in vitro* kinase assay. Remaining activity of recombinant mTOR kinase was determined in the presence of 1.25, 2.5, or 5 µM pomiferin relative to the DMSO control. Data are presented as means $\pm$ SEM (n 3, ** $p < 0.01$, *** $p < 0.001$). (B) Predicted computational model of the mTOR-pomiferin complex showing the interaction between pomiferin and specific amino acids within the ATP-binding pocket of mTOR. Pomiferin is shown in a stick model, protein residues in a ribbon model, and hydrogen-bonds appear as red lines.

Fig. 6. PT inhibits translation. (A) MCF7 cells were treated with either DMSO (*upper panel*), PT [20 μM] (*middle panel*), or rapamycin [100 nM] (*lower panel*) for 8 h. Cellular lysates were subjected to polysomal fractionation and representative spectra at 254 nm across the gradient are provided (sedimentation depth increases from left to right). (B) The ratio of the maximal height of the polysome-containing peak to the 80S peak was determined for each gradient and the data are presented as means $\pm$ SEM (n 3, * p < 0.05, ** p < 0.01).

Table 1

Medical Research Council Lipid Kinase Screen

Kinase	Pomiferin triacetate ^a	
	[5 μ M]	[10 μ M]
CHK α	98 $\pm$ 6	100 $\pm$ 0
CHK β	102 $\pm$ 2	102 $\pm$ 4
DGK β	104 $\pm$ 8	92 $\pm$ 13
DGK γ	102 $\pm$ 9	94 $\pm$ 20
DGK ζ	120 $\pm$ 1	103 $\pm$ 2
PI3K α	99 $\pm$ 1	91 $\pm$ 5
PI3K α E542K	65 $\pm$ 2	46 $\pm$ 1
PI3K α E545K	53 $\pm$ 1	38 $\pm$ 5
PI3K β	98 $\pm$ 5	97 $\pm$ 2
PI3K δ	109 $\pm$ 3	98 $\pm$ 8
PI3K γ	70 $\pm$ 0	70 $\pm$ 3
PI4K2 α	90 $\pm$ 5	85 $\pm$ 6
PIK4C α	102 $\pm$ 7	102 $\pm$ 10
PIK4C β	106 $\pm$ 7	94 $\pm$ 1
PIP5K2 α	73 $\pm$ 0	75 $\pm$ 1
SPHK1	85 $\pm$ 6	78 $\pm$ 2
SPHK2	89 $\pm$ 1	84 $\pm$ 3

^a numbers represent percent of kinase activity remaining relative to the DMSO-treated control $\pm$ SD.

Table 2

Medical Research Council Express Screen

Kinase	Pomiferin triacetate ^a		Kinase	Pomiferin triacetate ^a	
	[5 μ M]	[10 μ M]		[5 μ M]	[10 μ M]
AMPK (hum)	86 $\pm$ 1	79 $\pm$ 6	NEK6	100 $\pm$ 2	104 $\pm$ 5
Aurora B	93 $\pm$ 7	79 $\pm$ 2	p38 α MAPK	105 $\pm$ 2	94 $\pm$ 2
BTK	97 $\pm$ 2	88 $\pm$ 8	PAK4	88 $\pm$ 9	98 $\pm$ 2
CAMKK β	132 $\pm$ 0	129 $\pm$ 5	PDK1	87 $\pm$ 1	82 $\pm$ 1
CAMK1	107 $\pm$ 1	88 $\pm$ 1	PIM1	95 $\pm$ 1	100 $\pm$ 2
CHK2	72 $\pm$ 3	70 $\pm$ 0	PKA	99 $\pm$ 0	98 $\pm$ 7
CK1 δ	99 $\pm$ 7	85 $\pm$ 5	PKB α	102 $\pm$ 1	92 $\pm$ 1
CK2	110 $\pm$ 1	103 $\pm$ 1	PKC α	122 $\pm$ 1	109 $\pm$ 5
DYRK1A	92 $\pm$ 1	74 $\pm$ 1	PKD1	98 $\pm$ 1	102 $\pm$ 5
EPK2	113 $\pm$ 2	98 $\pm$ 3	PLK1	124 $\pm$ 2	96 $\pm$ 1
EPH-A2	102 $\pm$ 3	93 $\pm$ 6	PRK2	98 $\pm$ 8	82 $\pm$ 8
GSK3 β	96 $\pm$ 8	87 $\pm$ 2	RIPK2	100 $\pm$ 1	87 $\pm$ 2
HER4	108 $\pm$ 5	95 $\pm$ 4	ROCK 2	78 $\pm$ 1	69 $\pm$ 2
HIPK2	91 $\pm$ 1	95 $\pm$ 1	RSK1	92 $\pm$ 0	95 $\pm$ 0
IGF-1R	103 $\pm$ 1	93 $\pm$ 1	SGK1	93 $\pm$ 6	86 $\pm$ 1
IRAK4	90 $\pm$ 1	90 $\pm$ 1	S6K1	89 $\pm$ 0	99 $\pm$ 4
JAK2	117 $\pm$ 8	106 $\pm$ 2	SmMLCK	94 $\pm$ 3	89 $\pm$ 2
JNK1	84 $\pm$ 5	82 $\pm$ 5	SRPK1	102 $\pm$ 0	95 $\pm$ 3
Lck	93 $\pm$ 2	71 $\pm$ 3	Src	99 $\pm$ 3	88 $\pm$ 1
LKB1	110 $\pm$ 7	95 $\pm$ 2	SYK	81 $\pm$ 2	79 $\pm$ 4
MARK3	95 $\pm$ 1	81 $\pm$ 3	TAK1	107 $\pm$ 3	93 $\pm$ 2
MKK1	92 $\pm$ 6	77 $\pm$ 1	TBK1	112 $\pm$ 2	92 $\pm$ 1
MLK3	97 $\pm$ 6	91 $\pm$ 3	TrkA	88 $\pm$ 0	76 $\pm$ 5
MSK1	105 $\pm$ 4	99 $\pm$ 4	TTK	80 $\pm$ 1	83 $\pm$ 2
MST2	69 $\pm$ 2	76 $\pm$ 1	VEGFR1	86 $\pm$ 6	81 $\pm$ 1

^a numbers represent percent of kinase activity remaining relative to the control $\pm$ SD.