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UNIVERSITE DE LAUSANNE - FACULTE DE BIOLOGIE ET DE MEDECINE

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**The inhibition of MAPK potentiates the anti-angiogenic efficacy of  
mTOR inhibitors**

THESE

préparée sous la direction du Professeur Nicolas Demartines  
(avec la collaboration du Dr Olivier Dormond)

et présentée à la Faculté de biologie et de médecine de  
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***The inhibition of MAPK potentiates the anti-angiogenic efficacy of mTOR inhibitors***

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## Résumé du travail de thèse

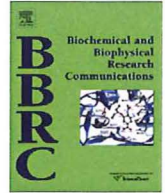
Malgré les nombreux progrès effectués dans la compréhension du cancer, cette maladie reste encore souvent incurable.

Récemment, il a été démontré qu'afin de progresser un cancer doit développer de nouveaux vaisseaux sanguins lors d'un processus appelé angiogenèse tumorale. Il a aussi été démontré que l'inhibition de ce processus réduisait la croissance tumorale et de ce fait représente une importante cible thérapeutique contre le cancer.

Les mécanismes impliqués dans l'angiogenèse tumorale ont été partiellement caractérisés et impliquent la prolifération, la survie et la migration des cellules endothéliales, cellules qui forment la paroi des vaisseaux sanguins. Quelques molécules régulant ces fonctions endothéliales ont été identifiées. Parmi celle-ci, une protéine intracellulaire appelée mTOR joue un rôle important dans l'angiogenèse tumorale. En effet, l'inhibition de mTOR par des molécules telle que la rapamycine, réduit l'angiogenèse dans de nombreux modèles expérimentaux ainsi que dans les tumeurs de patients traités par ces inhibiteurs.

Notre étude montre toutefois que l'inhibition de mTOR dans les cellules endothéliales induit l'activation d'autres molécules comme la MAPK qui favorise la prolifération et la survie endothéliale et de ce fait réduit la capacité anti-angiogénique des inhibiteurs de mTOR. De plus, nous avons montré que le traitement de cellules endothéliales par des inhibiteurs de mTOR en combinaison avec des inhibiteurs de MAPK diminuait la prolifération, la survie et la migration endothéliales de manière additive comparée à une inhibition de mTOR ou de MAPK seule. Nous avons obtenu des résultats similaires dans un modèle d'angiogenèse *in vitro*. Finalement, nos résultats ont été confirmés *in vivo* dans un modèle de xénotransplante tumorale chez la souris immuno-compromise. Un traitement combiné d'inhibiteurs de mTOR et de MAPK produisait un effet anti-angiogénique supérieur à un traitement d'inhibiteur de mTOR ou de MAPK seul chez les souris immuno-compromises porteuses de tumeurs sous-cutanées.

En résumé, nos résultats montrent que l'inhibition de mTOR dans les cellules endothéliales induit l'activation de MAPK qui compromet l'efficacité anti-angiogénique des inhibiteurs de mTOR. Ils démontrent également que la combinaison d'inhibiteurs de mTOR et de MAPK induit une efficacité anti-angiogénique supérieure à une inhibition de mTOR ou de MAPK seule. Nous proposons ainsi que l'utilisation de protocoles thérapeutiques qui bloquent à la fois mTOR et MAPK représente une approche prometteuse pour bloquer l'angiogenèse tumorale et donc la progression tumorale et mérite d'être évaluée chez les patients souffrant de cancers.



# The inhibition of MAPK potentiates the anti-angiogenic efficacy of mTOR inhibitors

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## ABSTRACT

The mammalian target of rapamycin (mTOR) which is part of two functionally distinct complexes, mTORC1 and mTORC2, plays an important role in vascular endothelial cells. Indeed, the inhibition of mTOR with an allosteric inhibitor such as rapamycin reduces the growth of endothelial cell *in vitro* and inhibits angiogenesis *in vivo*. Recent studies have shown that blocking mTOR results in the activation of other prosurvival signals such as Akt or MAPK which counteract the growth inhibitory properties of mTOR inhibitors. However, little is known about the interactions between mTOR and MAPK in endothelial cells and their relevance to angiogenesis. Here we found that blocking mTOR with ATP-competitive inhibitors of mTOR or with rapamycin induced the activation of the mitogen-activated protein kinase (MAPK) in endothelial cells. Downregulation of mTORC1 but not mTORC2 had similar effects showing that the inhibition of mTORC1 is responsible for the activation of MAPK. Treatment of endothelial cells with mTOR inhibitors in combination with MAPK inhibitors reduced endothelial cell survival, proliferation, migration and tube formation more significantly than either inhibition alone. Similarly, in a tumor xenograft model, the anti-angiogenic efficacy of mTOR inhibitors was enhanced by the pharmacological blockade of MAPK. Taken together these results show that blocking mTORC1 in endothelial cells activates MAPK and that a combined inhibition of MAPK and mTOR has additive anti-angiogenic effects. They also provide a rationale to target both mTOR and MAPK simultaneously in anti-angiogenic treatment.

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## 1. Introduction

Angiogenesis, the formation of new blood vessels from pre-existing ones, is a critical event in many physiological and pathological processes such as tumor growth and metastasis [1]. In fact, in order to grow, a tumor needs to develop new blood vessels once it reaches the dimension of 2 mm<sup>3</sup> [2]. Angiogenesis is a multi-step process which involves endothelial cell proliferation, survival, and migration. Several molecules implicated in angiogenesis have been identified including growth factors (e.g. vascular endothelial growth factor, fibroblast growth factors and angiopoietins), integrins, notch receptors and their ligands, as well as molecules involved in mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K)/mammalian target of rapamycin (mTOR) signaling pathways [3–5]. Since angiogenesis plays a key role in tumor growth, targeting tumor angiogenesis represents a

promising approach in cancer therapy. Accordingly, targeting the vascular endothelial growth factor (VEGF) has shown clinical efficacy and has been approved for the treatment of various cancers [6]. However, the benefits of anti-angiogenic therapies are transitory and most of the tumors eventually progress under therapy. Therefore, a strong need exists to design new therapeutic strategies that confer enduring anti-angiogenic effects.

mTOR is a key regulator of cell growth, proliferation, and survival as being part of two distinct complexes, mTORC1 and mTORC2. While mTORC1 is composed of five different components: mTOR, raptor, mLST8, PRAS40 and deptor, mTORC2 consists of mTOR, rictor, mSin1, mLST8, deptor and protor-1 [7]. mTORC1 phosphorylates, among others, S6K1 and 4E-BP1 resulting in the regulation of translation initiation and protein synthesis. mTORC2 phosphorylates Akt, SGK and PKC and is involved in cell proliferation, survival and cytoskeletal organization [8]. Numerous studies demonstrated that blocking mTOR with rapamycin reduces tumor angiogenesis [9]. Indeed, rapamycin inhibits the functions of endothelial cells relevant to angiogenesis *in vitro* and reduces angiogenesis in several models *in vivo* [10–12]. However, emerging evidence has shown that targeting mTOR also stops a negative feedback loop which results in the activation of proliferative signals such as Akt or MAPK that reduce the growth inhibitory properties of mTOR inhibitors [13]. The effect of mTOR inhibition on MAPK activity in

**Abbreviations:** MAPK, mitogen-activated protein kinase; PI3K, phosphoinositide 3-kinase; mTOR, mammalian target of rapamycin; VEGF, vascular endothelial growth factor; HUVEC, human umbilical vein endothelial cell; DMSO, dimethyl sulfoxide.

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endothelial cells as well as its relevance to angiogenesis has however not been determined. In this study, we evaluated the consequences of mTOR inhibition either by ATP-competitive inhibitors of mTOR or by rapamycin on MAPK activity in endothelial cells. We also explored the anti-angiogenic effects of mTOR inhibition in combination with MAPK inhibition both *in vitro* and *in vivo*.

## 2. Materials and methods

### 2.1. Antibodies and chemicals

NVP-BE2235, PP242, WYE-354, Ku-0063794 were from Chemdea. Rapamycin and UO126 were from LC laboratories. Antibodies directed against phospho-MAPK (Thr202, Tyr204), MAPK, phospho-Akt (S473), Akt, phospho-S6 ribosomal protein (Ser235/236), S6 ribosomal protein, raptor and rictor were from Cell Signaling.

### 2.2. Cell culture

Human umbilical vein endothelial cells (HUVEC) were purchased from Millipore and cultured in EndoGRO-VEGF complete medium (Millipore). HUVEC were used for the experiments between passages 2 and 5.

### 2.3. Cell transfection

HUVEC were transfected with siRNA as previously described [14].

### 2.4. Migration assay

Migration assay were performed as previously described [15].

### 2.5. MTS proliferation assay

HUVEC were plated on 96 well plates (Costar) at 10,000 cells per well and cultured in EndoGRO-VEGF complete medium. Twelve hours later, cells were either treated with dimethyl sulfoxide (DMSO) as a control or were treated with NVP-BE2235 (100 nM), PP242 (100 nM), rapamycin (10 nM) in combination or not with UO126 (10  $\mu$ M) for 48 h. Cellular proliferation was monitored after 48 h of treatment with the CellTiter 96<sup>®</sup> AQueous One Solution (Promega Corporation) colorimetric assay by following the manufacturer's instructions. Results are expressed as the relative absorbance compared to untreated HUVEC.

### 2.6. Apoptosis assay

The Cell Death Detection ELISA<sup>plus</sup> kit (Roche) was used to measure apoptosis. HUVEC were seeded in 96-well plates at 30,000 cells per well. Twelve hours later, cells were either treated with DMSO as a control or treated with NVP-BE2235 (100 nM), PP242 (100 nM), rapamycin (10 nM) in combination or not with UO126 (10  $\mu$ M) for 48 h. Subsequently cells were harvested and apoptosis was determined following the manufacturer's instructions. Results are represented as the mean enrichment factor (absorbance of the treated cells/absorbance of the control cells).

### 2.7. Tubulogenesis

HUVEC were treated with rapamycin, NVP-BE2235, PP242, UO126, or a combination of UO126 with mTOR inhibitors, or DMSO as a control for 4 h. HUVEC were subsequently harvested and cultured in matrigel-precoated 96-well plate for 6 h at 37 °C (1  $\times$  10<sup>4</sup>/well). Tubulogenesis was visualized with an Olympus in-

verted microscope and the numbers of branching points were counted. Points generating at least three tubules were counted.

### 2.8. Western blot

Western blot analysis was performed as previously described [16].

### 2.9. Tumor xenografts

Animal experiments were in accordance with the Swiss Federal Animal Regulations and approved by the local veterinary office. Female nude mice aged 8 weeks were purchased from Charles River (Charles River Laboratories, St. Germain sur l'Arbresle, France). One million LS174T cells were injected subcutaneously (s.c.) into the flank of nude mice. Once the tumor xenografts reached 25 mm<sup>3</sup>, mice were randomized into eight groups (*n* = 5 in each group) and treated with rapamycin (rapa, 1.5 mg/kg/d, i.p.), NVP-BE2235 (NVP, 30 mg/kg/d, p.o.), PP242 (60 mg/kg/d, p.o.) either alone or in combination with UO126 (40  $\mu$ mol/kg/d, i.p.). After 28 days of treatment, mice were sacrificed and tumors were harvested and processed for CD31 immunostaining. All mice received both p.o. and i.p. doses of vehicle to control for morbidity associated with treatment. NVP-BE2235 was solubilized in one volume of *N*-methylpyrrolidone and further diluted in nine volumes of PEG 300. PP242 was dissolved in PEG 300. Rapamycin was dissolved in ethanol and UO126 in DMSO.

### 2.10. CD31 immunostaining

Tumor xenografts were removed and frozen in OCT compound (Tissue-Teck) on dry ice. About 10  $\mu$ m transverse sections were cut on a cryostat (CM 1850, Leica), and processed for immunolabeling with an anti-CD31 antibody (1:20, MEC13.3, BD Biosciences). Slides were dried for 30 min. at room temperature, fixed in ice cold acetone for 15 min, hydrated in PBS, blocked with casein 0.5% for 1 h, and exposed to primary antibody overnight at 4 °C in PBS-BSA 1%. Primary antibody was visualized with Alexa Fluor 488 goat anti-rat antibody (Molecular Probes, Invitrogen). To reveal cell nuclei, cryosections were incubated 5 min. in DAPI solution (1:20000, Vector Laboratories), washed with PBS, and slides were coverslipped using Gel Mount<sup>TM</sup> (Sigma).

## 3. Results

### 3.1. ATP-competitive inhibitors of mTOR activate MAPK in endothelial cells

To assess the effects of mTOR inhibition on MAPK activity in endothelial cells, HUVEC were exposed to increasing concentrations of different ATP-competitive inhibitors of mTOR (Ku-0063794, WYE-354, PP242 or NVP-BE2235) for 4 h and Western Blot analysis was performed on cell lysates. While Ku-0063794 [17], WYE-354 [18] and PP242 [19] specifically block mTOR activity, NVP-BE2235 [20] also inhibits PI3K in addition to mTOR. We found that Ku-0063794, WYE-354 or PP242, blocked mTORC1 activity at 10 nM as observed by the dephosphorylation of S6 ribosomal protein (Fig. 1A). At higher concentrations (100 nM), PP242, WYE-354 or Ku-0063794 also inhibited mTORC2 as evidenced by the dephosphorylation of Akt. NVP-BE2235 already inhibited mTORC1/2 activity at 10 nM. In addition, we also observed that NVP-BE2235, PP242, WYE-354 or Ku-0063794 increased MAPK phosphorylation (Fig. 1A). This effect was already apparent after 4 h of treatment and persisted after 48 h (Fig. 1B). Taken together, these results show that ATP-competitive inhibitors of mTOR effec-

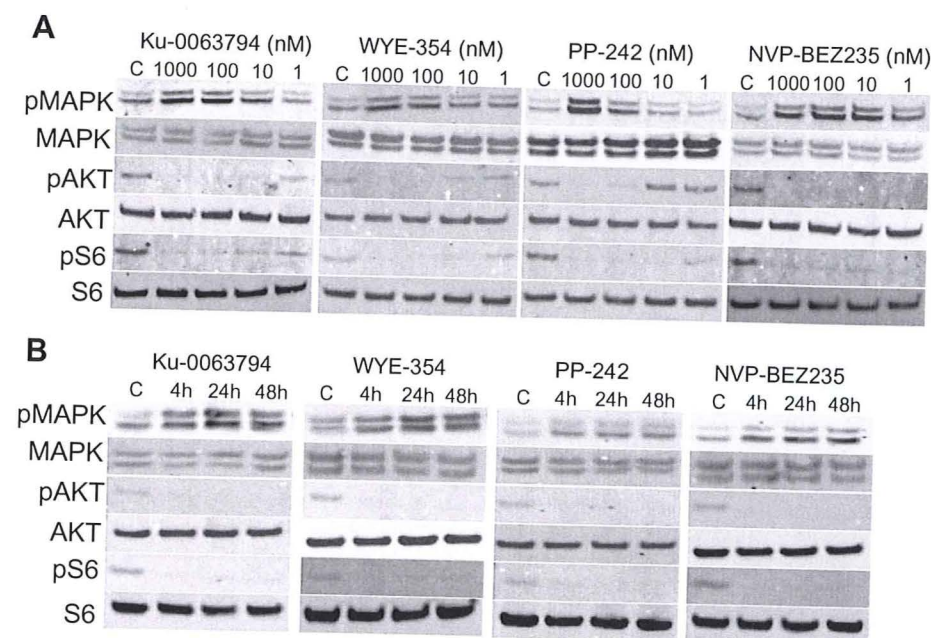


Fig. 1. Activation of MAPK by ATP-competitive inhibitors of mTOR in HUVEC. (A) HUVEC were treated with increasing concentrations of Ku-0063794, WYE-354, PP242 or NVP-BE2235 for 4 h or DMSO (C) as a control. Cells were subsequently lysed and lysates were examined for phospho-MAPK (Thr202/Tyr204), MAPK, phospho-AKT (Ser 473), AKT, phospho-S6 ribosomal protein (Ser234/235) or S6 ribosomal protein expression level by Western blot analysis. (B) HUVEC were treated with 100 nM of Ku-0063794, WYE-354, PP-242 or NVP-BE2235 or DMSO (C) as a control for the indicated times. Cells were processed and analyzed by Western blot as under panel A. The illustrated blots are representative of three similar experiments.

tively block mTORC1/2 activity in endothelial cells and increase MAPK phosphorylation.

### 3.2. The inhibition of mTORC1 activates MAPK in endothelial cells

We next determined whether the inhibition of mTORC1 or mTORC2 was responsible for the activation of MAPK by ATP-competitive inhibitors of mTOR in endothelial cells. To test this, HUVEC were transfected with siRNA targeting raptor or rictor to block mTORC1 or mTORC2 respectively. As a control HUVEC were also transfected with a scramble siRNA. We observed that downregulation of raptor in HUVEC increased MAPK phosphorylation (Fig. 2A). In contrast, downregulation of rictor decreased MAPK phosphorylation suggesting that the inhibition of mTORC1 by ATP-competitive inhibitors of mTOR increases MAPK phosphorylation in HUVEC.

Emerging data have shown that a subset of mTORC1 functions are resistant to the inhibition by rapamycin [21]. Therefore, we next analyzed whether the inhibition of rapamycin sensitive or resistant functions of mTORC1 increases MAPK phosphorylation. To test this, HUVEC were exposed for different period of time to rapamycin and MAPK phosphorylation was determined by Western Blot. Short-term exposure of HUVEC to rapamycin inhibited mTORC1 as observed by the dephosphorylation of S6 ribosomal protein (Fig. 2B). Long-term treatment of rapamycin also blocked mTORC2 as evidenced by the dephosphorylation of Akt and as previously reported [10]. Rapamycin increased MAPK phosphorylation already after 30 min of treatment (Fig. 2B). Taken together these results show that targeting rapamycin sensitive functions of mTORC1 increases MAPK phosphorylation in HUVEC.

### 3.3. Effect of combined mTOR and MAPK inhibition on endothelial cell proliferation, survival and migration and on vascular endothelial tube formation *in vitro*

Both mTOR and MAPK signaling pathways have been implicated in endothelial cell functions relevant to angiogenesis such as proliferation, survival, migration and tube formation [10] [22]. We

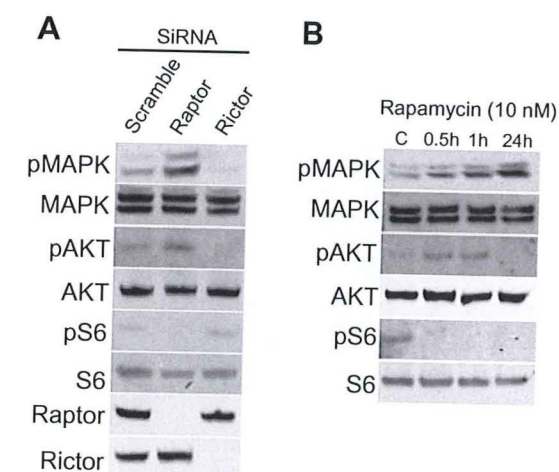
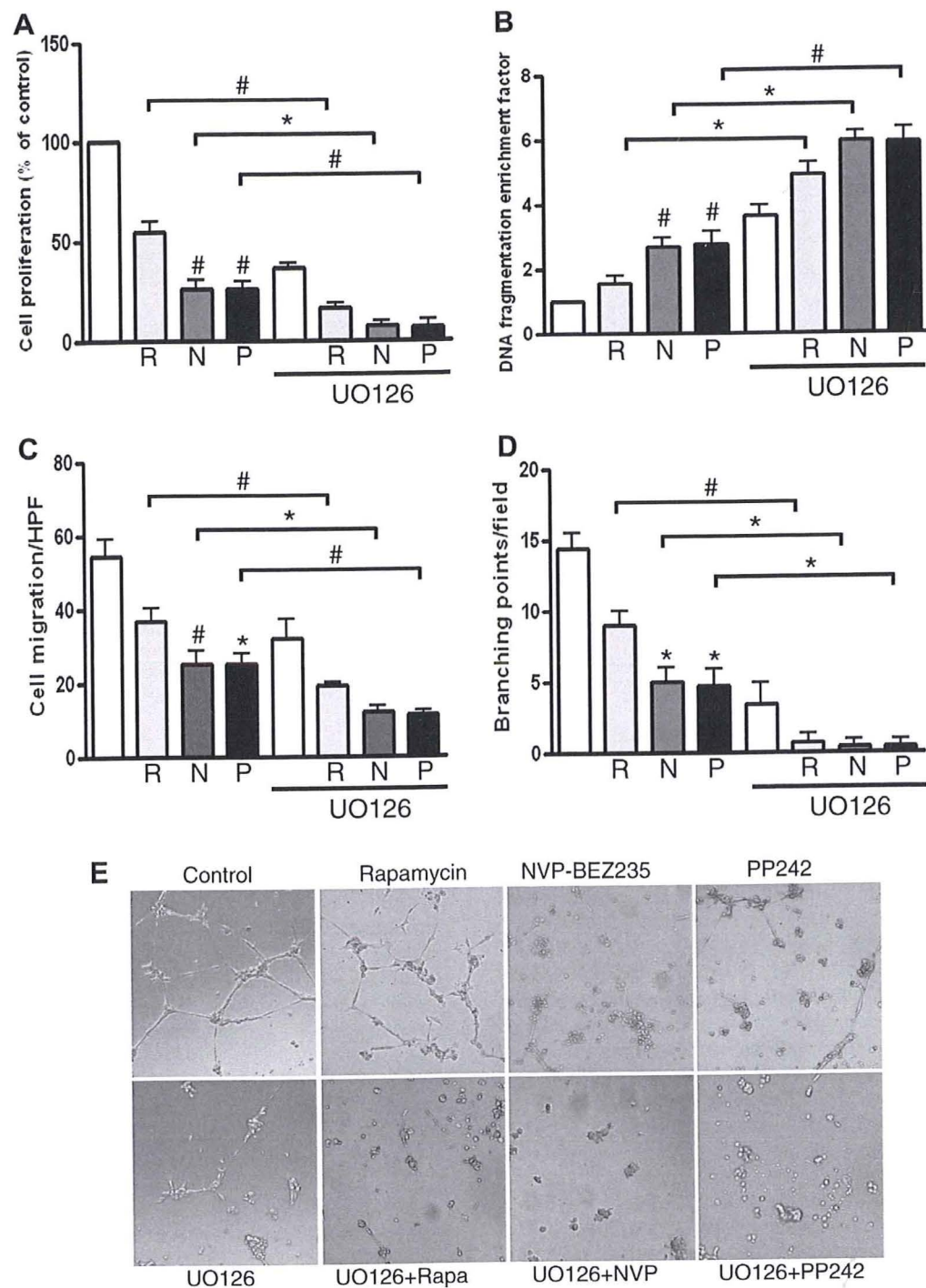


Fig. 2. The inhibition of mTORC1 increases MAPK phosphorylation in HUVEC. (A) HUVEC were transfected with scramble, raptor or rictor siRNA. Forty-eight hours post transfection HUVEC were lysed and lysates were examined for phospho-MAPK (Thr202/Tyr204), MAPK, phospho-AKT (Ser 473), AKT, phospho-S6 ribosomal protein (Ser234/235), S6 ribosomal protein, raptor or rictor expression level by Western blot analysis. (B) HUVEC were treated with rapamycin (10 nM) for the indicated times, lysed and lysates were analyzed by Western Blot for phospho-MAPK (Thr202/Tyr204), MAPK, phospho-AKT (Ser 473), AKT, phospho-S6 ribosomal protein (Ser234/235) or S6 ribosomal protein expression level by Western blot analysis. The illustrated blots are representative of three similar experiments.

therefore tested the effects of ATP-competitive inhibitors of mTOR on these functions. We found that mTOR inhibitors reduced endothelial cell proliferation (Fig. 3A). The effect of ATP-competitive inhibitors of mTOR was superior to rapamycin (Fig. 3A). No significant difference was observed between NVP-BE2235 the dual PI3K/mTOR inhibitor compared to PP242 which target mTOR alone. Similar findings were observed on endothelial cell survival and migration (Fig. 3B and C). We also hypothesized that the activation of MAPK by mTOR inhibitors would reduce the growth inhibitory



**Fig. 3.** Effect of mTOR and MAPK inhibitors on endothelial cell proliferation, survival, migration and tube formation. (A) MTS proliferation assay of HUVEC treated with rapamycin (R, 10 nM), NVP-BE2235 (N, 100 nM), PP242 (P, 100 nM) either alone or in combination with UO126 (10  $\mu$ M) for 48 h. Columns, mean cell proliferation of three individual experiments relative to control; bars, SD. (B) Apoptosis assay of HUVEC treated as under panel A for 48 h. Columns, mean enrichment factor at 405 nm of three individual experiments relative to control; bars, SD. (C) Migration assays of HUVEC that were pre-treated with rapamycin (R, 10 nM), NVP-BE2235 (N, 100 nM), PP242 (P, 100 nM) either alone or in combination with UO126 (10  $\mu$ M) for 4 h prior to the assay. Columns, mean cell count of migrated cells of three individual experiments; bars, SD. (D) HUVEC were treated for 4 h with rapamycin (R, 10 nM), NVP-BE2235 (N, 100 nM), PP242 (P, 100 nM) either alone or in combination with UO126 (10  $\mu$ M). HUVEC were subsequently harvested and plated onto matrigel for 6 h. Columns, mean number of branching points from three fields (magnification 100 $\times$ ); bars SD. Results are representative of three independent experiments. (E) Experiments were performed as in panel D and photomicrographs were taken (Olympus, magnification 100 $\times$ ). \*,  $P < 0.005$ ; #,  $P < 0.05$  compared to rapamycin or as specified by brackets.

properties of mTOR inhibitors. To test this, we treated HUVEC with mTOR inhibitors in combination or not with UO126 which inhibits MAPK by blocking MEK, a direct activator of MAPK [23]. Combining mTOR inhibitors with UO126 had additive effects compared to either drug alone on endothelial cell proliferation, survival and migration (Fig. 3A–C). Finally, we investigated the effects of

combined mTOR and MAPK inhibition on vascular endothelial tube formation *in vitro*. We found that ATP-competitive inhibitors of mTOR inhibited tube formation more efficiently than rapamycin. UO126 in combination with mTOR inhibitors had an additive effect (Fig. 3D and E). Taken together these results show that ATP-competitive inhibitors of mTOR reduced endothelial cell functions

relevant to angiogenesis more efficiently than rapamycin. They also show that combined mTOR and MAPK inhibition has additive anti-angiogenic effects *in vitro*.

### 3.4. UO126 potentiates the anti-angiogenic efficacy of mTOR inhibitors *in vivo*

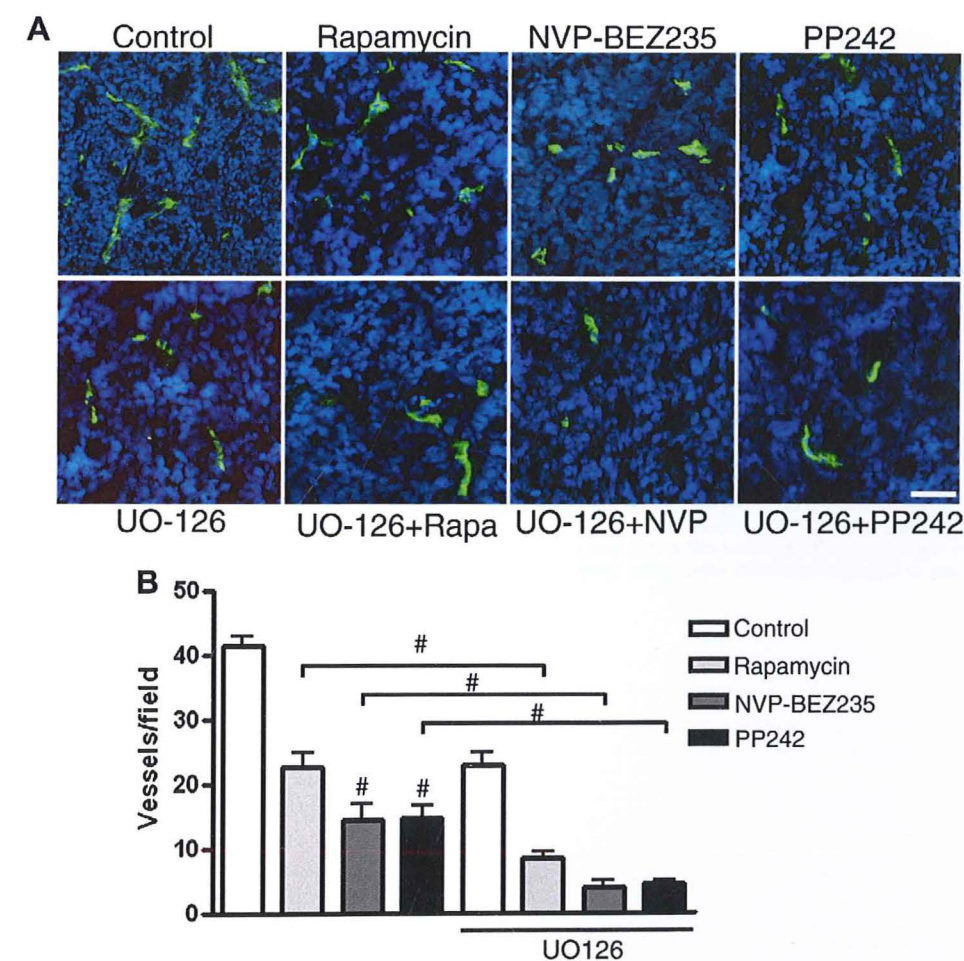
We next investigated whether the anti-angiogenic efficacy of mTOR inhibitors could be enhanced *in vivo* by the pharmacological blockade of MAPK signaling pathway. To test this, mice bearing LS174T colon cancer cell xenografts were treated with mTOR inhibitors either alone or in combination to UO126 to block MAPK signaling pathway. We observed that blocking mTOR or MAPK reduced tumor angiogenesis (Fig. 4A and B). The efficacy of ATP-competitive inhibitors of mTOR was superior to rapamycin. Combined mTOR and MAPK inhibition led to an additive effect consistent with our *in vitro* results. Taken together these results show that the combined inhibition of mTOR and MAPK produces a stronger anti-angiogenic effect compared to the inhibition of mTOR or MAPK alone.

## 4. Discussion

Despite encouraging experimental studies showing the anti-tumoral efficacy of rapamycin and rapamycin like drugs (rapalogs),

the clinical benefit has been less successful than expected. Part of it might be explained by the identification of multiple crosstalks between mTOR signaling pathway and other pathways implicated in cell growth. It has been well described that blocking mTORC1 by rapamycin stops a negative feedback loop resulting in the activation of proliferative and pro-survival signals such as the PI3K/Akt and the Ras/Raf-1/MEK/MAPK signaling pathways [13]. In turn, those signals counteract the growth inhibitory efficacy of rapamycin. Here we found that targeting mTOR in endothelial cells increased MAPK activity which reduced the anti-angiogenic efficacy of mTOR. Indeed the simultaneous inhibition of MAPK and mTOR had additive anti-angiogenic effects. Interestingly, the additive benefits of the use of MAPK and mTOR inhibitors have also been demonstrated on several cancer cells [24]. Tumor growth is more significantly reduced when MAPK and mTOR are used together compared to either agent alone as observed by reduced cancer cell proliferation and survival [24]. Therefore, the benefits of such therapeutic strategy are not restricted to the endothelium but also affect tumor cells.

Blocking tumor angiogenesis is a successful approach to reduce tumor growth. Therefore, the identification of the mechanisms regulating angiogenesis is crucial to develop new therapeutic strategies. Several evidences have shown that mTOR is an important mediator of cellular functions that are relevant to angiogenesis. Blocking mTOR with the allosteric inhibitor of rapamycin, which



**Fig. 4.** Effect of mTOR and MAPK inhibition on tumor angiogenesis. (A) Nude mice bearing LS174T xenografts were treated with rapamycin (rapa, 1.5 mg/kg/d), NVP-BE2235 (NVP, 30 mg/kg/d), PP242 (60 mg/kg/d), either alone or in combination with UO126 (40  $\mu$ mol/kg/d) for 28 days ( $n = 5$  in each group). Tumors were harvested and CD31 staining was performed on frozen sections of the tumors. Nuclei were counterstained with DAPI. Magnification 200 $\times$ . Scale bar 50  $\mu$ m. (B) Vessels were manually counted in five high-power field (HPF) in each tumor. Columns, mean vessel number per HPF ( $n = 3$  in each group); bars, SD. #,  $P < 0.05$  compared to rapamycin or as specified by brackets.

inhibits both mTORC1 and mTORC2 in endothelial cells, reduces endothelial cell proliferation and migration and induces apoptosis [10]. Furthermore, mTOR regulates VEGF expression and VEGF-mediated endothelial cell responses [10]. mTOR is also an important signaling intermediary in hypoxia-induced angiogenesis which is frequently encountered in tumors [25]. Consistently, targeting mTOR reduces tumor angiogenesis [11]. Our findings show that ATP-competitive inhibitors of mTOR are more efficient to block angiogenesis than rapamycin. Endothelial cell proliferation, survival, migration and tube formation were more significantly reduced by ATP-competitive inhibitors of mTOR compared to rapamycin. Furthermore, *in vivo*, ATP-competitive inhibitors of mTOR reduced tumor angiogenesis more effectively than rapamycin. Consistent with our observations on the endothelium, it has also been shown that the growth inhibitory properties of ATP-competitive inhibitors of mTOR are superior to rapamycin on various cancer cells including renal cell carcinoma [26]. Therefore, ATP-competitive inhibitors of mTOR represent promising agents in cancer therapy.

In summary, we show that ATP-competitive inhibitors of mTOR reduce angiogenesis more significantly than rapamycin. We also observe that the inhibition of mTOR in endothelial cells activates MAPK and that the simultaneous inhibition of mTOR and MEK has additive anti-angiogenic effects. Thus, we propose that blocking both mTOR and MAPK simultaneously is a promising approach to block angiogenesis.

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