A dual mTORC1 and mTORC2 inhibitor shows antitumor activity in esophageal squamous cell carcinoma cells and sensitizes them to cisplatin

Yu Huang, Qingsong Xi, Yu Chen, Jing Wang, Ping Peng, Shu Xia and Shiying Yu

The mammalian target of rapamycin (mTOR) signaling pathway is critical for the growth and proliferation of various malignant tumors, including esophageal squamous cell carcinoma (ESCC). Therefore, targeting of mTOR protein is a promising strategy for therapy in this disease. In the present study, we examined the antitumor effects of a specific mTOR kinase inhibitor, PP242, which blocks both mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) pathways, in two ESCC cell lines: Eca-109 and TE-1. We showed that PP242, but not rapamycin, attenuated the activities of both mTORC1 and mTORC2 signaling in ESCC. PP242 inhibited 4E-binding protein-1 phosphorylation and abrogated mTORC1-dependent PI3K/Akt feedback activation. Significantly, PP242 effectively suppressed ESCC cell proliferation, induced apoptosis, and arrested the cell cycle. Furthermore, PP242 promoted cisplatin-induced apoptosis and enhanced the antitumor efficacy of cisplatin in ESCC cells, which was likely to be associated with inhibition of Akt activity. Our results show that simultaneous targeting of both mTORC1 and mTORC2 pathways leads to effective antitumor actions in ESCC, and strongly suggest that dual mTORC1/2 inhibitors should be developed as potential agents for the treatment of ESCC. *Anti-Cancer Drugs* **24:889–898** © **2013 Wolters Kluwer Health | Lippincott Williams & Wilkins.**

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Introduction

The mammalian target of rapamycin (mTOR), which integrates signaling from the PI3K-Akt and AMP kinase pathways, plays crucial roles in cell growth, proliferation, and survival [1,2]. It is frequently deregulated in various malignant tumor cells and also related to chemoresistance in a variety of clinical settings, including esophageal squamous cell carcinoma (ESCC) [3,4]. Therefore, in recent years, interest has focused on its potential as an anticancer therapeutic target.

mTOR kinase exists in at least two distinctive cellular protein complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2), which have distinct molecular compositions, substrates, and mechanisms of activation [5–7]. mTORC1 consists of mTOR associated with Raptor, mLst8, FKBP38, Deptor, and PRAS40. The best-characterized substrates of mTORC1 are S6 kinase (p70S6K) and eukaryotic initiation factor 4E-binding protein-1 (4E-BP1), which mainly regulate protein synthesis. mTORC2 consists of mTOR associated with Rictor, mLst8, Sin1, and Protor, and is usually insensitive to rapamycin treatment. The best-known mTORC2 substrates are Akt (with mTOR-independent Akt phosphorylation occurring on serine 473), affecting Akt-mediated survival signaling, and other members of the AGC kinase protein family. mTORC2 mainly regulates survival, metabolism, and the cytoskeleton through phosphorylation of these AGC kinases. Taken together, both mTORC1 and mTORC2 play important roles in the biology of cell growth and proliferation, and therefore represent targets for drug development in oncology.

The first-generation mTOR inhibitor rapamycin and its analogs (rapalogs) partially suppress mTORC1 activity through an allosteric mechanism and slow the proliferation of cancer cells [8]. However, the rapalogs have achieved only limited success in the treatment of a few rare cancers, including renal cell carcinoma, mantle cell lymphoma, and pancreatic neuroendocrine tumors, at least when the agents are used in monotherapy settings [8–10]. The rapalogs have two mechanistic drawbacks. First, these compounds do not actually inhibit mTORC2 activity and the negative feedback loop that is suppressed upon mTORC1 inhibition elevates PI3K/Akt signaling and may promote the survival of cancer cells [8]. Second, rapamycin

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is an incomplete inhibitor of mTORC1 and has little effect on phosphorylation of 4E-BP1 [11,12].

Recently, a new generation of ATP-competitive mTOR kinase inhibitors has been developed that directly target the mTOR catalytic site and are able to completely suppress both mTORC1-mediated and mTORC2-mediated signaling, thereby suppressing the feedback activation of Akt [12,13]. Significantly, recent studies have shown that these agents are highly efficacious in reducing tumor growth *in vivo* and *in vitro* [6,9], and some of them are currently being tested in clinical trials [9,14].

PP242 is one such potent and selective active site-directed mTOR kinase inhibitor, and results in inhibition of both mTORC1 (including rapamycin-resistant 4E-BP1 phosphorylation) and mTORC2 activities [12,15]. Earlier studies showed greater inhibition of cell growth by PP242 compared with rapamycin in certain tumor models [9,16,17], thus highlighting a strong need to determine the efficacy of PP242 across a broad range of tumor types. Furthermore, little is known about the interactions of mTORC1/2 inhibitors with conventional chemotherapeutic drugs.

In the present study, we examined the antiproliferative and cytotoxic effects of PP242 in ESCC. We found that PP242 abrogated both mTORC1 and mTORC2 pathways in ESCC. PP242 also inhibited ESCC cell proliferation and cell cycle progression, and induced apoptosis. In addition, PP242 enhanced the chemosensitivity of ESCC cells to the chemotherapeutic drug cisplatin and modulated cisplatin-induced Akt activity.

Materials and methods

Cell culture and reagents

The human ESCC cell lines Eca-109 and TE-1 were purchased from the China Center for Type Culture Collection (Wuhan, China) and the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China), respectively. Eca-109 and TE-1 cells were cultured in RPMI 1640 medium (Gibco, Carlsbad, California, USA) supplemented with 10% fetal bovine serum.

PP242 and rapamycin were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Ku-0039674 was obtained from LC Laboratories (Woburn, Massachusetts, USA). These agents were each dissolved in dimethylsulfoxide and stored at −20°C. Cisplatin purchased from Sigma-Aldrich was prepared in 0.9% NaCl.

Cell proliferation assay

Cells were plated in 96-well plates at a density of 4000–5000 cells/well and treated with a range of drug concentrations of PP242 or rapamycin as indicated for 72 h. The effects of the drugs on cell proliferation were determined by a colorimetric assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich). IC_{50} values (the median inhibitory concentration) were calculated.

Colony-formation assay

For colony-formation assays, cells were seeded in six-well plates at a density of 600 cells/well (Eca-109) or 800 cells/well (TE-1). After 10–14 days of incubation, the colonies were fixed with 95% ethanol and stained with 0.1% crystal violet. The numbers of positive colonies (> 50 cells/colony) formed were counted, and all data on viability were calculated as percentages relative to untreated control cells.

Western blotting analysis

Following treatment, the cells were lysed in 100 μl of lysis buffer (20 mmol/l Tris-HCl pH 7.5, 1% Na-deoxycholate, 1% Triton X-100, 150 mmol/l NaCl, 1 mmol/l EDTA) containing a protease inhibitor cocktail (Roche, Mannheim, Germany). Protein extracts were normalized by the protein contents, resolved by 10% SDS-PAGE, and transferred to nitrocellulose membranes. The membranes were blocked for at least 1 h with 5% nonfat dried milk in TBST (TBS containing 0.1% Tween-20), followed by overnight incubation at 4°C with a diluted primary antibody. Subsequently, the membranes were washed three times with TBST and incubated with secondary antibodies at room temperature for 1 h. For immunodetection, the following primary antibodies were used: anti-phospho-mTOR (Ser2448), anti-phospho-Akt (Ser473), anti-phospho-p70S6K (Thr389), anti-phospho-4E-BP1 (Thr37/46), and anti-poly(ADP-ribose) polymerase (anti-PARP) from Cell Signaling Technology (Beverly, Massachusetts, USA); anti-Akt, anti-p70S6K, and anti-mTOR from Epitomics (Burlingame, California, USA). Anti-GAPDH and horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG were purchased from Boster (Wuhan, China).

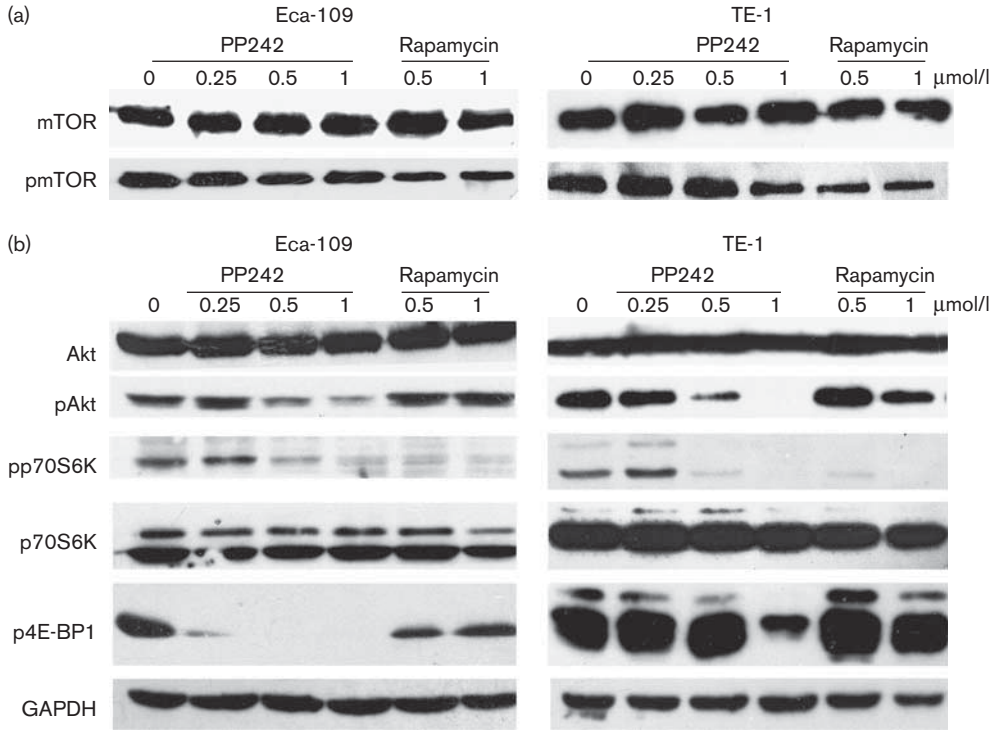
Apoptosis analysis

Cells were seeded in six-well plates at 1×10^5 cells/well, allowed to attach, and then treated with specified drugs. For apoptosis analyses, cells were harvested after 72 h of incubation. The harvested cells were washed with PBS, resuspended in binding buffer, and stained with annexin V–fluorescein isothiocyanate (FITC) and propidium iodide (PI) using a commercial kit (FITC Apoptosis Kit; KeyGEN Biotech, Nanjing, China). The fluorescence levels of apoptotic cells were determined by flow cytometry using a FACSort (Becton-Dickinson, Franklin Lakes, New Jersey, USA) and analyzed using CellQuest software (Becton-Dickinson). Apoptotic cells included both early apoptotic cells (positive for annexin V–FITC and negative for PI) and late apoptotic cells (positive for both annexin V–FITC and PI).

Cell cycle assay

For cell cycle analyses, cells were harvested after 48 h of treatment, washed with PBS, fixed with ice-cold 70%

Fig. 1



The mammalian target of rapamycin complex 1 (mTORC1)/mammalian target of rapamycin complex 2 (mTORC2) signaling pathways are blocked by the mammalian target of rapamycin (mTOR) kinase inhibitor PP242 in esophageal squamous cell carcinoma (ESCC) cells. Two human ESCC cell lines (Eca-109 and TE-1) were treated with increasing concentrations of PP242 (0.25, 0.5, and 1 μmol/l) or rapamycin (0.5 and 1 μmol/l) for 3 h, as indicated. The activities of mTOR, mTORC1, and mTORC2 were assessed by western blot analyses for the expressions of total mTOR, phosphorylated mTOR (pmTOR) (S2448) (a) and total p70S6K, phosphorylated p70S6K (pp70S6K) (T389), total Akt, phosphorylated Akt (pAkt) (S473), and phosphorylated 4E-binding protein-1 (p4E-BP1) (T37/46) (b). The experiment was repeated three times. GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

methanol, and incubated at 4°C overnight. The cells were then washed with cold PBS and treated with RNase for 30 min at 37°C. Cell nuclei were stained with PI for 1 h at 4°C in the dark, and detected by flow cytometry using the FACSort. The cell cycle profiles were analyzed using ModFit LT software (Becton-Dickinson).

Drug combination analysis

The combined effects of PP242 or rapamycin with cisplatin were determined using the Bliss additivism model. The Bliss value was expressed by the equation $E_{\text{Bliss}} = E_{\text{A}} + E_{\text{B}} - E_{\text{A}} \times E_{\text{B}}$, where E_{A} and E_{B} are the fractional inhibition effects of drug A and drug B alone at the desired concentrations, respectively. The E_{Bliss} value reflects the fractional inhibition that would be expected if the combination of the two drugs is exactly additive. If the experimentally measured fractional inhibition of the combined treatment is more than the expected E_{Bliss} value, the combination is considered to be synergistic, whereas if the experimentally combined effect is less than the expected E_{Bliss} value, the interaction is considered to be antagonistic. Otherwise, the combined effect is considered to be additive [18,19].

Statistical analysis

Each experiment was conducted in triplicate. The statistical significance of differences between two groups was evaluated using Student's *t*-test. SPSS 13.0 software (SPSS Inc., Chicago, Illinois, USA) was used to carry out all the statistical analyses. All *P*-values were two-sided, and values of *P* less than 0.05 were considered to indicate significance. All data were presented as the mean ± SD.

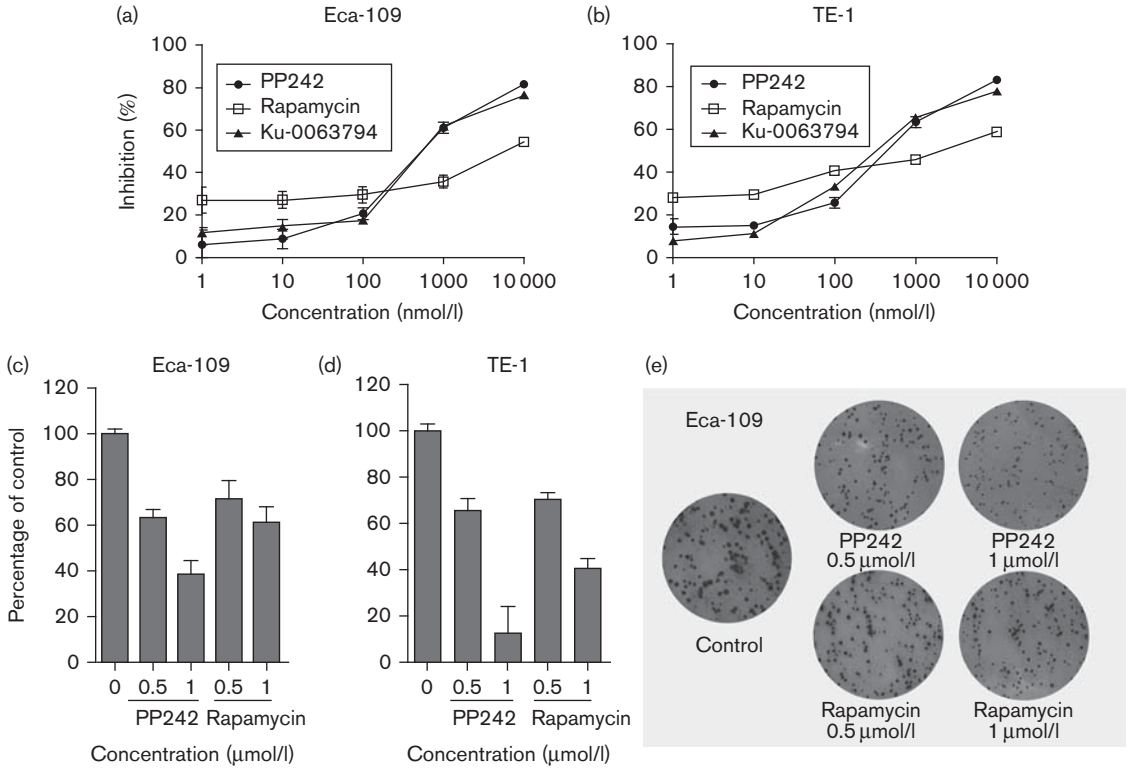
Results

PP242 inhibits mTORC1 and mTORC2 signaling in ESCC cells

We first identified the protein expression patterns of mTOR signaling in the two ESCC cell lines, Eca-109 and TE-1. Western blot analyses showed that mTOR was expressed and phosphorylated in both Eca-109 and TE-1 cells (Fig. 1a), suggesting that drugs targeting mTOR might affect ESCC biochemical activity. In both cell lines, the levels of phospho-mTOR (Ser2448) were inhibited by both rapamycin and PP242.

To investigate the effects of PP242 against mTORC1 and mTORC2, the two cell lines were treated with

Fig. 2



PP242 is more effective than rapamycin against esophageal squamous cell carcinoma (ESCC) cell lines. (a, b) Evaluation of the cell proliferation rates by MTT assays. Two ESCC cell lines (Eca-109 and TE-1) were treated with increasing concentrations of PP242 (1 nmol/l to 10 μmol/l), rapamycin (1 nmol/l to 10 μmol/l), or Ku-0063794 (1 nmol/l to 10 μmol/l) for 72 h. (c, d) Anticlonogenic effects of PP242 or rapamycin in ESCC cells determined by colony-formation assays. The cells were treated with the indicated doses of PP242 or rapamycin (0.5 and 1 μmol/l). (e) A representative picture of the colonies in Eca-109 taken using a digital camera is shown. The data were obtained from three experiments and represent means±SD.

PP242 or rapamycin at the indicated concentrations for 3 h. In contrast to rapamycin, PP242 strongly suppressed the phosphorylation of Akt (S473), an mTORC2 phosphorylation site. p70S6K phosphorylation (T389), which reflects mTORC1 activity, was inhibited efficiently by both PP242 and rapamycin. However, as expected, the rapamycin-insensitive site phosphorylation of 4E-BP1 (T37/46) was only decreased by the dual mTORC1/2 inhibitor PP242 (Fig. 1b). These findings indicate that PP242 effectively reduced mTORC1 and mTORC2 signaling in the ESCC cell lines, whereas rapamycin only partially inhibited the mTORC1 activity.

PP242 effectively suppresses ESCC cell proliferation

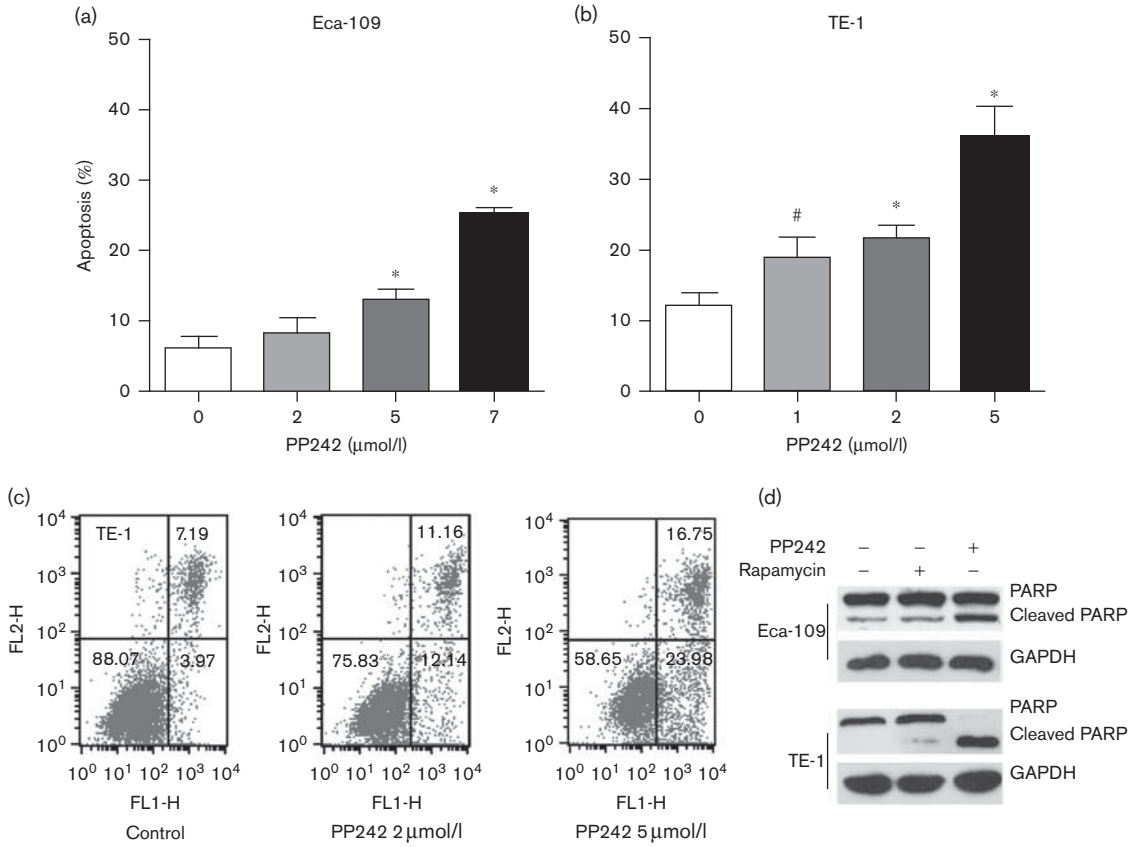
To assess the antitumor effects of PP242 compared with rapamycin on ESCC cells, Eca-109 and TE-1 cells were treated *in vitro* with increasing concentrations of the two drugs for 72 h and then analyzed by MTT assays. The results showed that both rapamycin and PP242 effectively suppressed the cell growth of the two cell lines (Fig. 2a and b). However, the rapamycin cytotoxicity dose–response curve reached a plateau in efficacy at 40–60% inhibition of cell growth. In contrast, PP242 could suppress cell growth by more than 80% in the treated cells at higher concentrations (IC_{50} = 0.679 μmol/l in Eca-109, IC_{50} = 0.554 μmol/l in TE-1 cells). The different shapes of these dose–response curves of rapamycin versus PP242 were very similar to those observed in previous studies [17,20]. Consistent with these results, PP242 inhibited the colony-formation ability of Eca-109 and TE-1 cells more effectively than rapamycin (Fig. 2c–e).

In addition, we used another selective mTORC1/2 inhibitor, Ku-0063794 [21,22], which is structurally unrelated to PP242, as a positive control for inhibition of the mTOR pathway. As shown in Fig. 2a and b, Ku-0063794 also showed greater antiproliferative potencies relative to rapamycin. These findings indicate the generally stronger anticancer effects of mTORC1/2 inhibitors.

PP242 induces apoptosis in ESCC cells

To understand the antitumor effects of the mTOR kinase inhibitor PP242 in ESCC, we investigated the impact of PP242 on apoptosis. In the present study, Eca-109 and TE-1 cells were incubated with PP242 for 72 h, followed by flow cytometric analysis of annexin V–FITC

Fig. 3



PP242 induces apoptosis in esophageal squamous cell carcinoma cells. (a–c) Eca-109 and TE-1 cells were treated with the indicated concentrations of PP242 for 72 h. The cells were then harvested and analyzed for apoptosis by annexin V–fluorescein isothiocyanate/propidium iodide staining and flow cytometry. The data in (a) and (b) represent the percentages of apoptotic cells as the sum of the early and late stages of apoptosis. (c) Shows representative flow data for the apoptotic effect of PP242 in TE-1 cells. (d) Eca-109 and TE-1 cells were incubated with 5 μmol/l PP242 or 5 μmol/l rapamycin for 24 h. Whole-cell lysates were subjected to western blot analyses for the expressions of total poly(ADP-ribose) polymerase (PARP) and cleaved PARP. The data were obtained from three experiments and represent means±SD. $^{*}P<0.01$, $^{\#}P<0.05$, versus control cells. GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

and PI staining. The results showed that PP242 induced apoptosis in the two ESCC cell lines, although only at relatively higher concentrations, as reported in other models [23,24]. PP242 induced significant increases in the percentages of apoptotic cells in Eca-109 cells compared with control cells at 5 and 7 μmol/l PP242 ($P < 0.01$ and $P < 0.01$, respectively; Fig. 3a), and in TE-1 cells at 1, 2, and 5 μmol/l PP242 ($P < 0.05$, $P < 0.01$, and $P < 0.01$, respectively; Fig. 3b and c). Consistently, PP242 significantly enhanced cleavage of PARP, an early marker of apoptosis, in both Eca-109 and TE-1 cell lines (Fig. 3d). However, we did not observe any proapoptotic activity of rapamycin in the two ESCC cell lines (Fig. 3d), consistent with a recently reported study in other solid tumors [16].

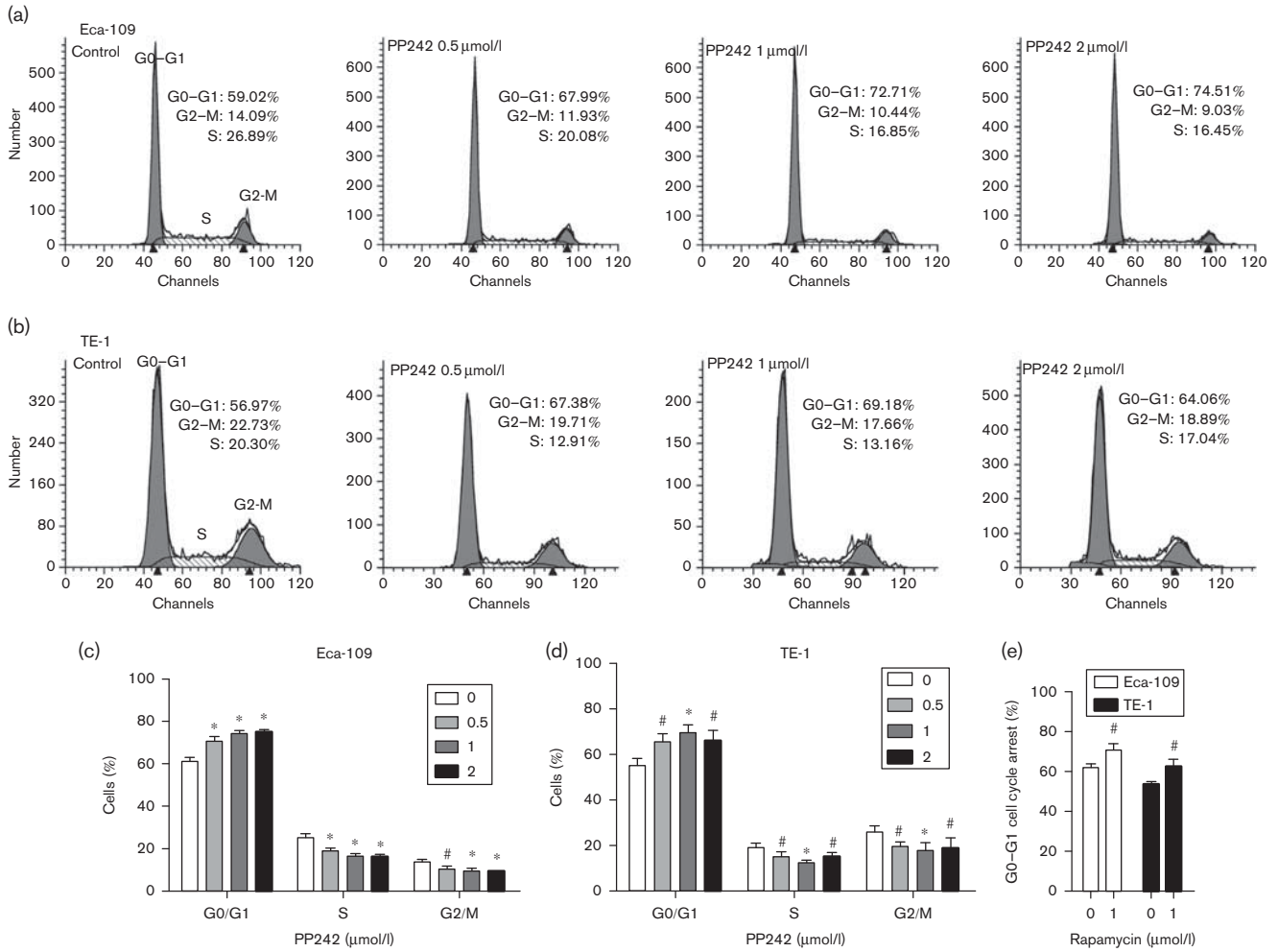
PP242 decreases ESCC cell cycle progression

Nest, we examined whether cell cycle arrest contributed toward the cell growth inhibition by PP242. For cell cycle analyses, the two ESCC cell lines were treated with PP242 at increasing concentrations for 48 h and then stained with PI. The results showed significant increases in G0–G1 cell cycle arrest in a dose-dependent manner, whereas the proportions of cells at S and G2/M were decreased ($P < 0.05$ vs. untreated cells; Fig. 4). In Eca-109 cells (Fig. 4a and c), the proportions of cells in the G0/G1 phase increased from 61.07% to 70.53, 74.21, and 75.02% with 0.5, 1, and 2 μmol/l PP242, respectively. Subsequently, significant inhibition of the S phase and the G2/M phase was observed. Similar results were obtained in the TE-1 cell line (Fig. 4b and d). Moreover, G0–G1 cell cycle arrest was observed in the two cell lines after treatment with rapamycin (Fig. 4e), but with less potency. Taken together, PP242 shows more effective antitumor effects than rapamycin in ESCC cells.

PP242 enhances the antitumor effects of cisplatin in ESCC cells

Cisplatin is a chemotherapeutic agent that is used commonly for many cancers. It has a narrow therapeutic window combined with severe side effects that limit its

Fig. 4



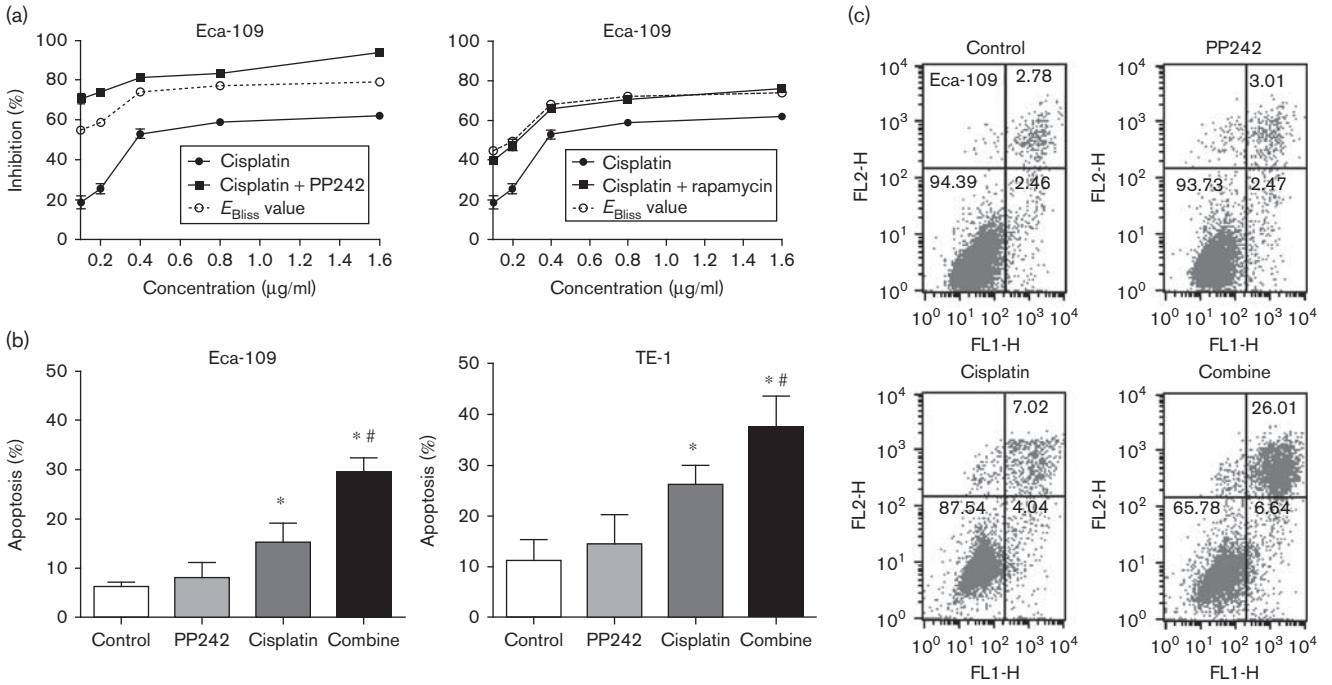
PP242 induces enhanced cell cycle arrest (G0–G1 phase) in Eca-109 and TE-1 cells. Esophageal squamous cell carcinoma cells were treated for 48 h with the indicated concentrations of PP242 (a–d) or rapamycin (e) and analyzed for cell cycle arrest by flow cytometry. (a, b) Show the effects of different treatments on the cell cycle distributions of Eca-109 and TE-1 cells. The values in (c)–(e) represent the percentages of cells in G0–G1, S, or G2/M cycle arrest. The data were obtained from three experiments and represent means±SD. $^{*}P<0.01$, $^{\#}P<0.05$, versus control cells.

broader use, including in ESCC. Thus, there is a strong need to reduce the dose of cisplatin, without compromising its anticancer effects. Combined treatment with a targeted therapy, such as an mTORC1/2 inhibitor, might potentiate its anticancer effects. A few recent studies have raised the possibility that mTORC1/2 inhibitors may serve as sensitizers for cisplatin [16,25]. To examine the interaction of the mTORC1/2 kinase inhibitor with cisplatin, we examined the effects of cisplatin alone or in combination with PP242 on the ESCC cell line Eca-109. We used the Bliss additivism model to evaluate the combined effects. Eca-109 cells were treated with cisplatin at low concentrations (0.1–1.6 μg/ml) alone or with PP242 (400 nmol/l). The proliferation curve and the Bliss theoretical curve expected if the combination was additive are shown in Fig. 5a. On comparing the experimental and theoretical curves, we found that the experimentally measured fractional inhibition of the combined treatment was more than the expected E_{Bliss} value, indicating that the combined treatment with cisplatin and PP242 had a synergistic effect on Eca-109 cells. In contrast, rapamycin did not show any synergistic interactions with cisplatin in Eca-109 cells, as no significant differences were observed between the experimental and theoretical values (Fig. 5a).

PP242 enhances cisplatin-induced apoptosis in ESCC cells

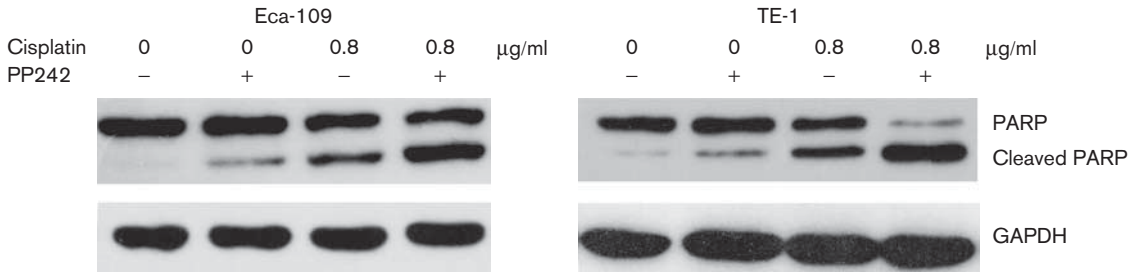
Cisplatin, a DNA-damaging agent, can induce apoptosis in a variety of cancer cell lines [26]. We further examined whether PP242 enhanced cisplatin-mediated cell apoptosis. For this purpose, Eca-109 and TE-1 cells were treated with PP242 (400 nmol/l) and cisplatin (0.8 μg/ml) alone or in combination for 72 h. Apoptosis was determined by flow cytometric analysis (Fig. 5b and c).

Fig. 5



PP242 sensitizes esophageal squamous cell carcinoma (ESCC) cells to cisplatin and potentiates cisplatin-induced apoptosis. (a) Combined treatment with PP242 and cisplatin shows a synergistic inhibitory effect on cell proliferation in Eca-109 cells. Eca-109 cells were treated with increasing concentrations of cisplatin (0.1–1.6 μg/ml) alone or in combination with PP242 or rapamycin at a fixed concentration (400 nmol/l). Proliferation curves versus the theoretical Bliss curve (dashed line) are shown. (b) PP242 potentiates cisplatin-induced ESCC cell apoptosis. Eca-109 and TE-1 cells were treated with cisplatin (0.8 μg/ml) alone or in combination with PP242 (400 nmol/l) for 72 h. (c) Representative flow cytometry data for Eca-109 cells. $^{*}P<0.01$, versus control cells; $^{\#}P<0.05$, versus cisplatin. The data were obtained from three independent experiments and represent means±SD.

Fig. 6



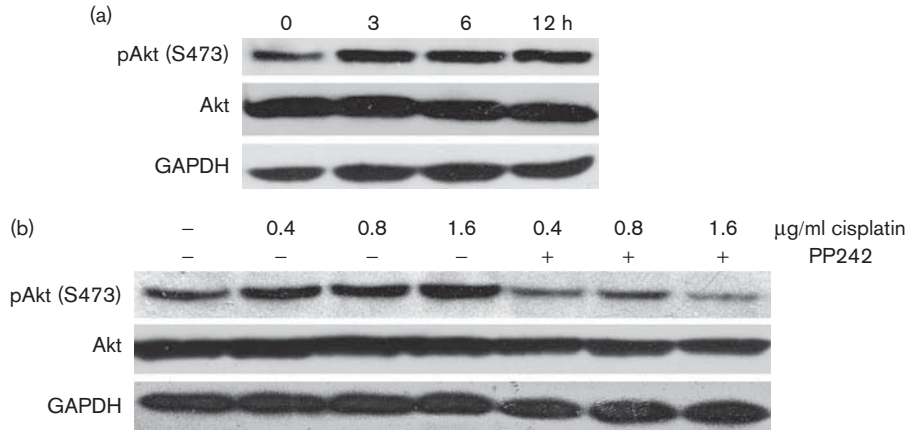
PP242 enhances cisplatin-induced cleavage of poly(ADP-ribose) polymerase (PARP) in esophageal squamous cell carcinoma cells. Eca-109 and TE-1 cells were treated with cisplatin (0.8 μg/ml) alone or in combination with PP242 (400 nmol/l) for 24 h. Whole-cell lysates were subjected to western blot analysis for the expression of total PARP and cleaved PARP. The experiment was repeated three times. GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

In Eca-109 cells, the percentage of apoptotic cells for the combined treatment (32.65%) was significantly higher than that for PP242 (5.48%) or cisplatin (11.06%) alone (Fig. 5c). Similar results were observed in TE-1 cells (Fig. 5b). Moreover, western blot analyses were carried out to characterize the apoptosis. After 24 h of treatment with the indicated drugs, PP242 enhanced cisplatin-induced cleavage of PARP in both Eca-109 and TE-1 cells (Fig. 6).

PP242 inhibits cisplatin-induced Akt phosphorylation in ESCC cells

It has been reported that the Akt pathway plays an important role in the response to chemotherapeutic

Fig. 7



PP242 reduces the level of phosphorylated Akt (S473) induced by cisplatin in Eca-109 cells. (a) Eca-109 cells were treated with cisplatin (0.8 μg/ml) for the indicated times (3, 6, and 12 h). (b) Eca-109 cells were treated with increasing concentrations of cisplatin (0.4, 0.8, and 1.6 μg/ml) alone or in combination with PP242 (400 nmol/l) for 3 h. Whole-cell lysates were subjected to western blot analyses for the expressions of phospho-Akt (pAkt) (S473) and total Akt. The experiment was repeated three times. GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

drugs [27,28]. Thus, we examined the pharmacological effects of the combined treatment on Akt activity to further investigate the possible molecular mechanism for the synergistic effect. As shown in Fig. 7, combined treatment with cisplatin and PP242 reduced the Akt phosphorylation level after 3 h. Meanwhile, in cisplatin-treated cells, Akt was constitutively activated (Fig. 7a and b). These findings indicate that Akt activity is likely to play an essential role in the synergism between PP242 and cisplatin in ESCC cells. Taken together, our data suggest that targeted inhibition of mTORC1/2 markedly potentiates the anticancer effects of cisplatin in ESCC.

Discussion

ESCC is one of the most frequently diagnosed cancers in developing countries. However, despite intensive therapies, the 5-year survival rate for ESCC remains poor [29–31]. Thus, new therapeutic approaches are urgently needed. The mTOR signaling cascade regulates downstream cellular events required for mRNA translation and plays critical roles in neoplastic cell proliferation and growth. Recent studies on ESCC have obtained evidence for aberrant activation of mTOR in ESCC [3,32–35], and have thus provided new targets for anticancer drug treatment in this disease.

In the present study, we have shown for the first time that the dual mTORC1/2 inhibitor PP242 shows more potent antitumor effects than rapamycin on ESCC *in vitro*, with confirmed blocking of both mTORC1 and mTORC2. PP242 inhibited 4E-BP1 phosphorylation and abrogated mTORC1-dependent PI3K/Akt feedback activation. We further showed that PP242 could promote cisplatin-induced apoptosis, and exerted a synergistic antitumor effect by combining this mTOR kinase inhibitor with cisplatin. In many other studies, the biochemical activity of dual mTORC1/2 kinase inhibitors appears more interesting than that of rapamycin, which only partly inhibits mTORC1 activity when used as an anticancer drug [9].

The mTOR signaling network has been reported to be activated in several models of ESCC. In the present study, the two cell lines expressed mTOR and its downstream effectors, supporting the notion that mTOR may be an important target for anticancer drug treatment in ESCC. There is a consensus that rapamycin is largely ineffective for inhibiting mTORC2 activity and that rapamycin-induced mTORC1 inhibition activates a feedback loop toward PI3K/Akt [6,9]. Consistent with this, our results showed that phospho-Akt (S473) was unregulated in Eca-109 and TE-1 cells after treatment with rapamycin. The typically identified feedback activation of Akt in rapamycin may arise through destabilization of insulin receptor substrate 1 (IRS1) by S6K1 phosphorylation [36]. However, as many downstream targets of mTORC1 remain to be identified, it is probable that there are further substrates that may also contribute toward this feedback loop, such as growth factor receptor-bound protein 10 (Grb10) [37,38]. In contrast, mTORC1/2 inhibitors are supposed to effectively inhibit rapamycin-insensitive mTORC2 activity and therefore prevent the occurrence of negative feedback loops [8,10]. Here, we found that PP242 effectively inhibited the phosphorylation of Akt. Akt activation could protect against apoptosis and may result in the limited efficacy of rapalogs when used as monotherapies. In fact, rapamycin did not fully inhibit the function of mTORC1. It was more effective as an mTOR inhibitor of p70S6K phosphorylation on a

molecular basis. Like many other studies [6,9], our data showed that PP242 exerted a greater inhibitory effect on the mTORC1 substrate 4E-BP1, which controls cap-dependent mRNA translation. 4E-BP1 is now recognized as an important rapamycin-insensitive mTORC1 complex [13]. The persistence of highly phosphorylated 4E-BP1 molecules in rapamycin-treated cells allows efficient protein translation, which results in intrinsic rapamycin resistance. Consequently, PP242 is a more potent inhibitor of cancer cell proliferation and growth.

Consistent with its biochemical activity, we have shown a greater antiproliferative potency of PP242 relative to rapamycin in ESCC cells. Significantly, PP242 decreased cell cycle progression and induced apoptosis in the ESCC cells examined in a dose-dependent manner, which was in agreement with the effects of other mTORC1/2 inhibitors in solid tumors or hematological tumors [17,23]. In many recently reported studies, the mTORC1/2 inhibitor PP242 or OSI-027 induced apoptosis in solid tumors, lymphoma, and acute myeloid leukemia [16,24,39]. Taken together with our results, we have established a critical role for mTORC1 and mTORC2 in the survival and proliferation of ESCC, and emphasized the antitumor effects of the mTORC1/2 inhibitor PP242 in suppressing ESCC cell growth.

mTORC1/2 inhibitors are also suggested for synergistic combinations in chemotherapy. However, evidence is scarce. In aggressive prostate hormone-refractory prostate cancer cells, the mTORC1/2 inhibitor Palomid 259 was able to sensitize the cancer cells to cisplatin and docetaxel [25]. Most chemotherapeutic agents, such as cisplatin, are major treatment agents for a wide range of tumor types. Improvements in the efficacy of cisplatin are urgently needed. However, the use of cisplatin is limited by its cytotoxic side effects: too high a dose is cytotoxic and too low a dose compromises its therapeutic effects [40–42]. Combined treatment with a 'sensitizer' may have the potential to considerably enhance its antitumor effects, while limiting its cytotoxic effects [40]. Here, we examined the dual mTORC1/2 kinase inhibitor PP242 in combination with cisplatin, which is commonly used in ESCC and is recognized as one of the most promising treatments for esophageal cancer [43,44]. However, considerable adverse effects exist that limit its efficacy. In this study, we showed that PP242 sensitized ESCC cells to cisplatin and markedly potentiated cisplatin-induced apoptosis, suggesting a better clinical approach using an mTORC1/2 kinase inhibitor for combination therapies in clinical trials. To understand the mechanism underlying the effects of the combined treatment, we examined the effects of PP242 combined with cisplatin on Akt. It has been shown previously that Akt signaling plays important roles in the survival and drug resistance of cancers [27,28]. Our data showed that Akt was activated after treatment with cisplatin alone, which might transmit survival signals and inactivate the apoptotic pathways [25]. In contrast, PP242 reduced the Akt activity in cisplatin-treated cells. This finding is in agreement with the previous report on prostate cancers [25]. It is plausible that PP242 may also target other signaling factors to exert its synergistic effect with cisplatin, and identification of such factors will require further studies.

In conclusion, our study has provided preclinical evidence that dual inhibition of mTORC1 and mTORC2 is a very effective approach for the treatment of ESCC as a monotherapy or when combined with chemotherapy. We strongly support further investigation of the potential of mTORC1/2 inhibitors alone or in combination with cisplatin for the treatment of ESCC in clinical trials.

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Conflicts of interest

There are no conflicts of interest.

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