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ORIGINAL ARTICLE

mTOR inhibition by TAK-228 is effective against growth, survival and angiogenesis in preclinical retinoblastoma models

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Abstract

We and others have shown that aberrant activation of the mammalian target of rapamycin (mTOR) signalling is essential for retinoblastoma progression and has potential therapeutic value. TAK-228 is a potent inhibitor of mTOR1 and 2 with preclinical activity in a variety of cancers. In this study, we report that TAK-228 is a dual inhibitor of retinoblastoma and angiogenesis. TAK-228 inhibits growth and induces apoptosis in a panel of retinoblastoma cell lines, with IC50 at ~0.2 μM. Under the same experimental conditions, TAK-228 was less effective in inhibiting growth and survival in normal retinal and fibroblast cells than retinoblastoma cells. In addition, TAK-228 inhibited retinal endothelial cell capillary network formation, migration, growth and survival. We further demonstrate that TAK-228 inhibits retinoblastoma and retinal angiogenesis through inhibiting mTOR signalling. Rescue studies confirm that mTOR is the target of TAK-228 in both retinoblastoma and retinal endothelial cells. Finally, we confirm the inhibitory effects of TAK-228 on tumor and angiogenesis in retinoblastoma xenograft mouse model. Our findings provide a preclinical rationale to explore TAK-228 as a strategy to treat retinoblastoma and highlight the therapeutic value of targeting mTOR in retinoblastoma.

KEYWORDS
angiogenesis, mTOR, retinoblastoma, TAK-228

1 | INTRODUCTION

Mammalian target of rapamycin (mTOR) is a serine/threonine-specific kinase complex and its-mediated pathway play key roles in cell growth, proteo-synthesis, ribosomal biogenesis, transcriptional regulation, lipid metabolism, and autophagy.[1] The mTOR signalling pathway is the second most frequently altered pathway in human cancers and its aberrant activation contributes to tumor proliferation, angiogenesis, invasion and survival.[2] Angiogenesis, a formation of new blood vessel from pre-existing ones, is required for solid tumor development.[3] Retinoblastoma is characterized with extensive vascularization and thus targeting mTOR signalling might represent a more effective therapeutic strategy for retinoblastoma as mTOR is critically involved in both tumor and angiogenesis. In support of this hypothesis, we previously demonstrated that temsirolimus, FDA-approved mTOR-targeted drug for the treatment

Abbreviations: mTOR, mammalian target of rapamycin; mTORC1, mTOR complex 1; mTORC2, mTOR complex 2; S6K1, ribosomal protein S6 kinase 1; 4E-BP1, eukaryotic initiation factor eIF4E-binding protein 1; NDRG1, N-Myc downstream regulated 1; RPE-1, retinoblastoma pigmented epithelial cell line; HREC, retinal microvascular endothelial cell; DMSO, dimethyl sulfoxide; NOD/SCID, NOD severe combined immunodeficient mice; SD, standard deviation.

Lanlan Tang, Yu Fu and Jiarun Song are co-first authors.

of advanced renal cell carcinoma,[4] is active against retinoblastoma growth, survival and tumor angiogenesis.

mTOR functions in two distinct multi-protein complexes designated as mTOR complex 1 (mTORC1) and complex 2 (mTORC2): mTORC1 is sensitive to nutrients while mTORC2 is regulated via PI3K and growth factor signaling.[5] Activation of mTORC1 leads to the phosphorylation of ribosomal protein S6 kinase 1 (S6K1) and the eukaryotic initiation factor eIF4E-binding protein 1 (4E-BP1) whereas activation of mTORC2 results in the phosphorylation of Akt and N-Myc downstream regulated 1 (NDRG1).[6] TAK-228 is an oral and selective dual inhibitor targeting both mTORC1 and mTORC2, and has recently been evaluated for solid tumor and hematology treatment under preclinical and clinical settings.[7-10] This work systematically evaluated the efficacy of TAK-228 on tumor cells and retinal endothelial cells in multiple preclinical retinoblastoma models, and attempted to identify the underlying mechanism of TAK-228's action.

2 | MATERIALS AND METHODS

2.1 | Cells, reagents, antibodies and western blot

Five human retinoblastoma cell lines, immortalized normal retinoblastoma pigmented epithelial cell line (RPE-1), and normal human fibroblast BJ-5ta were obtained from American Type Culture Collection or Chinese Academy of Sciences and were authenticated through short tandem repeat profiling analysis (Precision Biotechnology). Retinoblastoma and normal cells were maintained under the same culturing conditions as described in our previous studies.[11,12] Human primary retinal microvascular endothelial cell (HREC; Cell Systems) were cultured in basal M131 medium supplemented with microvascular growth supplement (Invitrogen). TAK-228 (Selleckchem, 99% purity with HPLC) and MHY1485 (Selleckchem, 99% purity with HPLC) were reconstituted in dimethyl sulfoxide (DMSO), sterile-filtered and stored in aliquots in −20°C. Antibodies against phospho-Akt(Ser473), -mTOR(Ser2448), -S6K1(Thr389), -rS6(Ser240/244), -4EBP1(Thr37/460), -NDRG1(Ser330) and their corresponding total were all obtained from Cell Signaling Technology. Denaturing sodium dodecyl sulfate–polyacrylamide gel electrophoresis and western blot were performed using the standard protocol.[13]

2.2 | Measurement of proliferation and apoptosis

5×10^3 cell/wells for proliferation assay and 5×10^6 cell/wells for apoptosis assay were treated with TAK-228 at 0.1, 0.2, 0.4, 0.8, and 1.6 μM. After 3 days, proliferation was determined by adding 20 μl MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt) was added to each well, incubating for 2–4 h and measuring absorbance at 490 nm. Apoptosis was determined by staining cells with Annexin V/7-AAD (BD Pharmingen) as per manufacture's protocol, followed by flow cytometry analysis on a Beckman Coulter FC50. Annexin V+/7-AAD- and Annexin V+/7-AAD+cells were considered as apoptotic cells under early and late stage of apoptosis, respectively.

2.3 | In vitro capillary network formation

150 μl/well of complete Matrigel (Chemicon International) was plated onto 96-well plate and placed in 37°C incubator. After gel solidification, 50 μl of 2×10^4 HREC cells, TAK-228 and medium mixture were gently plated onto each well. After 8 h incubation in cell culture incubator, capillary network was documented using an inverted microscope (Zeiss).

2.4 | Boyden chamber migration assay

Migration assay was performed using the Boyden chamber with 6.5-mm diameter tissue culture inserts and 8.0-μm pore size polycarbonate membranes. HREC and TAK-228 were placed in the gelatin-coated cell culture insert. Medium supplemented with 10 ng/ml vascular endothelial growth factor were placed on the lower chamber as chemoattractant. After 8 h incubation, unmigrated cells on the upper surface of the insert were removed with a cotton swab. 4% paraformaldehyde was used to fix the cells migrating the lower surfaces of the polycarbonate membranes. Then, the cells were stained with crystal violet and counted under a microscope (Zeiss).

2.5 | Endothelial cell adhesion assay

The VybrantTM Cell Adhesion assay kit was used to quantify cell adhesion as described in our previous study.[12] Briefly, HREC was pre-labelled with calcein and then seeded to onto 10× diluted Matrigel-coated plate. TAK-228 was concurrently added. After 1-h incubation, non-adherent cells were removed by gentle washing and adherent cells were quantified via measuring the calcine-absorbance on fluorescence microplate reader.

2.6 | In vivo retinoblastoma model and immunohistochemistry

All procedure was conducted in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals Committee of Wuhan University. 4–6 weeks old male NOD severe combined immunodeficient mice (NOD/SCID, Shanghai Laboratory Animal Center) were maintained in at a pathogen-free 12 h light/dark cycle environment. Ten million RB355 were harvested and re-suspended in phosphate-buffered solution for subcutaneous injection to mice flank under anesthesia condition. Tumors were allowed to form and mice with palpable tumors were randomized into groups (n = 10 each) receiving vehicle and TAK-228 at 0.5 mg/kg via oral

administration. Tumor size and general toxicity were monitored. Mice were euthanized when tumor size reached ~1500 mm^3 using CO^2 inhalation. Tumors were isolated and proceeded for blood vessel staining using immunohistochemistry of CD31 as described in our previous study.[12] Quantification of staining was performed using Image J software.

2.7 | Statistical analyses

Each in vitro experiment was performed at least thrice, and data were expressed as mean and standard deviation (SD). Student's t test for pair-wise comparisons for samples with normal assumptions, with $p < .05$ considered statistically significant.

3 | RESULTS

3.1 | TAK-228 inhibits proliferation and survival in retinoblastoma cells, and to a more extent than in normal cells

To determine the effect of TAK-228 on the proliferation and survival of retinoblastoma cells, we used five retinoblastoma cell lines that are frequently used to model retinoblastoma disease. Y79, RB355 and WERI-Rb27 are genetically related with similar, heterozygous rearrangements of their RB genes.[14] RB116 cells display RB expression without mutation, and expresses primitive stem cell and retinal progenitor cell markers.[15] WERI-Rb-1 cells retained retinal progenitor cell properties.[16] Cells were treated with TAK-228 at 72 h. Proliferation and apoptosis were assessed through measuring BrdU and Annexin V. All retinoblastoma cell lines were growth inhibited with varying IC_{50} at ~0.2 μM (Figure 1A). TAK-228 increased Annexin V percentage in retinoblastoma cells with the concentration starting from 0.4 μM (Figure 1B). Compared to retinoblastoma cells, TAK-228 at the same concentration either did not affect or led to less growth inhibition and apoptosis induction in normal retinal epithelial cells RPE-1 and fibroblast BJ-5ta cells (Figure 1), demonstrating that TAK-228 displays a preferentially toxicity to retinoblastoma compared normal cells.

3.2 | TAK-228 inhibits retinal angiogenesis via targeting multiple biological functions of retinal endothelial cells

Angiogenesis is a therapeutic target for retinoblastoma because retinoblastoma growth largely depends on angiogenesis.[17] It is therefore essential to determine whether TAK-228 also has anti-angiogenic activity. To mimic retinal angiogenesis model, we plated primary human

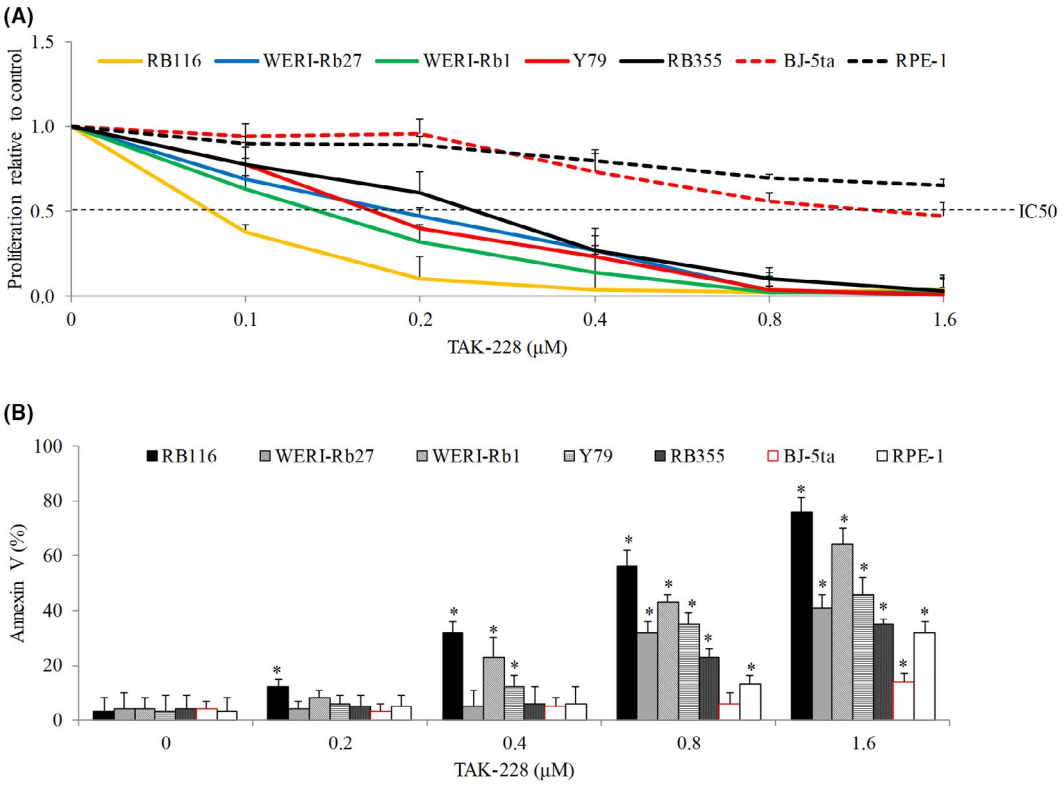

FIGURE 1 The inhibitory effects of TAK-228 on retinoblastoma and normal cells. The anti-proliferative (A) and pro-apoptotic (B) effects of TAK-228 on retinoblastoma cells (RB116, WERI-Rb27, WERI-Rb1, Y79 and RB355), normal fibroblast BJ-5ta and normal retinal pigment epithelial cell (RPE-1). IC50 was indicated at dash line

retinal microvascular endothelial cell (HREC) on extracellular matrix proteins and growth factors-enriched Matrigel which endothelial cells can rapidly form capillary network.[18] As expected, HREC forms extensive capillary network within 8 h in control (Figure 2A). In contrast, TAK-228 treated-HREC failed to form proper capillary network. Quantification of total capillary length showed that TAK-228 at nanomolar concentration dose-dependently inhibited retinal angiogenesis (Figure 2B).

The in vitro formation of endothelial cell capillary network is a multi-step and dynamic process including cell adhesion to Matrigel and cell migration. We further found that TAK-228 significantly inhibited HREC migration (Figure 2C) but not adhesion to diluted Matrigel (Figure 2D). Similar to retinoblastoma cells, TAK-228 also significantly decreased proliferation and induced apoptosis in HRECs (Figure 2E and F). Taken together, our results clearly demonstrate that TAK-228 inhibits retinal angiogenesis via targeting multiple biological functions of retinal endothelial cells.

3.3 | TAK-228 inhibits mTOR signalling in both retinoblastoma and HREC cells

As a specific dual inhibitor of mTORC1 and mTORC2, the anti-cancer activity of TAK-228 has been attributed to its ability in inhibiting mTOR signalling.[10,19] Given the importance of mTOR signalling in retinoblastoma and endothelial cell growth and survival,[20] we performed immunoblot analysis of molecules involved in mTOR signalling in both RB355 and HREC cells after TAK-228 treatment. As expected, TAK-228 at 0.2 to 1.6 μM remarkably decreased phosphorylation of mTOR at Ser2448 in retinoblastoma cells (Figure 3A and B). TAK-228 decreased phosphorylation of S6K1 and 4EPB1, the two well-characterized downstream substrates of TORC1, and inhibited AKT phosphorylation at Ser473 and NDRG1 at Ser330, the downstream substrate of TORC2 in retinoblastoma cells. The same inhibition of mTOR signalling by TAK-228 was also observed in HREC (Figure 3A and C). We These results demonstrate that TAK-228 inhibits mTOR signalling through disrupting mTORC1 and mTORC2 in both retinoblastoma and retinal endothelial cells.

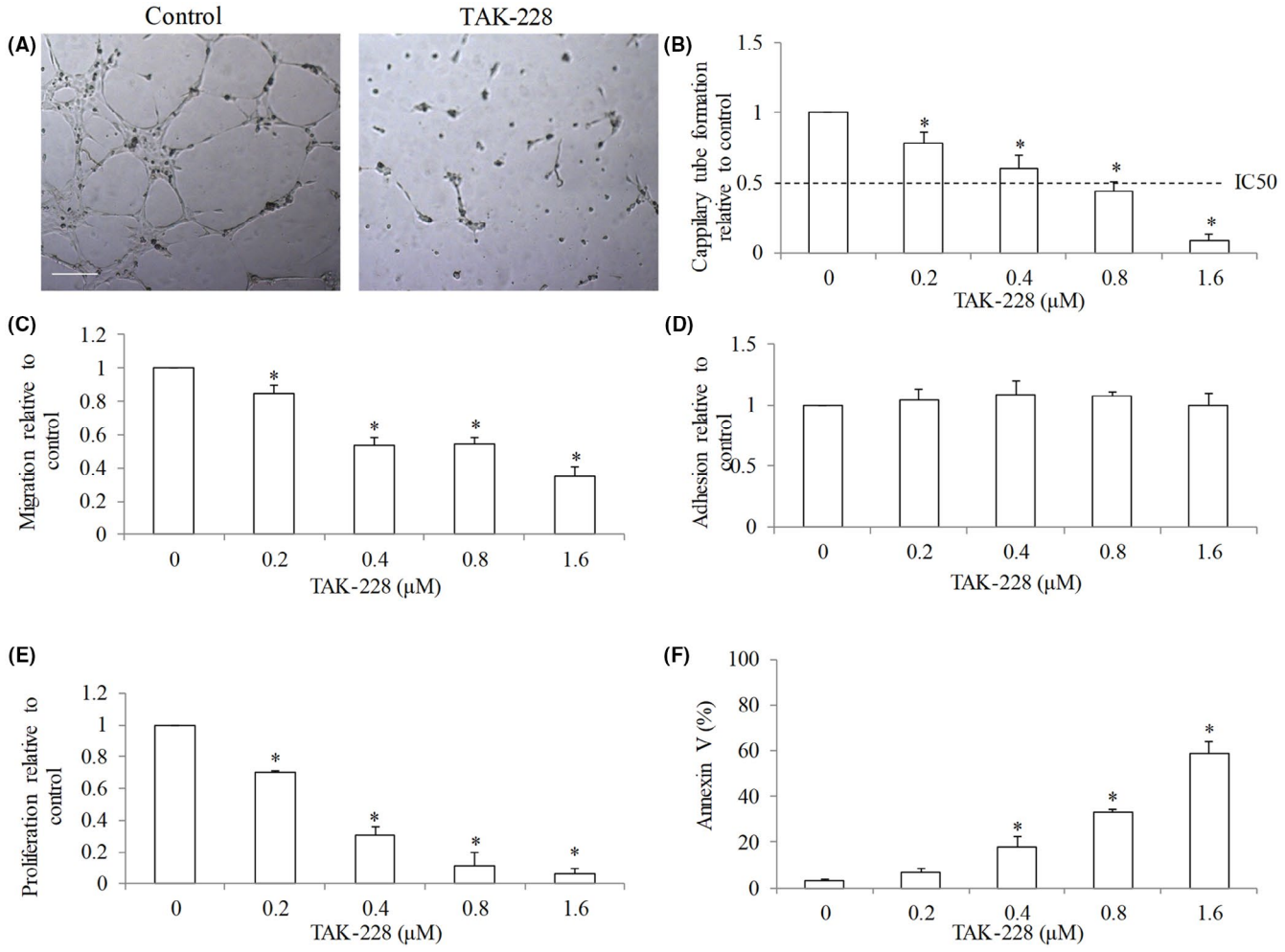

FIGURE 2 The anti-angiogenic activity of TAK-228 on retinal angiogenesis. (A and B) TAK-228 dose-dependently inhibits human retinal endothelial cell (HREC) capillary network formation. 20× magnification, scale bar = 0.5 mm. Complete Matrigel supplemented with various growth factors and cytokines were used for HREC tube formation. The inhibitory effects of TAK-228 on HREC migration (C), adhesion to matrix (D), proliferation (E) and survival (F). 10 ng/ml VEGF was used in migration assay. *$p < .05$, compared to control

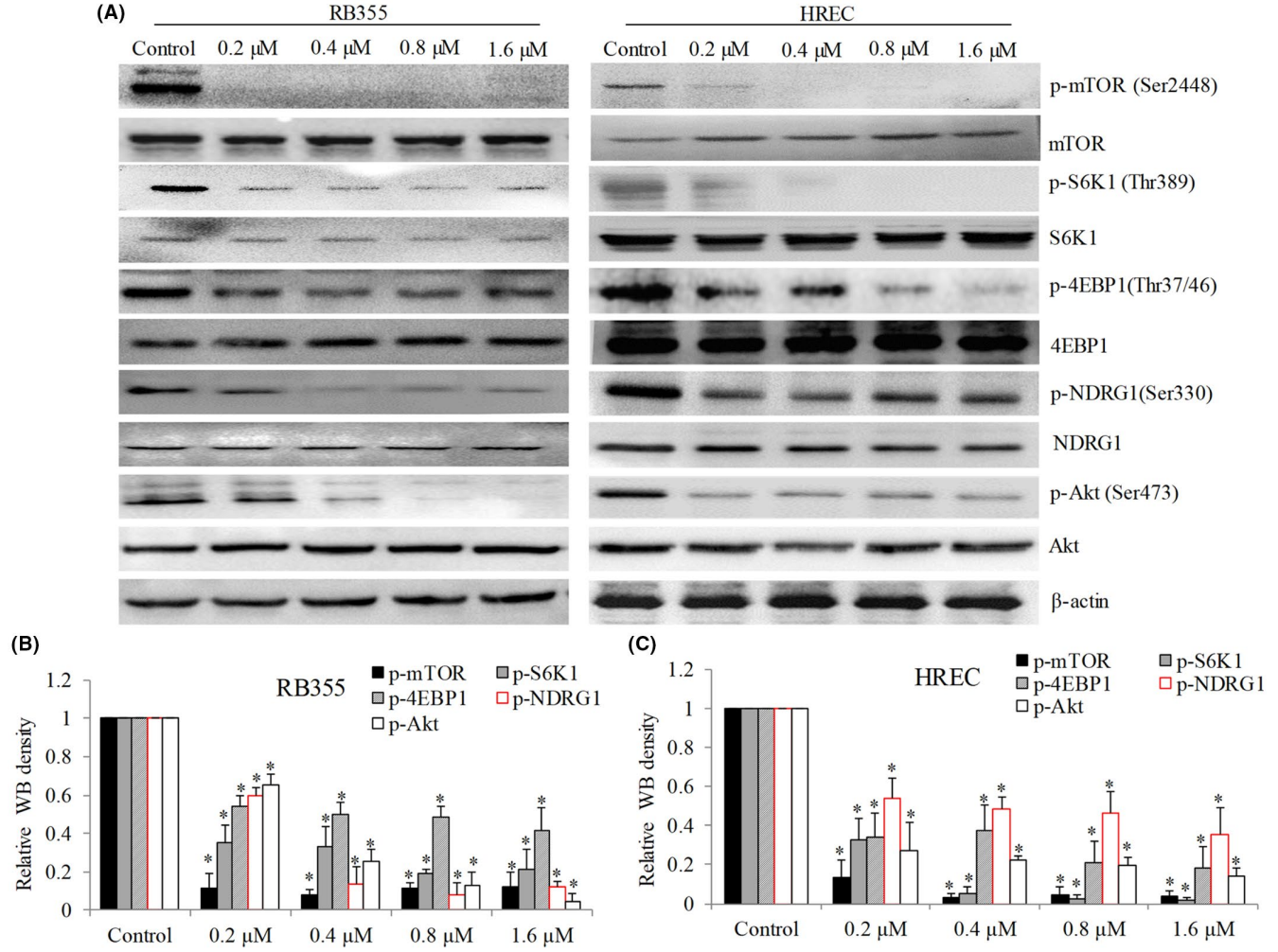

FIGURE 3 TAK-228 inhibits mTOR signaling in retinoblastoma and retinal endothelial cells. (A) Western blot of RB355 and HERC cells treated with TAK-228 for 24 h. Antibodies used in western blot analyses and representative western blot photos were shown. Quantification of p-mTOR, p-S6K, p-4EBP1, p-NDRG1 and p-Akt in RB355 (B) and HREC (C) cells exposed to TAK-228. Band density was measure by Image J and each phosphorylation was normalized with corresponding total, followed by β-actin normalization. $^*p < .05$, compared to control

To confirm mTOR as the target of TAK-228, we performed rescue studies using mTOR activator MHY1485. Consistent with other studies,[21,22] we showed that MHY1845 at 10 μM increased phospho-mTOR levels in RB355 and HREC cells (Figure 4A). We further showed that MHY1485 partially but significantly reversed the anti-proliferative and pro-apoptotic effects of TAK-228 in RB355 and HREC cells (Figure 4B–E). These clearly demonstrate that mTOR inhibition is the mechanism of TAK-228's action in retinoblastoma and retinal endothelial cells.

3.4 | TAK-228 inhibits retinoblastoma and angiogenesis in vivo

To further confirm the anti-angiogenic and anti-retinoblastoma activities of TAK-228, we established a retinoblastoma xenograft mouse model, monitored tumor size and assessed tumor angiogenesis in control and TAK-228 treatment groups. TAK-228 at 0.5 mg/kg was administrated to mice through oral gavage once per day for 24 days. Consistent with the previous findings,[10] we found that TAK-228 treated mice remained active and displayed no obvious toxicity or weight loss (data not shown). In contrast, TAK-228 at the same dose significantly delayed tumor growth beginning at 12 days of the initial treatment and its inhibitory effect was observed throughout the duration of treatment (Figure 5A). Immunohistochemistry staining of microvessel endothelial cell marker CD31 showed a significant reduction of CD31 staining in TAK-228-treated tumors (Figure 5B and C). These results demonstrate that TAK-228 at non-toxic dose is effective in inhibiting retinoblastoma and tumor angiogenesis in mice.

4 | DISCUSSION

TAK-228, a dual inhibitor of mTORC1 and mTORC2, has been recently shown to demonstrate a manageable safety profile with antitumor

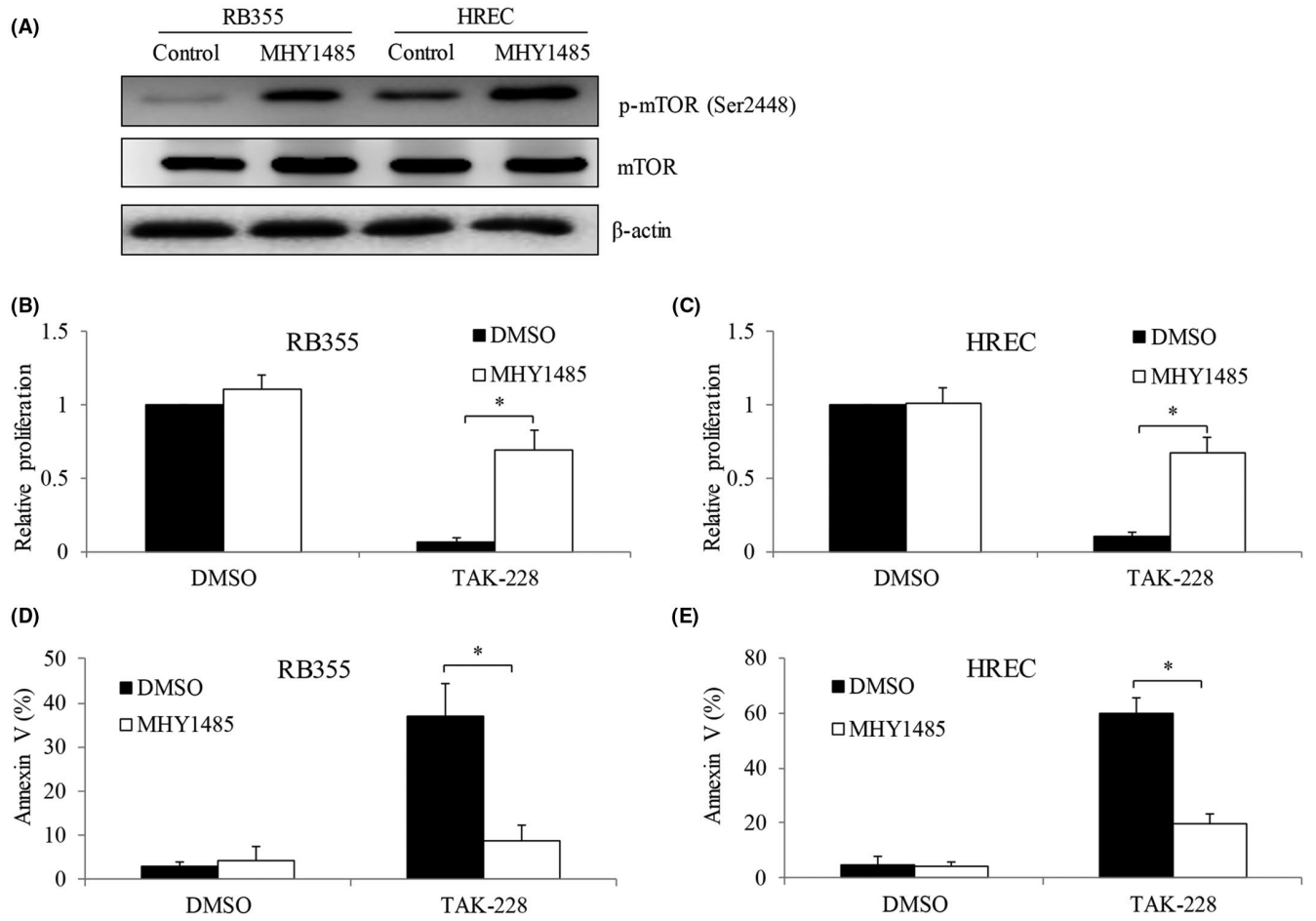

FIGURE 4 mTOR activator reverses TAK-228's effects in retinoblastoma and retinal endothelial cells. (A) Western blot of RB355 and HERE cells treated with 10 μM MHY1845. MHY1845 significantly reversed anti-proliferative (B and C) and pro-apoptotic (D and E) of TAK-228 in RB355 and HERC. *$p < .05$, compared to control

activity in advanced solid tumors.[8] It has been hotly investigated in many clinical trials either as single drug or in combination with standard of care drugs, for patients with a variety of cancers, such as metastatic triple negative breast cancer (NCT03193853), metastatic anaplastic thyroid cancer (NCT02244463) and recurrent epithelial ovarian (NCT03648489). However, no preclinical studies or clinical trials have assessed efficacy of TAK-228 on retinoblastoma. The poor prognosis of advanced retinoblastoma is partly attributed to a lack of effective targeted therapies. In this study, we highlight the therapeutic value of inhibiting mTOR signaling in retinoblastoma and reveal the anti-retinoblastoma activity of TAK-228 via suppressing both tumor and endothelial cells.

We show that TAK-228 is active in targeting retinoblastoma cells through inhibiting growth and inducing apoptosis, and furthermore that to a significantly more extent than in normal retinal cells and fibroblasts. The IC_{50} of TAK-228 in retinoblastoma is ~200 nM, which is similar to the IC_{50} in breast cancer and pancreatic cancer cells, and acute myeloid leukemia cells,[10,23,24] suggesting the potent efficacy of TAK-228 in many cancers. This is further supported by our in vivo studies that oral TAK-228 at 0.5 mg/kg is effective in delaying retinoblastoma growth in mice. Compared to other mTOR inhibitors, such as RAD001, temsirolimus and rapamycin,[12,25] we and others show that TAK-228 displays higher efficacy of anti-cancer activity. The combinatory efficacy of TAK-228 and carboplatin should be further validated using retinoblastoma xenograft models. Synergism has been observed between TAK-228 and CDK4/6 inhibitor palbociclib in pRb-expressing ER-negative breast cancer.[26] In addition, TAK-228 re-sensitizes platinum resistant ovarian cancer to platinum chemotherapy.[27]

Apart from targeting tumors, our work also reveals that TAK-228 is an angiogenesis inhibitor through disrupting retinal endothelial cell capillary network formation, inhibiting migration and growth, and inducing apoptosis. The dual inhibitory effects of TAK-228 on retinoblastoma and tumor angiogenesis has been confirmed in our established xenograft mouse model. Angiogenesis is required for retinoblastoma progression and angiogenesis inhibition by bevacizumab or pigment epithelium-derived factor is active against retinoblastoma without producing significant systemic toxicity.[28,29] Compared with angiogenesis inhibitors that have much less inhibitory effects on tumor cells, TAK-228 has advantages because TAK-228 targets both endothelial cell and tumor cells.

Mechanistically, we confirm that TAK-228 inhibits both mTORC1/2 activity as shown by the deactivation of downstream effectors of both mTORC1- and 2-mediated signaling in both

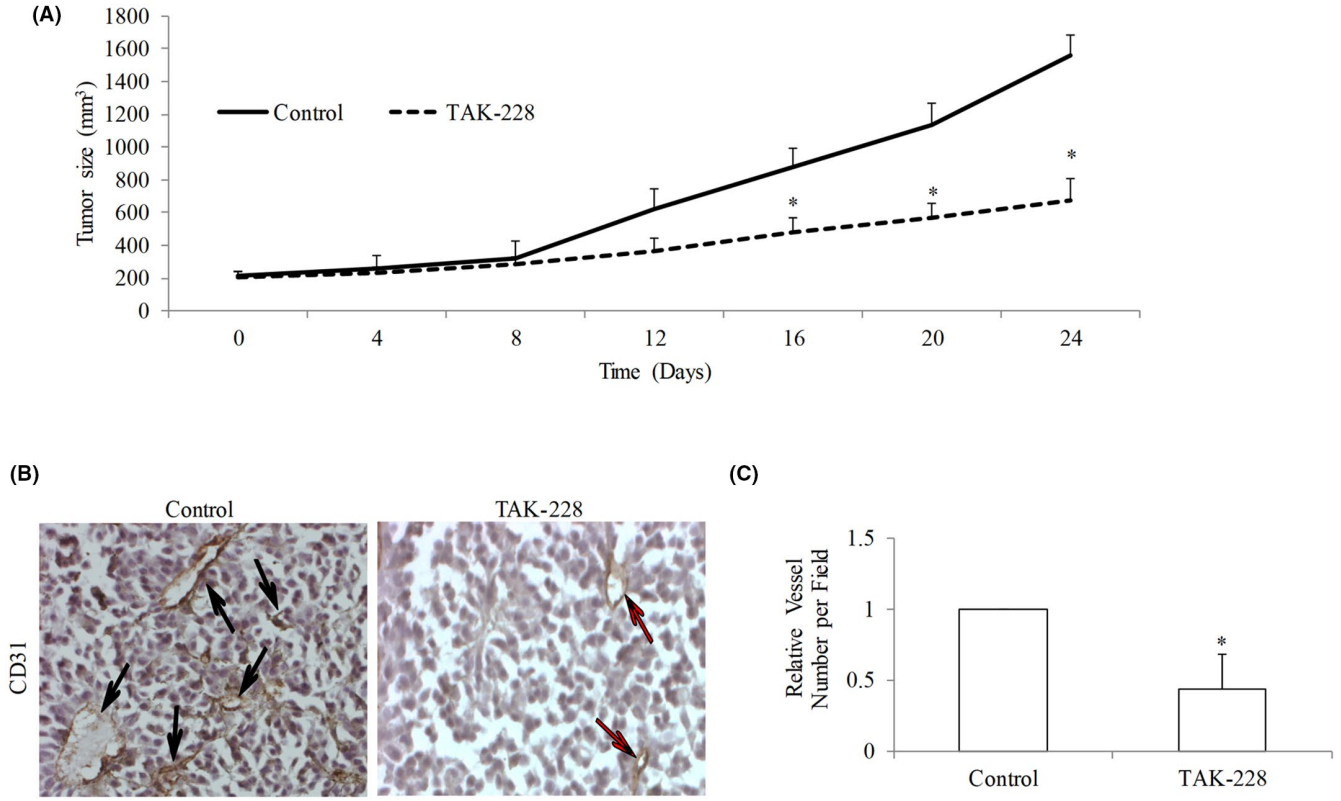

FIGURE 5 TAK-228 inhibits retinoblastoma growth and angiogenesis in vivo. (A) TAK-228 significantly inhibits RB355 tumor growth throughout the duration of treatment. SCID mice bearing RB355 tumor at the flanks were treated with equal volume of vehicles and oral TAK-228 at 0.5 mg/kg daily. (B) Representative immunohistochemical staining pattern of CD31 (indicated by arrow) in control and TAK-228-treated tumors. (D) Quantification of CD31 staining in control and TAK-228-treated tumors using Image J software. The average number of vessels per microscopic field, from three microscopic fields per tumor section were analyzed. Results were presented as relative to control. *$p < .05$, compared to control

retinoblastoma cells and retinal endothelial cells. Rescue studies using mTOR activator MY1485 confirm that mTOR is the molecular target of TAK-228. TAK-228 is superior to those mTOR inhibitors such as temsirolimus and RAD001 that are only effective in targeting mTORC1 without affecting mTORC2.[30] TAK-228 also overcomes the undesired effects of rapalogs, a PI3K/mTOR dual inhibitor, in the activation of Akt pathway. Mutations that constitutively hyperactivate PI3K/Akt/mTOR confer an advantage to cancer cells. Although there is low frequency of oncogenic mutations in the AKT1 and PIK3CA in retinoblastoma, PI3K/Akt is dysregulated possibly via different activating mechanism.[31] The development and application of mTOR inhibitors or compounds targeting the dysregulated PI3K/Akt/mTOR signalling are key areas of anti-cancer research.

In conclusion, TAK-228 exerts its inhibitory effect on both retinoblastoma and tumor angiogenesis through inhibiting mTOR1 and 2-mediated signalling. In addition, our work also emphasizes that targeting mTOR may represent a new therapeutic strategy against recurrent retinoblastoma.

ETHICS STATEMENT

The procedures with animal work were approved by the Ethics Committee of the Care and Use of Laboratory Animals of Wuhan University and were conducted in accordance with the recommendations.

ACKNOWLEDGEMENT

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DISCLOSURE

All authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Data will be made available from corresponding author upon request.

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