Mammalian target of rapamycin (mTOR) inhibition reduces cerebral vasospasm following a subarachnoid hemorrhage injury in canines

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ABSTRACT

Mammalian target of rapamycin (mTOR) pathway is a serine/threonine protein kinase that plays a vital role in regulating growth, proliferation, survival, and protein synthesis among cells. In the present study, we investigated the role of the mTOR pathway following subarachnoid hemorrhage brain injury — specifically investigating its ability to mediate the activation of cerebral vasospasm. Additionally, we investigated whether key signaling pathway molecules such as the mTOR, P70S6K1, and 4E-BP1 play a role in the process. Thirty dogs were randomly divided into 5 groups: sham, SAH (subarachnoid hemorrhage), SAH + DMSO (dimethyl sulfoxide), SAH + Rapamycin and SAH + AZD8055. An established canine double-hemorrhage model of SAH was used by injecting autologous arterial blood into the cisterna magna on days 0 and 2. Angiography was performed at days 0 and 7. Clinical behavior, histology, immunohistochemistry, and Western blot of mTOR, P70S6K1, 4E-BP1 and PCNA (proliferating cell nuclear antigen) in the basilar arteries were examined. In the SAH and SAH + DMSO groups, severe angiographic vasospasm was obtained (34.3 ± 19.8%, 38.4 ± 10.3%) compared with that in Sham (93.9 ± 5.0%) respectively. mTOR, P70S6K1, 4E-BP1 and PCNA increased in the sample of spastic basilar arteries (p < 0.05). In the SAH + RAPA and SAH + AZD8055 groups, Rapamycin and AZD8055 attenuated angiographic vasospasm (62.3 ± 15.9% and 65.2 ± 10.3%) while improving appetite and activity scores (p < 0.05) on days 5 through 7. Rapamycin and AZD8055 significantly reduced the level and expression of mTOR, P70S6K1, 4E-BP1 and PCNA (p < 0.05). In conclusion, our study suggests that the mTOR molecular signaling pathway plays a significant role in cerebral vasospasm following SAH, and that inhibition of the mTOR pathway has the potential to become an attractive strategy to treat vasospasm following SAH.

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Introduction

Subarachnoid hemorrhage (SAH) is a devastating stroke subtype with a significantly high morbidity and mortality rate (Shirao et al., 2011; Zhang, 2001). Despite promising therapeutic approaches such as triple-H therapy, calcium channel blockades, endothelin-receptor antagonists and sodium nitroprusside, successful treatment following SAH remains inadequate. This is partly attributed to the poor strategic approach when dealing with cerebral ischemia as a result of cerebral vasospasm (CVS), one of the major consequences seen following an SAH. Although much has been discovered with regard to the mechanistic understanding of CVS, it continues to puzzle most scientists (Kusaka et al., 2003; Zhang, 2001).

Mammalian target of rapamycin (mTOR) is a serine/threonine protein kinase that plays a vital role in regulating growth, proliferation, survival, and protein synthesis among cells. In the present study, we investigated the role of the mTOR pathway following subarachnoid hemorrhage brain injury — specifically investigating its ability to mediate the activation of cerebral vasospasm. Additionally, we investigated whether key signaling pathway molecules such as the mTOR, P70S6K1, and 4E-BP1 play a role in the process. Thirty dogs were randomly divided into 5 groups: sham, SAH (subarachnoid hemorrhage), SAH + DMSO (dimethyl sulfoxide), SAH + Rapamycin and SAH + AZD8055. An established canine double-hemorrhage model of SAH was used by injecting autologous arterial blood into the cisterna magna on days 0 and 2. Angiography was performed at days 0 and 7. Clinical behavior, histology, immunohistochemistry, and Western blot of mTOR, P70S6K1, 4E-BP1 and PCNA (proliferating cell nuclear antigen) in the basilar arteries were examined. In the SAH and SAH + DMSO groups, severe angiographic vasospasm was obtained (34.3 ± 19.8%, 38.4 ± 10.3%) compared with that in Sham (93.9 ± 5.0%) respectively. mTOR, P70S6K1, 4E-BP1 and PCNA increased in the sample of spastic basilar arteries (p < 0.05). In the SAH + RAPA and SAH + AZD8055 groups, Rapamycin and AZD8055 attenuated angiographic vasospasm (62.3 ± 15.9% and 65.2 ± 10.3%) while improving appetite and activity scores (p < 0.05) on days 5 through 7. Rapamycin and AZD8055 significantly reduced the level and expression of mTOR, P70S6K1, 4E-BP1 and PCNA (p < 0.05). In conclusion, our study suggests that the mTOR molecular signaling pathway plays a significant role in cerebral vasospasm following SAH, and that inhibition of the mTOR pathway has the potential to become an attractive strategy to treat vasospasm following SAH.

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Mammalian target of rapamycin (mTOR) is a serine/threonine protein kinase that plays a vital role in regulating growth, proliferation, survival, and protein synthesis among cells. In the present study, we investigated the role of the mTOR pathway following subarachnoid hemorrhage brain injury — specifically investigating its ability to mediate the activation of cerebral vasospasm. Additionally, we investigated whether key signaling pathway molecules such as the mTOR, P70S6K1, and 4E-BP1 play a role in the process. Thirty dogs were randomly divided into 5 groups: sham, SAH (subarachnoid hemorrhage), SAH + DMSO (dimethyl sulfoxide), SAH + Rapamycin and SAH + AZD8055. An established canine double-hemorrhage model of SAH was used by injecting autologous arterial blood into the cisterna magna on days 0 and 2. Angiography was performed at days 0 and 7. Clinical behavior, histology, immunohistochemistry, and Western blot of mTOR, P70S6K1, 4E-BP1 and PCNA (proliferating cell nuclear antigen) in the basilar arteries were examined. In the SAH and SAH + DMSO groups, severe angiographic vasospasm was obtained (34.3 ± 19.8%, 38.4 ± 10.3%) compared with that in Sham (93.9 ± 5.0%) respectively. mTOR, P70S6K1, 4E-BP1 and PCNA increased in the sample of spastic basilar arteries (p < 0.05). In the SAH + RAPA and SAH + AZD8055 groups, Rapamycin and AZD8055 attenuated angiographic vasospasm (62.3 ± 15.9% and 65.2 ± 10.3%) while improving appetite and activity scores (p < 0.05) on days 5 through 7. Rapamycin and AZD8055 significantly reduced the level and expression of mTOR, P70S6K1, 4E-BP1 and PCNA (p < 0.05). In conclusion, our study suggests that the mTOR molecular signaling pathway plays a significant role in cerebral vasospasm following SAH, and that inhibition of the mTOR pathway has the potential to become an attractive strategy to treat vasospasm following SAH.

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publications suggesting that mTOR inhibition can block proliferation of vascular smooth muscle cells, which our research has shown to play a vital role in arterial wall thickening and vasculature stiffening following SAH, we questioned whether rapamycin (a mTOR inhibitor) could prevent CVS from happening at all.

As a result in the present study, we investigated the role of the mTOR protein kinase following SAH brain injury in canines, specifically investigating its position as a key orchestrator of cerebral vasospasm. We hypothesize that rapamycin, an established inhibitor of mTOR, may reduce the production of key mTOR signal molecules (P70S6K1 and 4E-BP1) involved with vasculature wall thickness and subsequent vasospasm. In order to test these aims, we used an mTOR C1 inhibitor (rapamycin) and an mTOR C1/mTOR C2 simultaneous inhibitor (AZD8055) to explore potential mechanistic theories while measuring direct anti-proliferation activity through P70S6K1, 4E-BP1 and PCNA (proliferating cell nuclear antigen) expression.

Materials and Methods

Animals

All protocols for this study were evaluated and approved by the Animal Care and Use Committee at Peking University Health Sciences Center and Shandong University of China and with Guidelines for the Use of Animals in Neuroscience Research by the Society for Neuroscience. Thirty male/female dogs weighing 15 to 20 kg were housed in a 12-hour light/dark cycle at a controlled temperature and humidity with free access to food and water. All animals were randomly assigned to one of five groups — Sham (n = 6); SAH (n = 6); SAH + DMSO (dimethyl sulfoxide; n = 6); SAH + RAPA (rapamycin, mTORC1 inhibitor; n = 6); and SAH + AZD8055 (a mTORC1/mTORC2 inhibitor; n = 6).

Subarachnoid Hemorrhage Model and Angiogram

SAH was induced by the double-hemorrhage injection model as previously described in dogs (Kusaka et al., 2003; Varsos et al., 1983). Briefly, animals were anesthetized with a cepromazine (0.1 to 0.5 mg/kg), atropine (0.05 mg/kg), and xylazine (1.1 mg/kg) cocktail. They were then intubated and maintained on 1% isoflurane and oxygen while on mechanical ventilation. The mean arterial blood pressure, end-tidal CO2, and oxygen saturation were continuously monitored using the Surgivet V60046 monitor (Smiths Medical, USA) and were maintained within normal ranges by adjusting the flow of isoflurane.

A sterile catheter was inserted into the vertebral artery via the femoral artery under fluoroscopic control. Liodixanol (Visipaque; 7 mL) was injected to acquire an image of the basilar artery. After angiography, 0.5 mL/kg of blood taken from the femoral artery was injected into the cisterna magna at day 0 and then repeated at day 2. The animals were tilted at a 20° angle for 10 min with their heads down, in a prone position, to permit pooling of blood around the basilar artery. The animals in the sham group did not receive the blood injection.

Angiogram Assessment

The angiogram was repeated on day 7 prior to sacrificing the animals. The basilar artery on angiogram was measured by a computer-based image analyzer (NIH Image, version 1.62) and conducted exactly as described in our previous publication (Kusaka et al., 2003; Yamaguchi et al., 2004). Briefly, arterial diameters were measured in a double-blind fashion on magnified angiograms. To eliminate magnification differences on the angiograms, a radio detectable scale was placed on the dog’s chin during the angiography run. The same scale was used consistently and was put at the same point on each dog’s chin. Relative to the size of this scale as a standard, all arterial diametric values were adjusted. Two researchers independently measured the arterial diameters on the magnified angiograms at three points: the distal (just before the bifurcating superior cerebral arteries), the proximal (just after basilar union), and the central (the midpoint between the previous two points) portions of the basilar artery. The mean of these three measurements was calculated to yield the arterial diameter. The mean of the values measured by the two researchers was taken as the final diameter of the basilar artery. The caliber of the basilar artery on day 7 was calculated as a percentage of the mean basilar artery diameter on day 0 before blood injection in each dog.

Pharmacologic interventions

Rapamycin (Sirolimus; AT-22,989; RAPA; Rapamune), Formula: CS1H79NO13, MW: 914.2. Rapamycin is a mTORC1-selective inhibitor that was approved by the FDA in Sept 1999, which we purchased from AXXORA, LLC (San Diego, CA).

AZD8055 ($1555, AZD8055). Formula: C25H31NO13, MW: 465.54. AZD8055 is a potent and selective mTOR kinase inhibitor that acts on both mTORC1 and mTORC2 which we purchased from Selleck Calbiochem (San Diego, CA).

Dosages for both drugs were 1 mg in 1 mL of 2% dimethyl sulfoxide (DMSO, Sigma). These values were appropriately calculated in order to reach the same level of drug in each animal’s cerebrospinal fluid (CSF) — assuming that canine CSF volume is 2 mL/kg (Kusaka et al., 2003; Zoghbi et al., 1985). The first injection was given 30 min following the first blood injection and was continued daily for an additional 3 days.

Clinical assessment

Three behavioral examinations were modified from a previous study (Kusaka et al., 2003; Yamaguchi et al., 2004) and performed daily after SAH to record appetite, activity, and neurological deficits (Fig. 2). Points were given using the following criteria: appetite: finished meal = 2, left meal unfinished = 1, scarcely ate = 0; activity: active, barking, or standing = 2, lying down, will stand and walk with some stimulation = 1, almost always lying down = 0; neurological deficit: no deficit = 2, unstable walk because of ataxia or paresis as = 1, and impossible to walk and stand because of ataxia or paresis = 0.

Assessment of histology

Three animals from each group were perfused under deep anesthesia with cold phosphate-buffered saline (PBS, pH 7.4), followed by infusion of 4% paraformaldehyde. The brains were then removed and fixed in formalin at 4 °C for a minimum of 3 days. The brains were then dehydrated with 30% sucrose in phosphate-buffered saline (PBS, pH 7.4) and the frozen coronal slices (10 μm thick) were then sectioned in cryostat (CM3050S; Leica Microsystems).

H&E Staining

Sections were stained in hematoxylin for 2 min and eosin for 1 min. They were then dehydrated and mounted by Permount.

Immunohistochemical Staining

Immunohistochemistry was performed using the following primary antibodies: rabbit polyclonal anti-mTOR (Protor-2, sc-101956), goat polyclonal anti-P70S6K1 (sc-101768), rabbit polyclonal anti-4E-BP1 (sc-25280), and mouse polyclonal anti-PCNA (sc-25280) (1:200; Santa Cruz Biotechnology). The histological sections of the basilar artery were then incubated with goat anti-rabbit (donkey anti-goat or goat anti-mouse) IgG as a secondary antibody (1:200) for 30 min, placed in
an avidin–peroxidase complex solution containing avidin–peroxidase conjugate for 30 min, and then mounted, dried, and cover-slipped.

Some sections were double and triple labeled (Zhou et al., 2004, 2005) with rabbit anti mTOR (protor-2) (red, goat anti-rabbit IgG-Texas Red 1:200), rabbit anti P70S6K1 (green, donkey anti-goat IgG-FITC, 1:200, Santa Cruz Inc.) and mouse anti PCNA (blue, goat anti-mouse IgG-AMCA (aminomethylcoumarin acetate, 1:200, Jackson Immuno Research Inc., Pennsylvania, USA) fluorescence staining, respectively. Sections were cover-slipped with 30% glycerin and examined under an OLYMPUS BX51 microscope with fluorescence light.

Western blotting analysis

Samples (50 μg protein; Bradford dye-binding procedure, Bio Rad Laboratories, Italy) of basilar arteries from each group (n = 3), were separated onto SDS-polyacrylamide membranes as previously described (Kusaka et al., 2003), and probed with the rabbit polyclonal anti-mTOR (protor-2), goat polyclonal anti-P70S6K1, rabbit polyclonal anti-4E-BP1, and mouse polyclonal anti-PCNA (1:200; Santa Cruz Biotechnology). A polyclonal antibody against β-actin (1:4000, Santa Cruz Biotechnology) was used as a control for protein gel loading. Blots were analyzed using the NIH-Image software. Data was normalized to those of β-actin and expressed as OD integration.

Data analysis

Values are presented as means ± SEM. Statistical comparisons between groups were performed using one-way analysis of variance (ANOVA) followed by either a Dunnett’s or a Tukey’s post-hoc test, the former for comparisons to a single control group, the latter to compare across multiple groups. For neurobehavioral tests, one-way repeated measures ANOVA after rank transformation. A probability of p < 0.05 was considered statistically significant.

Results

SAH extent and cerebral vasospasm

Severe SAH was particularly pronounced around the Circle of Willis and along the ventral brainstem following injury (Fig. 1A). The animals in the SAH and SAH+DMSO groups developed severe vasospasm (p < 0.05 vs Sham) as shown by angiography on day 7 (Fig. 1B). The mean values of the residual diameter of the basilar artery on day 7, as a percentage of that on day 0, were 34.3 ± 19.84% in SAH, 38.4 ± 10.26% in SAH + DMSO, and 93.9 ± 5.01% in Sham respectively (Fig. 1C). In the SAH + RAPA and SAH + AZD8055 groups, a moderate vasospasm, 62.3 ± 15.92% and 65.2 ± 10.34% was observed (p < 0.05 versus SAH and SAH + DMSO, Fig. 1B). No statistical difference was observed between the SAH + APA and SAH + AZD8055 groups (p > 0.05, ANOVA).

mTOR inhibitors improve appetite and activity following SAH injury

The behavior scores for appetite, activity, and neurological deficits are shown in Fig. 2. The appetite score in both Rapamycin and AZD8055 treatment groups were better than in the SAH group from days 2 to 4, even though statistical significance was achieved at days 5 to 7 (p < 0.05, ANOVA; Fig. 2A). No statistical difference was found between SAH + Rapamycin and SAH + AZD8055 groups (p > 0.05 ANOVA). The activity scores in Rapamycin and AZD8055 treatment groups were significantly better than in the SAH and SAH + DMSO groups (p < 0.05, ANOVA) at days 6 and 7 (Fig. 2B). Most animals did not show any signs of serious neurological deficits which
was evident by the lack of statistical significance among the observed groups (Fig. 2C).

**Vasospastic changes improve following both mTOR inhibitor treatments**

Morphological vasospasm was observed in animals assigned to the SAH group. This was characterized by corrugation of the internal elastic lamina, contraction of smooth muscle cells, and increased thickness of the vessel wall, which was a sign of severe vasospasm (Fig. 3A2). Treatment with vehicle of DMSO did not get any improvement (Fig. 3A3). Moderate vasospasm was observed in the SAH + RAPA (Fig. 3A4) and SAH + AZD8055 animal groups (Fig. 3A5). Sham animals were the only group that did not show any signs of vasospasm (Fig. 3A1).

mTOR inhibitors attenuate the notable immunoreactive of mTOR and proliferation in basilar artery

In the SAH and SAH + DMSO groups, notable staining of mTOR, P70S6K1, 4E-BP1, and PCNA was observed across all layers of the basilar artery, especially among smooth muscle cells (Figs. 3B2–E2 and B3–E3) compared to Sham animals (Figs. 3B1–E1). Animals treated with both Rapamycin and AZD8055 where characterized by a notable
reduction in staining of mTOR, P70S6K1, 4E-BP1, and PCNA (Figs. 3B4–E4 and B5–E5).

Double and triple fluorescence immunohistochemistry staining revealed a marked elevation in staining with mTOR (red, Fig. 4A1) P70S6K1 (green, Fig. 4A2) and PCNA (blue, Fig. 4A3) across all layers of the basilar artery, especially in smooth muscle layer; merging double and triple of these images indicated that mTOR co-localized with P70S6K1 (Fig. 4A4) and PCNA (Figs. 4A5; A6). Using a high magnification zoom, the stain demonstrated that mTOR, P70S6K1 and PCNA co-localized in the smooth muscle cells (Figs. 4B1–B6).

Western blotting revealed increased expression of mTOR pathway molecular biomarkers in the vasospastic artery.

The expressions of mTOR, P70S6K1, 4E-BP1, and PCNA in the basilar artery samples in Sham, SAH, SAH + DMSO, SAH + RAPA and SAH + AZD8055 groups were shown in Fig. 5. When the values in the sham basilar arteries were regarded as 100%, there was a significant enhancement of expression of mTOR, P70S6K1, 4E-BP1, and PCNA in the spastic basilar arteries from the SAH and SAH + DMSO animals sacrificed at day 7 (p<0.05 vs. Sham). Representative bands are shown at the top of Figs. 5A, B, C and D. Treatment with Rapamycin and AZD8055 significantly suppressed the expression of mTOR, P70S6K1, 4E-BP1, and PCNA (p<0.05 vs. Sham; Figs. 5A, B, C, D). No significant differences were noted between the two inhibitor treatment groups and between the SAH and SAH + DMSO groups.

Discussion

Subarachnoid hemorrhage (SAH) is a devastating stroke subtype that can lead to a variety of consequences including cerebral ischemic damage secondary to cerebral vasospasm. In the present study, we investigated the role of rapamycin, an established mTOR inhibitor, on cerebral vasospasm following SAH injury in canines and explored possible mechanisms behind its actions. We made the following novel observations: (1) mTOR and PCNA (proliferating cell nuclear antigen) expression significantly increased in the VSMCs (vascular smooth muscle cells) in vasospastic basilar arteries at day 7 following SAH injury; (2) both Rapamycin (inhibits mTORC1) and AZD8055 (inhibits both mTORC1 and mTORC2) abolished the expression of mTOR and its downstream P70S6K1 and 4E-BP1, as well as the proliferation marker PCNA; and (3) both inhibitors attenuated angiographic vasospasm and improved clinical scores in our subject population. These results suggest that the mTOR pathway may in fact play a significant role in cerebral vasospasm following SAH injury and its mediation may be through blockade of vascular cell proliferation.

mTOR is a major biological switch, coordinating an adequate response to changes in energy uptake, growth signals and environmental stress. Additionally, it can regulate cell cycle progression, protein translation, metabolism as well as cellular proliferation. Given its fundamental role in tumorigenesis, it is not surprising that a huge effort has been made to develop mTOR inhibitors as a potential cancer therapy (Carew et al., 2011; Ciuffreda et al.,...
2010). As a protein kinase, mTOR forms two distinct multiprotein complexes called mTOR1 and mTORC2. The C1 prototype is responsible for activating p70S6K, which in turn phosphorylates the ribosomal protein S6 and 4E-BP1—which is inhibited by rapamycin (Corradetti et al., 2006; Gera and Lichtenstein, 2011). On the other hand, AZD8055 is a potent and selective mTOR kinase inhibitor that acts on both the C1 and C2 prototypes, along with other downstream substrates (Chresta et al., 2010). However, in addition to their anti-tumorigenesis properties, previous research has suggested their role as an inhibitor of vascular smooth muscle cell proliferation (Marx et al., 1995). Specifically, studies have suggested that the mTOR/P70S6K1/4E-BP1 pathway may be specifically responsible for the proliferation (Liao et al., 2010). However, screenings of the mechanism of vasospasm after SAH by targeting mTOR pathway for antiproliferation have not been investigated extensively. Accordingly, in our study, we found that the mTOR/P70S6K1/4E-BP1 pathway was significantly increased in the vasospastic artery at day 7 following SAH injury [(Figs. 3, 5), the triple fluorescence labeling of mTOR, P70S6K1 and PCNA (Fig. 4)] suggesting that mTOR proliferation in VSMCs may play a role in cerebral vasospasm. Previous research has alluded to the notion that death of endothelial cells resulting in its denudation exposes the smooth muscle cell layer to neurotransmitters, toxins, and other vasoactive agents circulating in the blood stream (Debdi et al., 1992; Zhou et al., 2004). This may potentially explain how the activity of mTOR molecular pathway mediates the proliferation (vasospasm) in basilar artery, and why we witnessed an improvement in CVS following our therapeutic interventions i.e. Rapamycin and AZD8055.

Additionally, recent works have suggested that mTOR inhibitors can also decrease hypoxia inducible factor 1α (HIF-1α) levels (Carew et al., 2011; Faivre et al., 2006). Back in 2006, we also made similar conclusions and found that with the use of HIF-1α inhibitors (2ME2 and D609), we could reduce the cerebral vasospasm following SAH injury in rats and attenuate the expression of one of its downstream target genes i.e. vascular endothelial growth factor (VEGF) (Yan et al., 2006). Accordingly, in the present study although we acknowledge we did not measure HIF nor VEGF in our subject population, those animals that were treated with Rapamycin or AZD8055 experienced an attenuation of angiographic vasospasm and an improvement in clinical behavioral scores. These results suggest that in addition to anti-proliferation properties, mTOR inhibitor could potentially provide some protection through other therapeutic targets including anti-HIF-1α/VEGF; this is
because VEGF is a potent stimulant of angiogenesis and is involved in the pathogenesis of cerebral vasospasm (Bhardwaj, 2003; Borel et al., 2003; McGirt et al., 2002).

In conclusion, our study suggests that vascular smooth muscle cell proliferation mediated by the mTOR pathway may in fact play a significant role in cerebral vasospasm following SAH injury. By blocking the activation of the mTOR pathway, the attenuation of angiographic vasospasm was attributed to the anti-proliferation that ensues following cerebral vasospasm. More studies evaluating the exact mechanistic target of the mTOR pathway within vasospasm are warranted.

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References


Fig. 5. mTOR, P70S6K1, 4E-BP1 and PCNA activation in the basilar artery of SAH. Top, Western blotting bands of antibodies, bottom, density of the protein band from each group. A. mTOR activation in the basilar artery demonstrated an elevated expression of mTOR on day 7 following SAH in SAH and SAH + DMSO animals. The mTOR inhibitors, Rapamycin and AZD8055 reduced the enhanced expression of mTOR (p<0.05, ANOVA). B. P70S6K1 activation. C. 4E-BP1 activation. D. PCNA activation. Rapamycin and AZD8055 significantly decreased the expression of P70S6K1, 4E-BP1 and PCNA compared with the SAH and SAH + DMSO (p<0.05, respectively; ANOVA). There was no significant difference between the two inhibitors in mTOR, P70S6K1, 4E-BP1 and PCNA expression.


