Verteporfin suppresses cell survival, angiogenesis and vasculogenic mimicry of pancreatic ductal adenocarcinoma via disrupting the YAP-TEAD complex

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Pancreatic ductal adenocarcinoma (PDAC) is one of the most aggressive human malignancies. The Yes-associated protein-1 (YAP) plays a critical role in cell proliferation, apoptosis and angiogenesis. Verteporfin is a photosensitizer used in photodynamic therapy and also a small molecular inhibitor of the Hippo-YAP pathway. However, little is known about whether verteporfin could inhibit YAP activity in PDAC cells. Our present results showed that verteporfin suppressed the proliferation of human PDAC PANC-1 and SW1990 cells by arresting cells at the G1 phase, and inducing apoptosis in dose- and time-dependent manners. Verteporfin also inhibited the tumor growth on the PDAC xenograft model. Treatment with verteporfin led to downregulation of cyclinD1 and cyclinE1, modulation of Bcl-2 family proteins and activation of PARP. In addition, verteporfin exhibited an inhibitory effect on angiogenesis and vasculogenic mimicry via suppressing Ang2, MMP2, VE-cadherin, and α-SMA expression in vitro and in vivo. Mechanism studies demonstrated that verteporfin impaired YAP and TEAD interaction to suppress the expression of targeted genes. Our results provide a foundation for repurposing verteporfin as a promising anti-tumor drug in the treatment of pancreatic cancer by targeting the Hippo pathway.
TEAD interactions and preventing YAP induced oncogenic growth.\(^{(22)}\) Verteporfin was shown to inhibit angiogenesis and the growth and migration of human retinoblastoma cells via disrupting the YAP-TEAD-associated downstream proto-oncogenes.\(^{(23)}\) Furthermore, verteporfin effectively inhibited the expression of YAP and endothelial growth factor receptor (EGFR) and enhanced the effects of cytotoxic drugs in killing cells from esophageal cancer\(^{(24)}\) and lung cancer.\(^{(25)}\) The results from a phase I/I study of verteporfin photodynamic therapy in locally advanced pancreatic cancer indicated that verteporfin was feasible and safe, and might represent a useful supplement for the treatment of pancreatic cancer.\(^{(26)}\)

In this study, we have shown that verteporfin inhibited the proliferation of PDAC cells by arresting cells at G1 phase via reducing the expression of cyclinD1 and cyclinE1, and inducing apoptosis by activating the intrinsic apoptotic signaling pathway. Verteporfin also inhibited tumor angiogenesis by downregulating angiopoietin-2 (Ang2) through inhibiting YAP activity, and suppressed VM by downregulating MMP2, VE-cadherin and α-SMA expression. The above mechanism studies on YAP regulation by verteporfin provide insights into potential approaches with verteporfin in PDAC therapy.

**Materials and Methods**

**Cell culture.** Human umbilical vein endothelial cells (HUVECs) and human pancreatic ductal cells (HPDE6) were purchased from Fuheng biology (Shanghai, China). Human PDAC PANC-1 and SW1990 cells were obtained from CTCC (Shanghai, China). Cells were cultured in Dulbecco’s modified Eagle’s medium (Hyclone, Logan, Utah, USA) containing 10% fetal bovine serum (Gibco, Carlsbad, CA, USA) in a humidified incubator containing 5% CO\(_2\) at 37°C.

**Transmission electron microscopy.** Conventional electron microscopy was used to visualize PANC-1 and SW1990 cells morphology at the ultrastructural level. The cells after verteporfin treated were fixed with 2.5% glutaraldehyde, and post-fixed in 1% phosphate-buffered osmium tetroxide. After

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**Fig. 1.** Verteporfin inhibits cell proliferation and cell cycle progression in vitro. (a, b) PANC-1 and SW1990 cells were incubated with vehicle or verteporfin at various concentrations for 24, 48, and 72 h. Cell viability was assessed (%). (c) Proportion of PANC-1 and SW1990 cells at different phases of cell cycle was measured by flow cytometry. (d) Representative histograms depicting cell cycle profiles of PANC-1 and SW1990 cells were shown. (e, f) Representative colonies formed by PANC-1 or SW1990 cells treated with vehicle or verteporfin were shown in the left panel, and the quantified results in the right panel. The experiments were performed in triplicate. Data were presented as mean ± SD. *\(P < 0.05; \)**\(P < 0.01; \)**\(P < 0.001.\)
being embedded, sectioned, and double-stained with uranyl acetate and lead citrate, images were captured with a transmission electron microscope (JEM-1200EX, JEOL Ltd, Tokyo, Japan).

**Flow cytometry for cell cycle and apoptosis assay.** For cell cycle assay, $1 \times 10^6$ cells were harvested, washed, and fixed with ice-cold 70% ethanol overnight at 4°C. After washing again, cells were incubated with DAPI and RNaseA for 30 min at room temperature, and then analyzed cytometrically. For cell apoptosis assay, cells were harvested and incubated with 1 μL of Zombie violet stain and 5 μL of FITC-conjugated Annexin V for 30 min (Biolegend, San Diego, CA, USA), and then analyzed cytometrically. Three independent repeated experiments were performed.

**Gene expression vectors and transfection.** Full-length human YAP ectopic expression lentivirus vector and the negative control (GeneChem, Shanghai, China) were transfected into HUVECs according to the manufacturer’s instruction. YAP shRNA (5’-CCCAGTTAAATGTTCACCAAT-3’) and scrambled control shRNA (GenePharma, Shanghai, China) were transfected into HUVECs with the PolyJet DNA in vitro transfection reagent (SignaGen, Ijamsville, MD, USA).

**Transwell assay.** The assay was performed as previously described. Briefly, cells were added to the upper compartment of the chamber. After incubation for 24 h, cells in the lower compartment were fixed with methanol, and stained with 0.1% crystal violet. The number of migrated cells in five randomly selected fields (magnification, 100×) was counted under...

**Fig. 2.** Verteporfin induces cell apoptosis in pancreatic cancer cells. (a) Ultrastructure of PDAC cells treated with vehicle control or verteporfin (Magnification ×10,000, bar = 1 μM) was examined by electron microscopy. (b) PDAC cells were incubated with vehicle control or verteporfin and then stained by TUNEL (Magnification ×200, bar = 50 μM). TUNEL positive cells were quantified. (c) Representative dot plots were from cytometrically analyzed apoptotic cells. (d) Percentages of apoptotic cells (%) were quantified. The experiments were performed in triplicate. Data were presented as mean ± SD. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. © 2017 The Authors. Cancer Science published by John Wiley & Sons Australia, Ltd on behalf of Japanese Cancer Association.
microscopy. The experiments were repeated at least in triplicate.

**In vitro tube formation assay.** The assay was performed as previously described. Briefly, 24-well culture plates were coated with 200 μL of Matrigel matrix (Corning, Bedford, MA, USA). Cells (2.5 × 10^4/well) were seeded in plates. Twenty-four hours after treatments, images were captured with an Olympus microscope. The number of tubular structures in five randomly chosen fields (magnification, 40×) was quantified with the Image-Pro Plus 6.0 software (Media Cybernetics, Rockville, MD, USA).

**Chicken chorioallantoic membrane (CAM) assay.** Chorioallantoic membrane assay was performed as described previously. Groups of 10 fertilized chicken eggs were incubated at 37.8°C and 60–70% relative humidity for 8 days. A small hole was then drilled on the broad end of each egg and a window was carefully created on the eggshell. The chorioallantoic membranes were treated with either vehicle or verteporfin. The windows were then sealed with cellophane tape. After incubation for further 48 h, the CAMs were photographed. Angiogenesis was quantified by counting the number of branching blood vessels.

**Animal experiments.** The animal experiments were approved by the Animal Care and Use Committee of Shandong University. Six-week-old male BALB/c nude (nu/nu) mice were purchased from Beijing HFK Bioscience (Beijing, China). The experimental protocol has been described previously. Briefly, the PANC-1 or SW1990 cells (10^5 cells in 100 μL PBS) were subcutaneously inoculated into the right axilla of mice after a week of acclimatization. Tumor growth was measured daily with calipers. The tumor volume was calculated as a^2 × b × π/6, where b is the length in millimeters and a is the width in millimeters. When tumors reached a mean volume of 70–150 mm^3, the animals were randomized to two groups. The mice in the verteporfin group were intraperitoneally injected with verteporfin (100 mg/kg body weight) every 2 days for 3 weeks. The control mice received the intraperitoneal injection with an equal volume of PBS. The tumors were harvested at the end of the experiments.

**Statistical analysis.** The data were expressed as the mean ± standard deviation. Comparisons were made with one-way ANOVA followed by Dunnett’s t-test. The GraphPad Prism 5 software (La Jolla, CA, USA) was used to evaluate statistical significance. A value of P < 0.05 was considered to indicate a statistically significant difference.

**Antibody and reagents, Cell viability assay, Colony formation assay, TUNEL assay, Quantitative real-time PCR, Western blot, immunohistochemistry and periodic acid-schiff staining.** Please see details in Supplemental additional materials and methods.

### Results

Verteporfin inhibits cell proliferation and cell cycle progression. Incubation of verteporfin reduced the viability of PANC-1 and SW1990 cells in dose- and time-dependent manners. The IC50 of verteporfin was 6.8, 2.6, and 1.4 μM in PANC-1 cells after incubation for 24, 48 and 72 h, respectively (Fig. 1a). Similarly, the IC50 in SW1990 cells was 7.7, 3.6 and 1.7 μM after incubation for 24, 48, and 72 h, respectively (Fig. 1b). Flow cytometry assays revealed that verteporfin increased the percentage of cells in G1 phase and decreased the percentage of cells in S phase (Fig. 1c,d). Additionally, the number of colonies formed by verteporfin-pretreated PDAC cells was significantly less than those by control cells (Fig. 1d,e).

Verteporfin induces cell apoptosis. Under electron microscopy, control cells displayed regular oval shape with prominent nucleus, distinct nucleolus and maintained the integrity of cellular membranes; while verteporfin-treated cells featured significant cytoplasmic vacuolization, large vacuoles, autophagosome, and swollen mitochondria (Fig. 2a). V–FITC/Zombie violet apoptosis assay showed that the apoptosis rates of PANC-1 and SW1990 cells treated with verteporfin were approximately 30% and 24%, respectively (Fig. 2c,d). Similarly, verteporfin also inhibited the viability and induced the apoptosis of HPDE6 and HUVECs in relatively more doses (Fig. S1).

Verteporfin regulates the expression of proteins involved in cell cycle progression and apoptosis via disrupting YAP-TEAD interaction. Mizuno et al. found that YAP1 knockdown led to an accumulation of cells in G0 and G1 phase as well as a significant loss of S phase cells in mesothelioma cell lines. Mechanism studies demonstrated that YAP directly regulated the
expressions of cyclinD1 and foxhead box m1 (FOXM1). (31)
Here, verteporfin inhibited the expression of cyclinD1 and cyclinE1 in a dose-dependent manner (Fig. 3a). Verteporfin also increased the expression of Bax, decreased the expression of Bcl-2, and activated PARP (Fig. 3b). Verteporfin also suppressed necrosis-associated protein expression in PDAC cells (Fig. S2). It has been shown that verteporfin abrogated the interaction of YAP and TEAD. (22) As expected, verteporfin inhibited the expression of total YAP, phosphoYAP and TEAD in a dose-dependent manner (Fig. 3c). The results indicate that verteporfin may induce cell G1 phase arrest and apoptosis via disrupting the YAP-TEAD interaction.

Verteporfin suppresses tumor growth in PDAC xenograft model. In subsequent study, we further observed the effect of verteporfin on the PDAC xenograft model. Tumors from verteporfin-treated mice were significantly smaller than those from vehicle-treated controls (Fig. 4a,b). The weights of tumors harvested from mice at the end of experiments showed a similar trend to the volume of tumors (Fig. 4c). Immunohistochemical analysis of tumor sections showed that verteporfin decreased the expression of YAP, cyclinD1 and cyclinE1 (Fig. 4d). Fewer Ki-67 positive-cells were observed in tumors treated with verteporfin compared with control tumors. TUNEL assay showed increased apoptosis in verteporfin-treated tumors (Fig. 4e).

**YAP transcriptionally regulates Ang2 expression to enhance angiogenesis.** Ang2 is the critical marker for vasculogenic events, and we have reported that Ang2 contributed to neoangiogenesis. (27) In addition, YAP plays an important role in angiogenic sprouting and remodeling of HUVECs, and the expression of Ang2 correlates to YAP expression. (32) Here, we showed that depletion of YAP inhibited, while overexpression of YAP increased, Ang2 expression (Fig. 5a,b). Accordingly, depletion of YAP inhibited, while overexpression of YAP promoted, the migration of HUVECs (Fig. 5c,d). Depletion of YAP impaired, while overexpression of YAP, promote the capillary tube formation ability of HUVECs (Fig. 5e,f).

**Verteporfin inhibits angiogenesis by downregulating Ang2 expression.** Western blot analysis showed that verteporfin suppressed Ang2 expression in a dose-dependent manner.
Verteporfin inhibited the migration and tube formation of HUVECs (Fig. 6a,b). In a CAM angiogenesis model, verteporfin exhibited an anti-angiogenic activity as well (Fig. 6c). In the xenograft model, verteporfin-treated tumors expressed less Ang2 (Fig. 6d) and CD31 (Fig. 6e) than control tumors as detected by IHC (Fig. 6d).

Verteporfin suppresses the migration of PDAC cells and formation of VM via downregulating MMP2, VE-cadherin and α-SMA expression. The formation of VM by tumor cells contributes to tumor rapid progression, drug resistance and metastasis. Verteporfin treatment inhibited the migration ability of PDAC cells as examined by transwell assays (Fig. 7a). PANC-1 and SW1990 cells were able to form tube-like structures, which was disrupted by verteporfin treatment (Fig. 7b). Verteporfin treatment inhibited the expression of MMP2, VE-cadherin and α-SMA in a dose-dependent manner (Fig. 7b). To quantify VM, we double stained tumor sections with antibodies against CD31 and PAS. CD31 is a well-known marker for endothelial cells, while PAS is used to detect basement membrane structures and VM networks in tumors. Verteporfin treatment resulted in less detectable VM (branching networks of PAS+ structures and lacking CD31 staining) compared to the controls (Fig. 7d). Verteporfin-treated tumors had lower expression levels of MMP2, VE-cadherin and α-SMA than control tumors as detected by IHC (Fig. 7e). Similar results were observed in the SW1990 xenograft model (Fig. S3).

Discussion

Yes-associated protein-1 is highly expressed in various malignant tumors including pancreatic cancer, and its activation is an independent predictor of the survival of cancer patients. Although initially regarded as a photo-sensitizer in photodynamic therapy, verteporfin has recently been reported to exhibit anti-tumor activities by disrupting YAP-
TEAD interaction. In accordance with previous reports that verteporfin inhibited the growth of cells from esophageal cancer and lung cancer, our present results demonstrated that verteporfin also suppressed the growth of pancreatic cancer cells and induced apoptosis in vitro and in vivo. Verteporfin treatment led to downregulation of cyclinD1 and cyclinE1, modulation of Bcl-2 family proteins, and activation of PARP. Verteporfin had also displayed a profound effect on angiogenesis through suppressing Ang2 expression. Verteporfin suppressed the migration of PDAC cells and the formation of VM by downregulating MMP2, VE-cadherin and α-SMA (Fig. 8). The encouraging results warrant a further investigation of verteporfin as an anti-tumor agent in PDAC.

Recent studies suggest that the Hippo-YAP pathway is implicated in cell proliferation, apoptosis, chemo-resistance and angiogenesis. YAP regulates the transcription of cyclinD1 and FOXM1, which are important factors for G1 progression and G1/S transition. Hyperactivated YAP also regulates the insensitivity to apoptosis of cancer cells. Activated YAP resulted in gemcitabine resistance in pancreatic cancer. Thus, YAP has become a promising target for seeking novel cancer therapeutics. It has been reported that verteporfin induced the sequestration of YAP in the cytoplasm by increasing 14-3-3 expression, thereby inhibiting YAP activation. Verteporfin interfered with the YAP-TEAD pathway, resulting in downregulation of the downstream proto-oncogenes, such as c-myc and Axl, thus inducing growth inhibition, apoptosis, and G0/G1-phase cell cycle arrest of human retinoblastoma cells. In the present study, we showed that verteporfin inhibited the proliferation and induced apoptosis of PDAC cells by regulating cell cycle- and apoptosis-related proteins via suppressing YAP-TEAD interaction.

Fig. 6. Verteporfin inhibits angiogenesis by downregulating Ang2 expression. (a–c) Human umbilical vein endothelial cells (HUVECs) were pre-treated with verteporfin at 4 μM concentration for 24 h. Cell migration was analyzed by transwell assays (Magnification ×100, bar = 100 μM) (a), and angiogenesis by tube formation assays (Magnification ×40) (b) and by chorioallantoic membrane assays (c). (d, e) The expression of Ang2 and CD31 was detected by IHC (Magnification ×400, bar = 25 μM). Data were presented as mean ± SD. *P < 0.05; **P < 0.01; ***P < 0.001.
Hippo signaling is also involved in tumor progression, metastasis, and angiogenesis. In cholangiocarcinoma, hyper-activated YAP enhanced the expression of pro-angiogenic MFAP5. The YAP-TEAD complex regulates the expression of genes participating in angiogenesis and cell migration, such as CTGF, CYR61 and VEGF-A, which could be inhibited by verteporfin. Ang2 is one member of the angiopoietin family which induces destabilization, disassociation from the endothelial cell basement membrane, migration and sprouting of endothelium cells, thus regulating tumor angiogenesis and progression. High expression of Ang2 is associated with poor prognosis of patients with breast cancer, colorectal...
Verteporfin suppresses PDAC via Hippo pathway

Verteporfin is an FDA-approved photosensitizer for treating age-related macular degeneration and exhibits limited toxicity, and could inhibit tumorigenesis via disrupting YAP-TEAD complexes. Our present results have demonstrated that verteporfin suppressed the growth of pancreatic cancer cells by inducing G1 phase arrest and apoptosis \textit{in vitro} and \textit{in vivo}. The mechanisms accounting for its activities include downregulation of cyclinD1 and cyclinE1, modulation of Bcl-2 family proteins and activation of PARP. Verteporfin has also displayed an inhibitory effect on angiogenesis by suppressing Ang2 expression, and the migration of PDAC cells and VM via downregulating MMP2, VE-cadherin and α-SMA expression. The encouraging results warrant a future investigation of verteporfin as a novel therapeutic in the treatment of pancreatic cancer.

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Conflict of Interest
We have no conflict of interest.

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Verteporfin suppresses necrosis-associated protein expression in PDAC cells.

Verteporfin suppresses vasculogenic mimicry in SW1990 xenografts.

Supporting Information
Additional Supporting Information may be found online in the supporting information tab for this article:

Fig. S1. Verteporfin inhibits the viability and induces the apoptosis of HPDE6 and HUVECs.

Fig. S2. Verteporfin suppresses necrosis-associated protein expression in PDAC cells.

Fig. S3. Verteporfin suppresses vasculogenic mimicry in SW1990 xenografts.

Appendix S1. Materials and methods.