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The anti-esophageal cancer cell activity by a novel tyrosine/phosphoinositide kinase inhibitor PP121

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ABSTRACT

Here we explored the potential effect of PP121, a novel dual inhibitor of tyrosine and phosphoinositide kinases, against human esophageal cancer cells. We showed that PP121 exerted potent cytotoxic effect in primary (patient-derived) and established (Eca-109, TE-1 and TE-3 lines) esophageal cancer cells, possibly through activating caspase-3-dependnent apoptosis. PP121 was, however, non-cytotoxic to the normal human esophageal epithelial cells (EECs). At the molecular level, we showed that PP121 blocked Akt-mTOR (mammalian target of rapamycin) activation in esophageal cancer cells, which was restored by introducing a constitutively-active Akt (CA-Akt). Yet, CA-Akt only partly inhibited cytotoxicity by PP121 in Eca-109 cells. Importantly, we showed that PP121 inhibited nuclear factor kappa B (NFκB) signaling activation in esophageal cancer cells, which appeared independent of Akt-mTOR blockage. *In vivo*, oral administration of PP121 remarkably inhibited Eca-109 xenograft growth in nude mice, and significantly improved mice survival. Further, the immunohistochemistry (IHC) and Western blot assays analyzing xenografted tumors showed that PP121 inhibited Akt-mTOR and NFκB activations *in vivo*. Together, we demonstrate that PP121 potently inhibits esophageal cancer cells *in vitro* and *in vivo*, possibly through concurrently inhibiting Akt-mTOR and NFκB signalings.

1. Introduction

Esophageal cancer is the third most common tumor in the digestive tracts [1–3]. It also ranks the sixth leading cause of cancer-related mortality around the world [1–3]. Yet, the incidence of esophageal cancer is increasing at an alarming rate, particularly in the Eastern countries [3,4]. In the past decades, clinical and basic research of esophageal cancer has achieved significant progresses, however there has been no extremely effective chemotherapeutic agents for this devastating disease [1,2]. Thus, there is an urgent need to develop novel and effective anti-esophageal cancer agent [1,2].

The phosphatidylinositide 3-kinase (PI3K)-Akt-mammalian target of rapamycin (mTOR) pathway plays a pivotal role in cancer initiation and progression [5,6]. Over-activation of this pathway promotes cancer cell survival, proliferation, apoptosis-resistance as well as tumorigenesis, and metastasis [5,6]. Recent studies have shown that this pathway is over-activated in esophageal cancers, which represents a valuable therapeutic target for effective treatment [7–9].

Recent studies have developed a novel tyrosine and phosphoinositide kinase dual inhibitor, named PP121 [10,11]. Its activity in cancer cells, however, has not been extensively studied [11]. One exemption is a study by Che and colleagues, who demonstrated that PP121 inhibited anaplastic thyroid carcinoma cell proliferation *in vitro* and *in vivo* through blocking PI3K/Akt signalings [11]. In the current study, we investigated the activity of PP121 in human

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esophageal cancer cells. In particular, we examined molecular changes by PP121 by focusing on Akt-mTOR and nuclear factor kappa B (NFκB) signalings.

2. Material and methods

2.1. Reagents and antibodies

PP121, rapamycin, RAD001 and LY294002 were obtained from Selleck (Shanghai, China). The caspase inhibitors z-VAD-fmk and z-DVED-fmk were obtained from Calbiochem (Shanghai, China). All antibodies utilized in this study were purchased form Cell Signaling Tech (Denver, MA). All cell culture agents were obtained from Gibco (Shanghai, China).

2.2. Cell culture

Established human esophageal cancer cell lines, Eca-109, ET-1 and ET-3, were obtained from the Cell Bank of Chinese Academy of Sciences (Shanghai, China). Cells were cultured in RPMI-1640/DMEM medium plus 8–10% fetal bovine serum (FBS), penicillin (100 units/mL), and streptomycin (100 μg/mL) in an atmosphere of 5% CO_2. The human esophageal epithelial cells (EECs) [12] were obtained from Wuhan PriCells Biomedical Technology Co. (Wuhan, China). The cell line is derived from the esophageal tissues of a 4-month-old female aborted fetus, which expresses cytokeratin, a marker of normal (non-cancerous) esophageal epithelial cells [12]. EECs were cultured in RPMI-1640 medium supplemented with 10% FBS [12].

2.3. Primary esophageal cancer cell isolation and culture

The surgery-isolated fresh esophageal epithelial carcinoma specimens were thoroughly washed in PBS and 2 mM DTT (Sigma). The cancer tissues were then minced, followed by digestion with the 0.25% (w/v) collagenase I (Sigma) at 37 °C for 1 h. Acquired cancer cell pellets were thoroughly washed, and re-pelleted at 500 g for 5 min. Individual cells were pelleted, rinsed, re-suspended in cell culture medium (DMEM, 10% FBS, 10 mg/Ml transferrin, 2 mM glutamine, 1 mM pyruvate, 10 mM HEPES, 100 units/mL penicillin/streptomycin, 0.1 mg/mL gentamicin, 0.2 units/mL insulin, and 2 g/L fungizone). All patients received no thermotherapy or radiation prior to surgeries, and all signed the written informed consents. All clinical investigations were conducted according to the principles expressed in the Declaration of Helsinki, and were approved by the institutional review board of all authors' institutions.

2.4. Cell survival assay (MTT assay)

As reported previously [13], cell survival was measured by the 3-[4,5-dimethylthylthiazol-2-yl]-2,5 diphenyltetrazolium bromide (MTT) method.

2.5. Soft agar colony formation assay

Cells were seeded onto 6-well plates (4×10^3 cells per well). After treatment, cells were trypsinized and suspended in RPMI 1640 medium containing 0.33% agar and 10% FBS, and then layered on RPMI 1640 containing 0.6% agar and 10% FBS in a 6-well plate. PP121 containing medium was switched every 2 d. Colonies were counted 10 days (d) later.

2.6. LDH release assay

Cell death was tested by lactate dehydrogenase (LDH) release through a commercially available enzymatic reaction LDH assay kit (Takara, Tokyo, Japan). The percentage of released LDH was calculated by the following formula: % LDH release = LDH released in conditional medium/(LDH released in conditional medium + LDH in cell lysates). Cells were lysed by Triton X-100 (1%).

2.7. TUNEL staining

The cell apoptosis was detected by the TUNEL (Terminal deoxynucleotidyl transferase dUTP nick end labeling) In Situ Cell Death Detection Kit (Roche Molecular Biochemicals, Indianapolis, IN) according to the manufacturer's instructions. Cells were also stained with 4′,6'-diamino-2-phenylin-dole (DAPI, blue fluorescence; Molecular Probes) to visualize the cell nuclei. Cell apoptosis rate was calculated by the TUNEL percentage (TUNEL vs. DAPI) detected under a fluorescence microscope (Zeiss).

2.8. Histone DNA ELISA assay

Cell apoptosis after applied treatment was also detected by the Histone DNA ELISA assay as described in our previous study [13].

2.9. Caspase-3 activity assay

After applied treatment, cell lysates (20 μg/sample) were assayed for caspase-3 activity utilizing a DEVDpNA peptide substrate, and incubated for 6 h at 37 °C. Caspase-3 activity was measured using a colorimetric assay kit from R&D Systems (Minneapolis, MN). The activities were quantified spectrophotometrically at a wavelength of 405 nm.

2.10. Western blots

As previously reported [13], equal amount of protein samples (30–40 μg/sample) were separated by electrophoresis in SDS-PAGE, transferred to the PVDF membrane and detected with the specific antibody. The immuno-reactive proteins after incubation with appropriately labeled secondary antibody were detected with the enhanced chemiluminescence (ECL) detection kit (Amersham, Buckinghamshire). Band intensity was quantified by ImageJ software (NIH) after normalization to the corresponding loading control.

2.11. Measuring NFκB (p65) DNA-binding activity

After treatment, nuclear proteins were extracted through the nuclear extraction kit (Sigma, Shanghai, China). NFκB (p65) DNA-binding activity was examined using the TransAM ELISA kit (Active Motif, Carlsbad, CA) according to the manufacturer's protocol. In brief, 1.0 μg of nuclear extract was subjected to the binding of NFκB to an immobilized consensus sequence in a 96-well plate, and the primary and secondary antibodies were added. After the colorimetric reaction, OD value of samples was measured in an ELISA reader at 450 nm.

2.12. Constitutively active Akt1 (CA-Akt) transfection and stable cell selection

The plasmid encoding a constitutively active Akt1 (CA-Akt) as well as the empty vector were gift from Dr. Zhu's group [14]. Cells were seeded onto a 6-well plate with 60–70% confluence. Lipofectamine 2000 was applied to transfected the CA-Akt or the

empty vector with recommended procedure [14]. Stable cells were selected by puromycin (2.5 μg/mL, Sigma).

2.13. Mice xenograft assay

Eca-109 cells were injected into the axillary regions of nude mice (5×10^6 cells/mouse). When the tumor volumes reached around 200 mm^3, the mice were randomly separated to three groups: Untreated control, PP121 (30 mg/kg) [11] and vehicle (10% 1-methyl-2-pyrrolidinone and 90% PEG 300) group [11] ($n = 10$ per group). Tumor volumes and the mice body weights were measured every 10 d. The tumor volumes were calculated with the formula (mm^3) = width × width × length × 0.5. Two weeks after initial treatment, two mice per group were euthanized, and tumors were isolated, and were subjected to Western blots and Immunohistochemistry (IHC) staining assay. All care and treatment of experimental animals were performed according to the animal care and use committee guidelines.

2.14. Immunohistochemistry (IHC)

The slides were de-paraffinized and hydrated with 100% ethanol twice for 10 min, 95% ethanol twice for 10 min, and deionized water for 2 min. For antigen unmasking, slides were placed in a container, covered with 10 mM sodium citrate buffer, pH 6.0, and heated in a steamer for 1 h. The slides were then washed and blocked with 5% normal goat serum for 30 min, and were then incubated with the appropriate dilutions of primary antibodies (anti-p-Akt Ser 473, 1:100; anti-p-IKKα/β, 1:100), and subsequently stained them with horseradish peroxidase (HRP)-coupled secondary antibodies (Santa Cruz). The slides were then visualized using 3-amino-9-ethyl-carbazol (AEC) and counterstained tissues with MAYER'S solution (Merck).

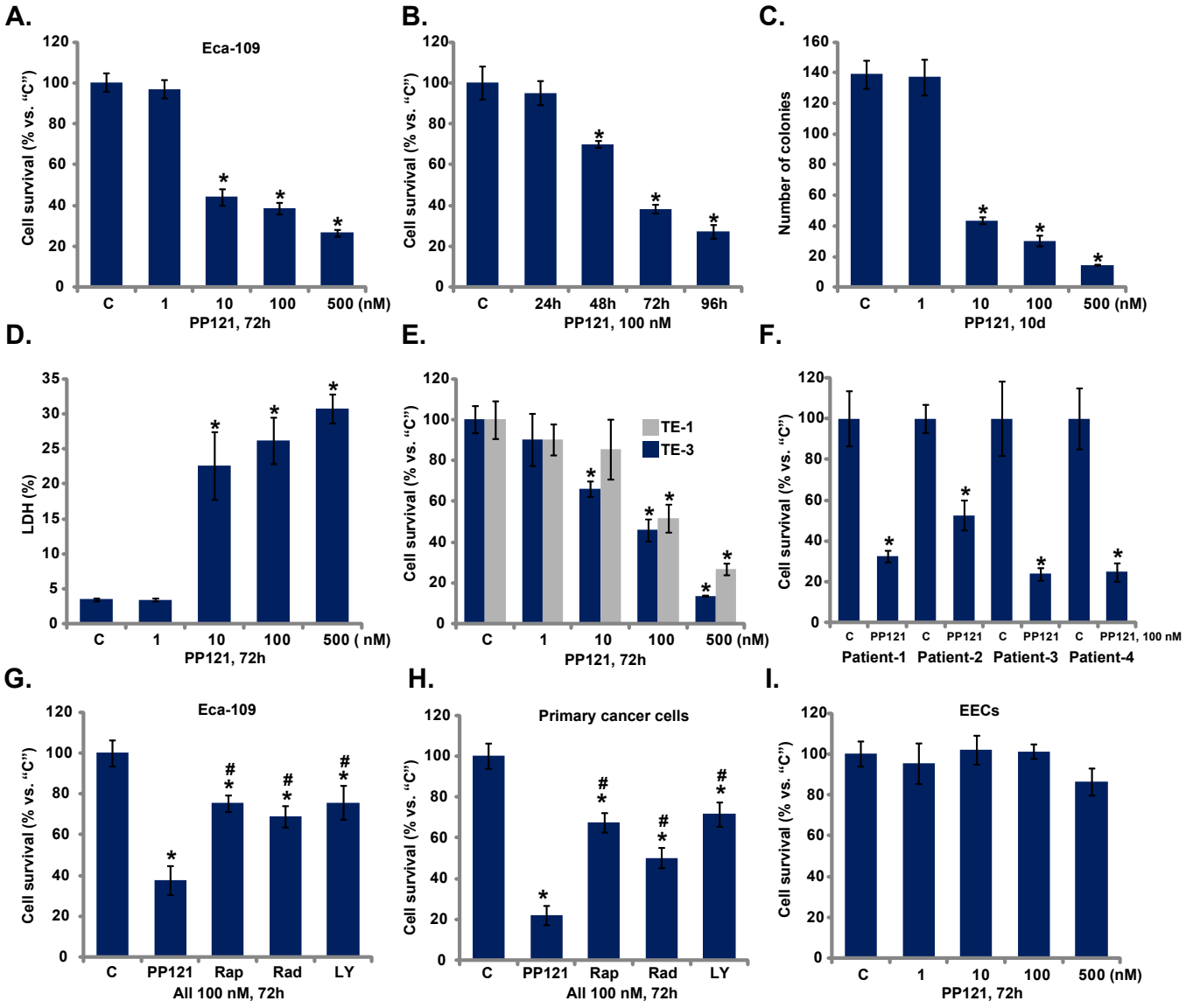

Fig. 1. PP121 exerts potent cytotoxic effect against esophageal cancer cells. Eca-109 (**A**–**D**, **G** and **H**), TE-1 (**E**) and TE-3 (**E**) human esophageal cancer cell lines, as well as the primary human esophageal cancer cells (**F**) and the normal human esophageal epithelial cells (EECs, **I**) were either left untreated ("C"), or stimulated with applied concentrations of PP121, or the known PI3K/mTOR inhibitors [rapamycin ("Rap"), RAD001 ("Rad") or LY294002 ("LY")] for indicated time, cell survival was tested by MTT assay (**A**, **B**, **E**, **F**, **G**–**I**); Colony formation assay (**C**, for Eca-109 cells) and LDH release assay (**D**, for Eca-109 cells) were also performed. Data were means ± SD of three independent experiments. *P < 0.05 compared with the "C" group. #P < 0.05 compared with the PP121 only group (**G**–**I**).

2.15. Statistics

The results were expressed as the mean ± standard deviation (SD). Statistical significance ($P < 0.05$) was evaluated by one-way ANOVA followed by Bonferroni post hoc test (SPSS 18.0, Chicago, IL). All experiments were performed in triplicate, and similar results were obtained. The time point of each experiment was chosen based on the pre-experimental results.

3. Results

3.1. PP121 exerts potent cytotoxic effect against esophageal cancer cells

This study aimed to explore the potential activity of PP121 on esophageal cancer cells. Eca-109, an immortalized human esophageal cancer cell line, was applied [15]. MTT cell survival assay results showed that PP121 potently inhibited Eca-109 cell survival (Fig. 1A and B). The effect of PP121 was both dose-dependent (Fig. 1A) and time-dependent (Fig. 1B). Meanwhile, the colony formation assay results in Fig. 1C further confirmed the cytotoxic effect of PP121 against Eca-109 cells, as the number of survival Eca-109 colonies was dramatically decreased after applied PP121 (10–500 nM) treatment (Fig. 1C). Further, we showed that the level of medium LDH, an indicator of Eca-109 cell death, was significantly increased after PP121 (10–500 nM) treatment (Fig. 1D). Results in Fig. 1E showed PP121 was cytotoxic to two other human esophageal cancer cell lines (TE-1 and TE-3). More importantly, as shown in Fig. 1F, PP121 at 100 nM significantly inhibited survival of primary human esophageal cancer cells, which were derived four

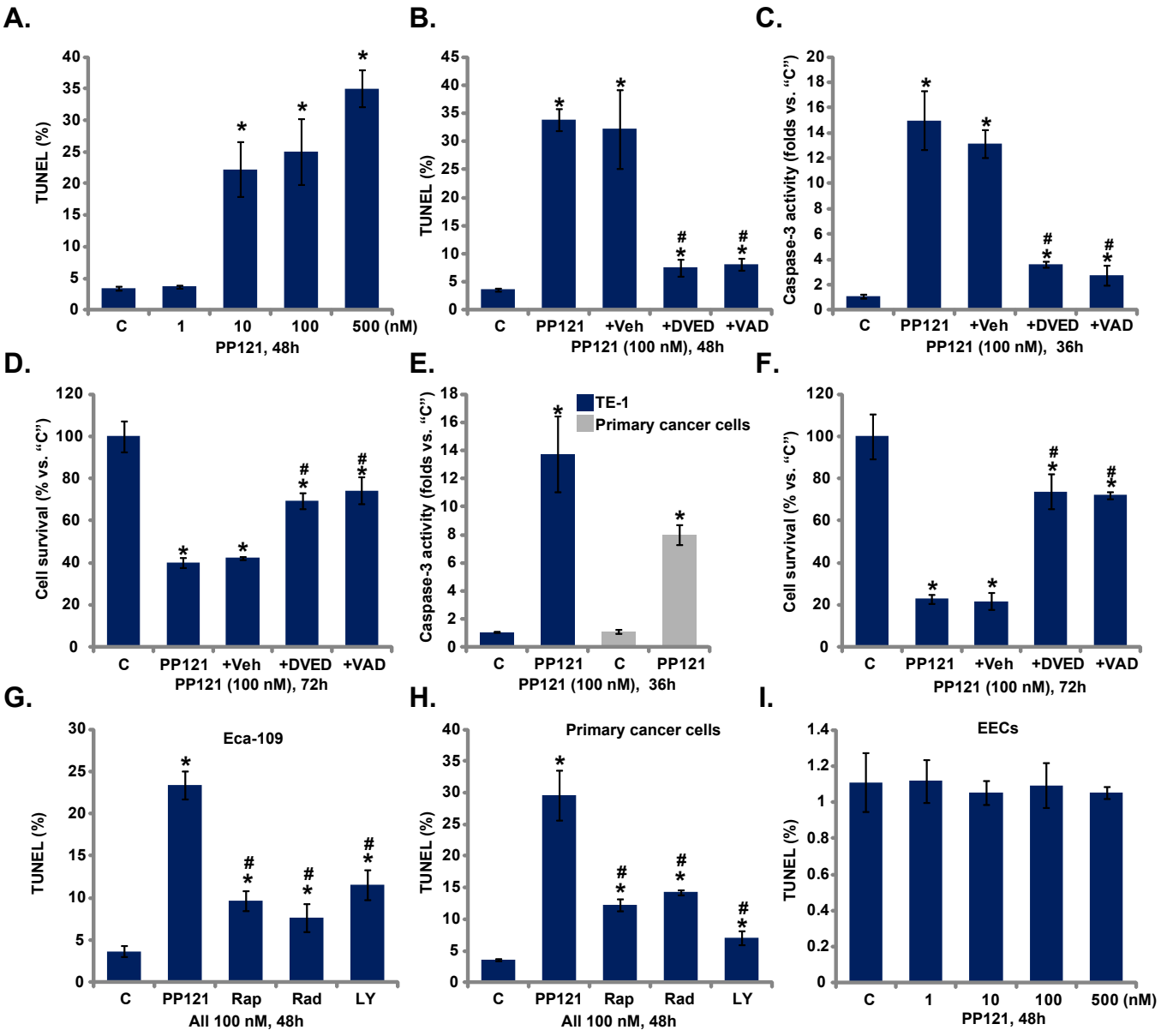

Fig. 2. PP121 induces caspase-3-dependnent apoptosis in esophageal cancer cells. Eca-109 (**A** and **G**), TE-1 (**E**), the primary human esophageal cancer cells (**E** and **H**) or the EECs (**I**) were either left untreated ("C"), or stimulated with applied concentrations of PP121, rapamycin ("Rap"), RAD001 ("Rad") or LY294002 ("LY") for indicated time period, cell apoptosis was tested by TUNEL staining assay. Eca-109 (**B**–**D**) or the primary human esophageal cancer cells (**F**), pretreated with the caspase-3 inhibitor z-DVED-fmk (DVED, 50 μM) or the pan caspase inhibitor z-VAD-fmk (VAD, 50 μM) for 1 h, were stimulated with PP121 (100 nM) for applied time, cell apoptosis was tested by TUNEL staining assay (**B**) or the caspase-3 activity assay (**C**), and cell survival was tested by MTT assay (**D** and **F**). Data were means ± SD of three independent experiments. *$P < 0.05$ compared with the "C" group. #$P < 0.05$ compared with the PP121 only group (**B**–**D**, **F**–**H**).

independent esophageal cancer patients. PP121 was remarkably more potent than the same concentration (100 nM) of traditional mTOR complex 1 (mTORC1) inhibitors [Rapamycin (Rap) and RAD001 (Rad)], or the PI3K-Akt-mTOR pan inhibitor LY294002 (Fig. 1G and H). The same PP121 treatment showed almost no effect against normal human esophageal epithelial cells (EECs) (Fig. 1I). Together, these results demonstrate that PP121 exerts significant cytotoxic effect against human esophageal cancer cells.

3.2. PP121 induces caspase-3-dependnent apoptosis in esophageal cancer cells

Above results showed that PP121 efficiently inhibited survival of human esophageal cancer cells. Next, we tested whether apoptosis played a role. Results showed that the number of TUNEL positive ("apoptotic") cells was significantly increased after applied PP121 treatment (Fig. 2A), which was largely inhibited by the specific caspase-3 inhibitor z-DVED-fmk and the pan caspase inhibitor z-VAD-fmk (Fig. 2B). Further, the caspase-3 activity was also increased by PP121 in Eca-109 cells (Fig. 2C). z-DVED-fmk and z-VAD-fmk, which expectedly blocked caspase-3 activation (Fig. 2C), also significantly inhibited PP121-indued Eca-109 cytotoxicity (Fig. 2D). Note that PP121 also induced caspase-3 activation in TE-1 and primary human esophageal cancer cells (Fig. 2E). The two caspase inhibitors also alleviated cytotoxicity by PP121 in primary esophageal cancer cells (Fig. 2F). These caspase inhibitors alone showed no activity on esophageal cancer cell survival or apoptosis (Data not shown). Once again, PP121 was more efficient than rapamycin, RAD001 and LY294002 in inducing esophageal cancer cell apoptosis (Fig. 2G and H), but was non-effective in EECs (Fig. 2I). Collectively, these results suggest that PP121 induces caspase-3-dependnent apoptosis in esophageal cancer cells.

3.3. PP121 blocks Akt-mTOR activation in esophageal cancer cells

Next, we studied the signaling changes after PP121 treatment in esophageal cancer cells. Western blots were utilized to tested Akt-mTOR activation in PP121-treated cells. Results demonstrated that PP121 almost completely blocked activation of mTORC1 and mTOR complex 2 (mTORC2) in both Eca-109 (Fig. 3A) and primary esophageal cancer cells (Fig. 3B). Note that mTORC1 activation was reflected by phosphorylated (p-) p70S6 Kinase 1 (S6K1) and p-S6 [16,17], while p-Akt Ser-473 was tested as an indicator of mTORC2 activation [16,17]. The mTORC1-inhibitory activity by PP121 was

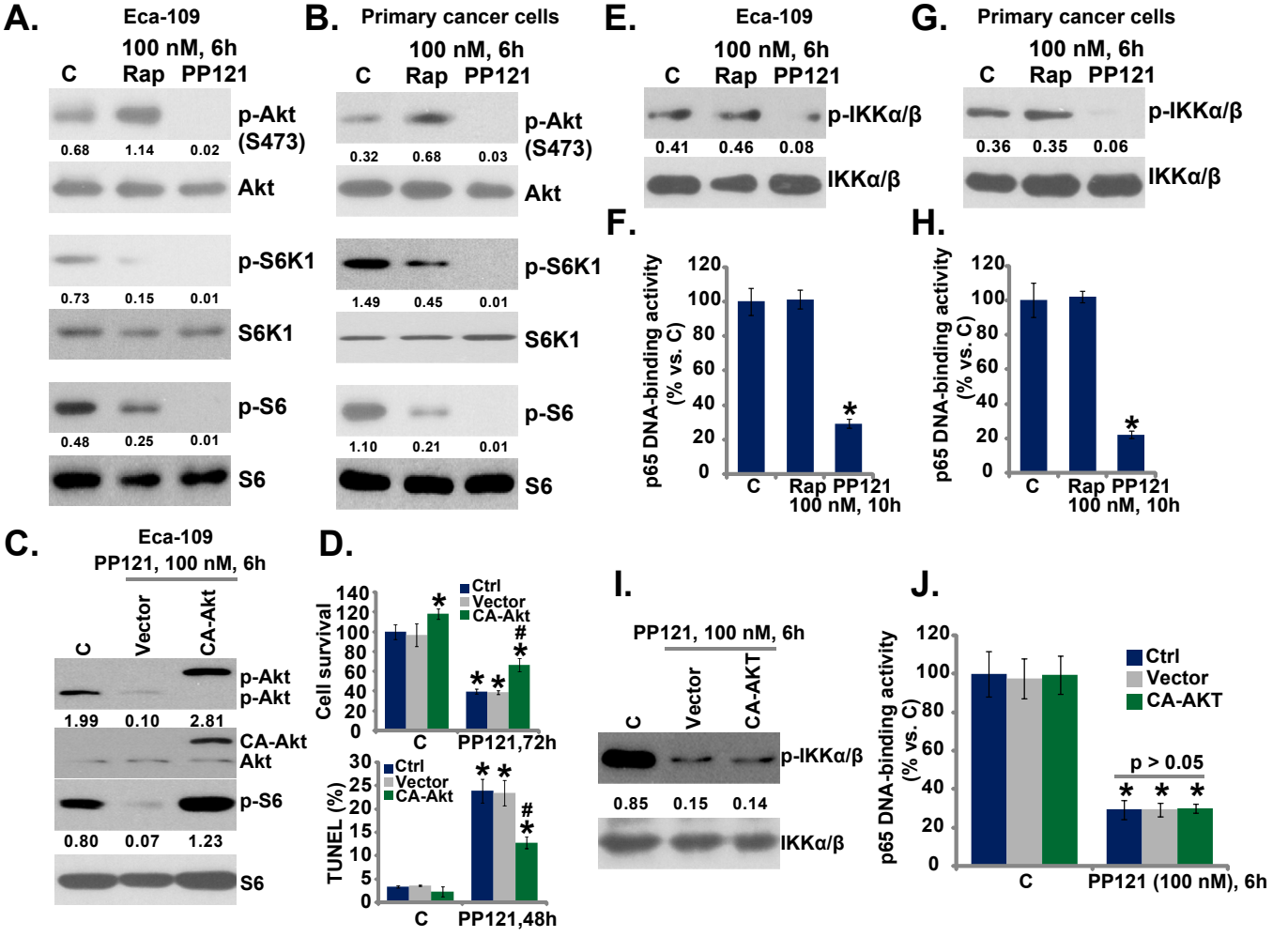

Fig. 3. PP121 blocks Akt-mTOR activation in esophageal cancer cells. Eca-109 or primary human esophageal cancer cells were treated with rapamycin (Rap, 100 nM) or PP121 (100 nM) for 6 h, expression of listed proteins were tested by Western blots (**A**, **B**, **E**, and **G**), NFκB (p65) DNA-binding activity was also tested (**F** and **H**). Stable Eca-109 cells expressing CA-Akt or the empty vector (pSuper-puro) were treated with PP121 (100 nM) for applied time, listed proteins were tested by Western blots (**C** and **I**), cell survival and apoptosis were tested by MTT assay and TUNEL staining assay, respectively (**D**), NFκB (p65) DNA-binding activity was also tested (**J**). Data were means ± SD of three independent experiments. *P < 0.05 compared with the "C" group. #P < 0.05 compared with the PP121 only group (**D**). For all the blots, kinase phosphorylations were quantified.

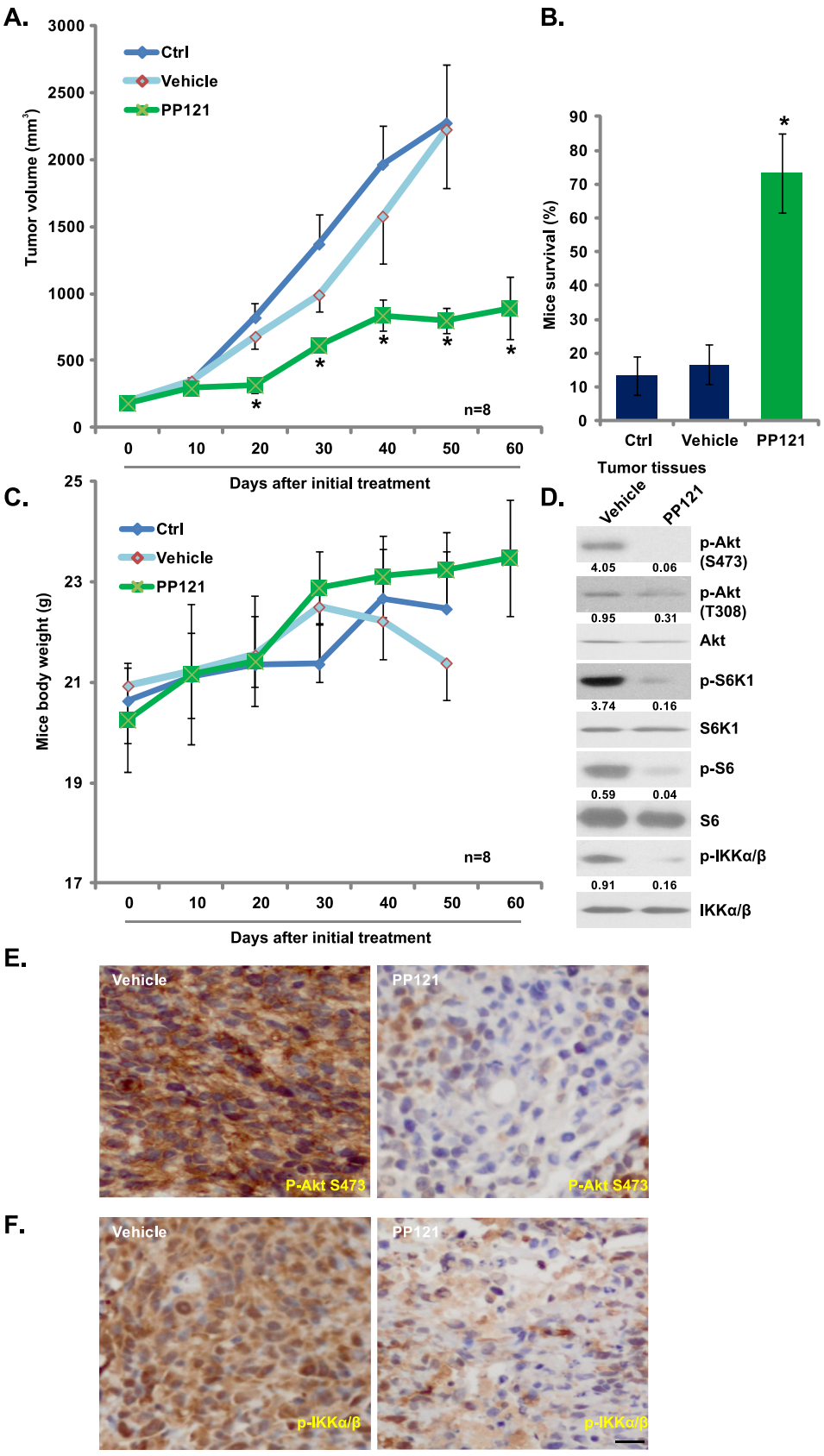

Fig. 4. PP121 inhibits Eca-109 cells *in vivo*. The growth curve of Eca-109 xenografts in nude mice administrated with PP121 (30 mg/kg, *oral* gavage, daily, 30 d) or vehicle (10% 1-methyl-2-pyrrolidinone and 90% PEG 30, *oral* gavage, daily, 30 d) was shown (**A**), mice survival at the termination of the experiments (60 d) was presented (**B**); Mice body weights were also recorded (**C**). Two weeks after initial treatment, two xenograft tumors per group were isolated, and expressions of listed proteins were tested by Western blots (**D**) or IHC staining assay (**E** and **F**). *In vivo* experiments were repeated twice, and similar results were obtained. *$P < 0.05$ vs. "vehicle" group (**B**). Kinase phosphorylations were quantified (**D**). Bar = 25 μm (**E** and **F**).

more potent than the traditional mTORC1 inhibitor rapamycin (Fig. 3A and B). Note that rapamycin induced feedback activation of mTORC2 (p-Akt Ser 473), while PP121 blocked it (Fig. 3A and B). Next, we introduced a constitutively-active (CA) Akt [14,18] into Eca-109 cells. CA-Akt was able to restore activation of mTORC1 (p-S6) and mTORC2 (p-Akt Ser 473) in PP121-treated cells (Fig. 3C). However, CA-Akt only partly inhibited PP121-medaited cytotoxicity again Eca-109 cells (Fig. 3D). In another word, Eca-109 cell death and apoptosis by PP121 were attenuated, but not completely blocked, by CA-Akt (Fig. 3D). These results indicate that other mechanisms besides Akt-mTOR blockage might also contribute to PP121-mediated activity against esophageal cancer cells.

3.4. PP121 inhibits NFκB activation in esophageal cancer cells

Importantly, we showed that PP121 treatment significantly inhibited NFκB activation in Eca-109 (Fig. 3E and F) and primary esophageal cancer cells (Fig. 3G and H). NFκB activation was tested by Western blots analyzing p-IκB kinase complex α/β (IKKα/β, Ser176/180), and by the p65 DNA-binding assay (Fig. 3E–H). Note that rapamycin had almost no effect on NFκB activation in esophageal cancer cells (Fig. 3E–H), nor did the pan PI3K/Akt/mTOR inhibitor LY209004 (Data not shown). Further, CA-Akt showed no rescue effect on NFκB activation in PP121-treated Eca-109 cells (Fig. 3I and J). These results indicate that NFκB inhibition could be the unique effect by PP121 in esophageal cancer cells, independent of Akt-mTOR blockage.

3.5. PP121 inhibits Eca-109 cells in vivo

The *in vivo* activity of PP121 was also tested utilizing an Eca-109 nude mice xenograft model. Eca-109 cells were inoculated into nude mice, and tumor growth was monitored. As shown in Fig. 4A, oral administration of PP121 remarkably inhibited Eca-109 xenograft growth. Compared to the control mice, the tumor volumes of PP121-administrated mice were significantly lower (Fig. 4A). Importantly, mice survival was significantly improved with PP121 oral administration (Fig. 4B). The fast majority of mice were dead 60 d after initial PP121 treatment. However, over 70% of mice were still alive with PP121 administration (Fig. 4B). As expected, vehicle control (10% 1-methyl-2-pyrrolidinone and 90% PEG 30) had no effect on tumor growth or mice survival (Fig. 4A and B). Results in Fig. 4C showed that the mice body weights were not significantly affected by PP121 or the vehicle treatment. No signs of apparent toxicities (*i.e.* vomiting, diarrhea and fever) in the tested animals were observed during the experimental duration (Data not shown). Importantly, as shown in Fig. 4D, PP121 oral administration dramatically inhibited activations of Akt-mTOR and NFκB in xenograft tumors. Further, IHC images showed that p-Akt Ser 473 and p-IKKα/β were both inhibited by PP121 administration. Thus, in line with the *in vitro* findings, PP121 oral administration dramatically inhibited Akt-mTOR and NFκB activation in xenografted tumors, which might be responsible for its anti-tumor activity *in vivo*.

4. Discussion

Studies have shown that PI3K/Akt/mTOR signaling is highly-activated in esophageal cancer, which is often associated cancer progression and poor prognosis [8,9]. Our results demonstrated that PP121 was effective in inhibiting esophageal cancer cell survival and proliferation, and inducing cell apoptosis. The potential anticancer mechanism for PP121 might be due to its inhibitory effect on activation of Akt-mTOR and NFκB pathways. More importantly, PP121 is effective in suppressing Eca109 xenograft tumor growth *in vivo*. Thus, PP121 could be further investigated as a novel and efficient anti-esophageal cancer agent.

Recent studies have explored the complex relationship between Akt and mTOR in promoting cancer progression [19]. Thus far, two mTOR complexes, mTORC1 and mTORC2, have been identified [20,21]. Unlike the traditional mTORC1, which is composed of mTOR, Raptor and mLST8 [16], the latter mTORC2 is assembled with mTOR, Rictor, Sin1 and possible several others [16]. Activated Akt serves as one upstream kinase for mTORC1 activation [19]. Akt phosphorylates and inhibits Tuberous sclerosis 2 (TSC2), thus removing TSC2 from inhibiting mTORC1, causing mTORC1 activation and subsequent phosphorylation of S6K1 and eIF4E-binding protein 1 (4E-BP1) [16]. On the other hand, mTORC2 is required for Akt activation through functioning as the Akt Ser-473 kinase [16]. Both complexes were shown to play positive roles in regulating cancer behaviors, such as protein translation, energy metabolism, cell survival and proliferation, among others [20,21]. In the current study, we showed that PP121 blocked activation of Akt as well as mTORC1 and mTORC2 in esophageal cancer cells. That might explain its superior activity against esophageal cancer cells. As a matter of fact, we showed that PP121 was significantly more potent than traditional mTORC1 inhibitors (rapamycin and Rad001) and the pan PI3K-Akt-mTOR inhibitor LY294002 in inhibiting esophageal cancer cells.

Another important signaling that plays vital roles in esophageal cancer progression is NF-κB [22]. It has been shown that NF-κB is constitutively-active in both esophageal cancer cell lines and human esophageal cancer tissues [22]. On the other hand, inhibition NF-κB pathway repressed esophageal cancer proliferation, enhanced the sensitivity to chemotherapeutic drugs and, more importantly, suppressed esophageal cancer tumor growth and metastasis [22]. One importantly finding of this study is that PP121 dramatically inhibits NF-κB activation in esophageal cancer cells. PI3K/Akt could exert a positive role in NF-κB activation. In the current study, we showed that PP121-mediated NF-κB inhibition is independent of PI3K/Akt blockage. CA-Akt, which restored Akt/mTOR activation in PP121-treated cells, showed no effect on NF-κB signaling. Further, traditional PI3K/Akt/mTOR inhibitors (rapamycin and LY294002) showed no effect on NF-κB activation in esophageal cancer cells. We propose that NF-κB inhibition could be the unique effect by PP121, which could be another reason of its superior activity. Together, we show that PP121 significantly inhibits esophageal cancer cells *in vitro* and *in vivo*, and its activity could be related to concurrent inhibition of Akt-mTORC1/2 and NFκB signalings.

Conflict of interests

No conflict of interests were stated by authors.

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Transparency document

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