

Anti-angiogenic Effects of Mammalian Target of Rapamycin Inhibitors in a Mouse Model of Oxygen-Induced Retinopathy

Rina Yagasaki, Tsutomu Nakahara,* Hiroko Ushikubo, Asami Mori, Kenji Sakamoto, and Kunio Ishii

Department of Molecular Pharmacology, Kitasato University School of Pharmaceutical Sciences; 5–9–1 Shirokane, Minato-ku, Tokyo 108–8641, Japan.

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Ocular pathologic angiogenesis is a causative factor for retinopathy of prematurity, diabetic retinopathy, and age-related macular degeneration. In the present study, we examined the effects of rapamycin and everolimus, inhibitors of mammalian target of rapamycin (mTOR), on retinal pathologic angiogenesis in mice with oxygen-induced retinopathy (OIR), an animal model of proliferative ischemic retinopathy. Mice were exposed to 80% oxygen from postnatal day (P) 7 to P10, and were then brought into room air and subcutaneously injected with rapamycin and everolimus. The neovascular tufts, the size of the central avascular zone, and the immunoreactivity for phosphorylated ribosomal protein S6 (pS6), a downstream indicator of mTOR activity, were evaluated in flat-mounted retinas. Retinal neovascular tufts and vascular growth in the avascular zone were observed in P15 mice with OIR. In addition, intense immunoreactivity for pS6 was detected in the neovascular tufts and in endothelial cells located at the vascular–avascular border. Both rapamycin and everolimus reduced the extent of retinal neovascular tufts and pS6 immunoreactivity, but they also increased the size of the avascular zone. Thus, activation of the mTOR pathway in endothelial cells contributes to retinal pathologic angiogenesis, and mTOR inhibitors that target proliferating endothelial cells are promising candidates as anti-angiogenic agents for the treatment of vasoproliferative retinal diseases.

Key words mammalian target of rapamycin; pathologic angiogenesis; retina; vascular endothelial growth factor

Ocular pathologic angiogenesis contributes to the pathogenesis of vision threatening eye diseases, such as retinopathy of prematurity, diabetic retinopathy, and age-related macular degeneration.¹⁾ The vascular endothelial growth factor (VEGF) and its receptors (VEGFRs) system play an important role in ocular pathologic angiogenesis, and inhibiting this system is a promising therapeutic strategy for these potentially blinding diseases.^{2–6)} In addition, compounds that modulate signaling pathways upstream and downstream of VEGF represent promising anti-angiogenic strategies for vasoproliferative retinal diseases.

One possible candidate agent to oppose the VEGF-induced signaling pathways is rapamycin, an inhibitor of mammalian target of rapamycin (mTOR).^{7,8)} Rapamycin is a macrolide antibiotic that was originally developed as an antifungal agent, but it was later found to have potent immunosuppressive effects and antiproliferative effects on tumor cells. In addition to inhibiting tumor cell growth, rapamycin also exhibits anti-angiogenic effects.^{9,10)} The anti-angiogenic action of rapamycin could be mediated by decreasing VEGF production¹¹⁾ and by attenuating the response of endothelial cells to VEGF.^{9,12)} In the field of ophthalmology, the anti-angiogenic effects of rapamycin have been demonstrated in animal models,^{13,14)} but the mechanisms are not fully understood.

Here, we examined the effects of two mTOR inhibitors, rapamycin and everolimus, on retinal pathologic angiogenesis in a mouse model of oxygen-induced retinopathy (OIR). We also evaluated the status of the mTOR pathway in endothelial cells under pathologic angiogenesis using immunohistochemistry to detect phosphorylated ribosomal protein S6 (pS6), a downstream indicator of mTOR activity.

MATERIALS AND METHODS

Animals Adult ICR (Institute of Cancer Research) mice were obtained from the Charles River Breeding Laboratories (Tokyo), the mice were provided a standard diet (Oriental Yeast, Tokyo, Japan) and tap water *ad libitum*. Female animals were placed with males, and pregnant females were segregated and kept in separate cages with their litters. The day of birth was determined by daily inspections and defined as postnatal day (P)0.

All animal procedures were performed at Kitasato University in accordance with the Association for Research in Vision and Ophthalmology Statement on the Use of Animals in Ophthalmic and Vision Research and the Regulations for the Care and Use of Laboratory Animals in Kitasato University adopted by the Institutional Animal Care and Use Committee for Kitasato University.

A Mouse Model of OIR OIR was induced in mouse pups according to the protocol presented in Fig. 1A. Briefly, P7 mice were placed under hyperoxic conditions (80% O₂) in an oxygen-regulated chamber with their nursing mother for 3 d to produce retinal vasoobliteration. Oxygen concentrations were measured with a sensor placed inside the cage and regulated by an oxygen controller (ProOx110; Biospherix, Redfield, NY, U.S.A.). Then, the mice were returned to normoxia (room air) for 5 d to induce ischemic retinal neovascularization (Fig. 1A). The formation of central avascular zones and neovascular tufts, typical pathological changes in the OIR mouse,¹⁵⁾ were observed under our experimental conditions.

Drug Treatments According to protocol presented in Fig. 1C, mice were treated with rapamycin or everolimus and sacrificed using sodium pentobarbital (Nacalai Tesque, Kyoto, Japan). The eyes were enucleated and fixed for 0.5–1 h at 4°C

The authors declare no conflict of interest.

* To whom correspondence should be addressed. e-mail: nakaharat@pharm.kitasato-u.ac.jp

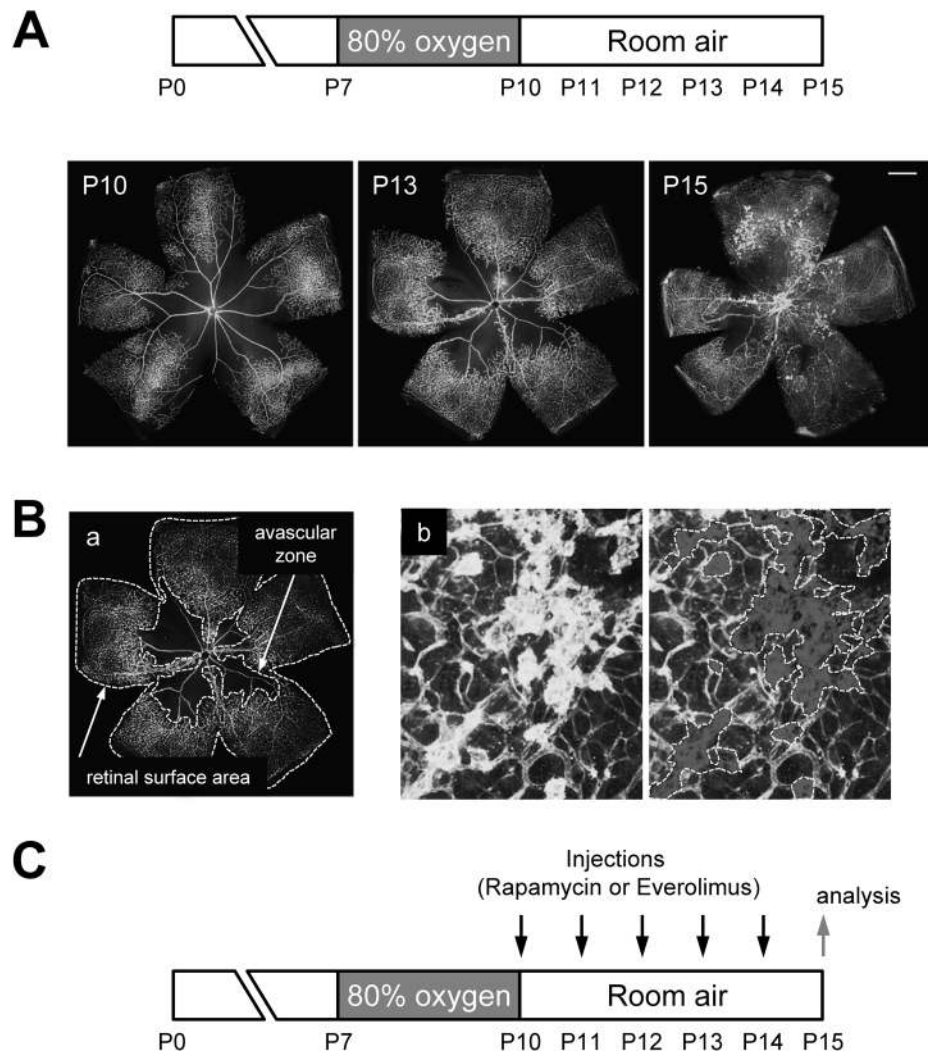


Fig. 1. Scheme of the OIR Model, Methods of the Image Analysis and Schedules for Drug Treatments

A: Neonatal mice were exposed to 80% oxygen for 3 d, from postnatal day (P)7 to P10. At P10, mice were returned to room air. Representative images of the flat-mounted retinas stained with an anti-CD31 antibody to detect endothelial cells. Immediately after returning the mice to room air, a large avascular area was observed in the center of the retina (P10). The central avascular retina becomes hypoxic, triggering both normal vessel regrowth and a pathologic formation of retinal neovascularization (P13). The maximum severity of neovascularization was reached at P15. Scale bar, 500 μ m. B: Representative analysis images. To evaluate the central avascular area, areas of central avascular zone and total retinal surface were measured (a). To quantify the neovascular tufts, neovascular tufts and clusters were manually outlined, and the areas were measured (b). Scale bar, 500 μ m (a), 57 μ m (b). C: Schedule for injections with rapamycin and everolimus. Eyes were obtained at P15 for analyses.

in 4% paraformaldehyde (Nacalai Tesque) in phosphate-buffered saline (PBS). After fixation, the cornea, lens, uvea, and sclera were removed. The remaining retinas were post-fixed and stored in methanol at -20°C .

Rapamycin (LC Laboratories, Woburn, MA, U.S.A.) and everolimus (LC Laboratories) were dissolved in dimethyl sulfoxide (DMSO) (25 mg/mL), diluted in 4% EtOH/5% Tween 80/5% PEG400, and administered subcutaneously in a volume of 10 μ L/g body weight. Controls animals were treated with the vehicle in a similar manner.

Immunohistochemistry Immunohistochemical analyses were performed according to the methods described in our previous report.¹⁶ Briefly, retinas were incubated in blocking solution (5% normal goat serum) in PBS containing 0.5% Triton X-100 (PBS-T) for 0.5–1 h at room temperature. To visualize vascular endothelial cells, retinas were incubated with a rat monoclonal anti-CD31 antibody (1 : 500, BD Biosciences, San Diego, CA, U.S.A.) overnight. For co-staining of endothelial cells and pS6, the tissues were incubated with a rat monoclonal anti-CD31 antibody (BD Biosciences) and a rabbit

monoclonal anti-pS6 antibody (1 : 200; Cell Signaling, Danvers, MA, U.S.A.). After several rinses with PBS-T, the tissues were incubated for 4 h at room temperature with species-specific secondary antibodies (1 : 400; Jackson ImmunoResearch, West Grove, PA, U.S.A.). Then, tissues were rinsed in PBS-T, and flat-mounts were prepared using Vectashield (Vector Laboratories, Burlingame, CA, U.S.A.). The retinas incubated in the absence of primary antibodies were used as controls; these retinas were processed and evaluated for specificity or background levels of staining. Images were collected using a fluorescent microscope system BZ-9000 (Keyence, Osaka, Japan) or a confocal laser scanning microscope LSM 510 Meta (Zeiss, Oberkochen, Germany).

Evaluation of Pathological Angiogenesis The pathological angiogenesis in the OIR were evaluated by quantifying the central avascular area of the retina and neovascular tufts. First, images of whole-mount retinas were obtained using a fluorescent microscope system (BZ-9000). And then, areas of central avascular zone and total retinal surface were measured using ImageJ software (NIH, Bethesda, MD, U.S.A.;

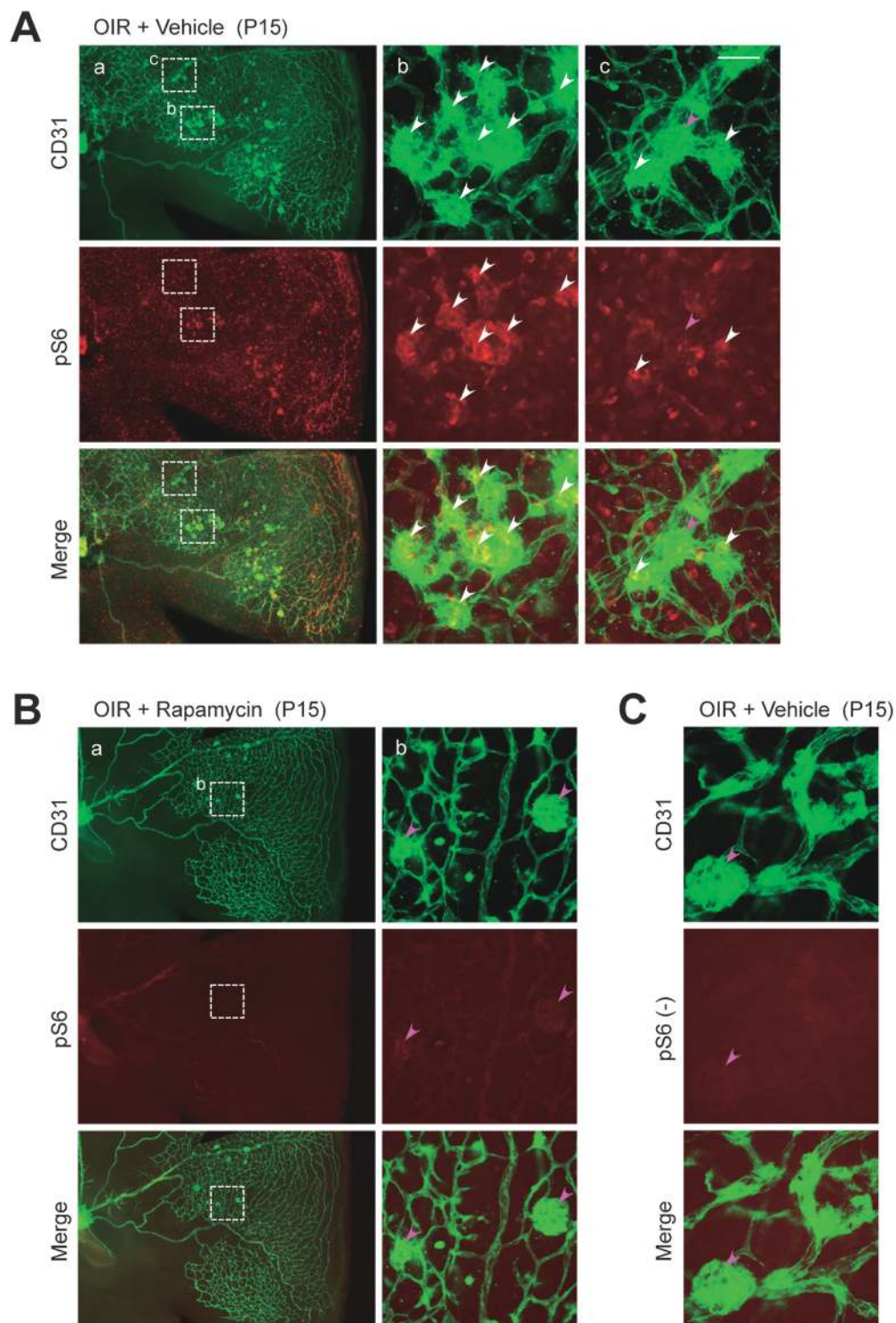


Fig. 2. Effects of Rapamycin on Retinal Neovascularization in OIR Mice

A, B: Representative images of flat-mounted retinas labeled with antibodies for CD31 and phosphorylated S6 (pS6) protein at postnatal day (P)15 OIR mice treated with either vehicle (A) or rapamycin (B) according to protocol presented in Fig. 1C. Insets with higher magnification images are shown in the right panels. Strong pS6 staining was observed in some neovascular tufts (white arrows), but not in others (pink arrows). The formation of neovascular tufts and the immunoreactivity for pS6 were markedly diminished in rapamycin-treated mice compared to vehicle-treated mice. Scale bar, 366 μ m (a), 100 μ m (b, c). C: The retinal image of negative control without anti-pS6 antibodies [pS6 (-)]. Scale bar, 100 μ m.

<http://rsb.info.nih.gov/ij/>) (Fig. 1Ba). The avascular area was expressed as a percentage of the total retinal surface area. To quantify the neovascular tufts, neovascular tufts and clusters were manually outlined in 5 selected regions. The area of neovascular tufts relative to that of selected region was measured (Fig. 1Bb).

Statistical Analysis Statistical comparisons were performed using ANOVA, followed by a Tukey–Kramer post-test (GraphPad Prism 5; GraphPad Software, San Diego, CA, U.S.A.). A *p* value less than 0.05 was considered statistically significant. All values are presented as the mean \pm S.E.

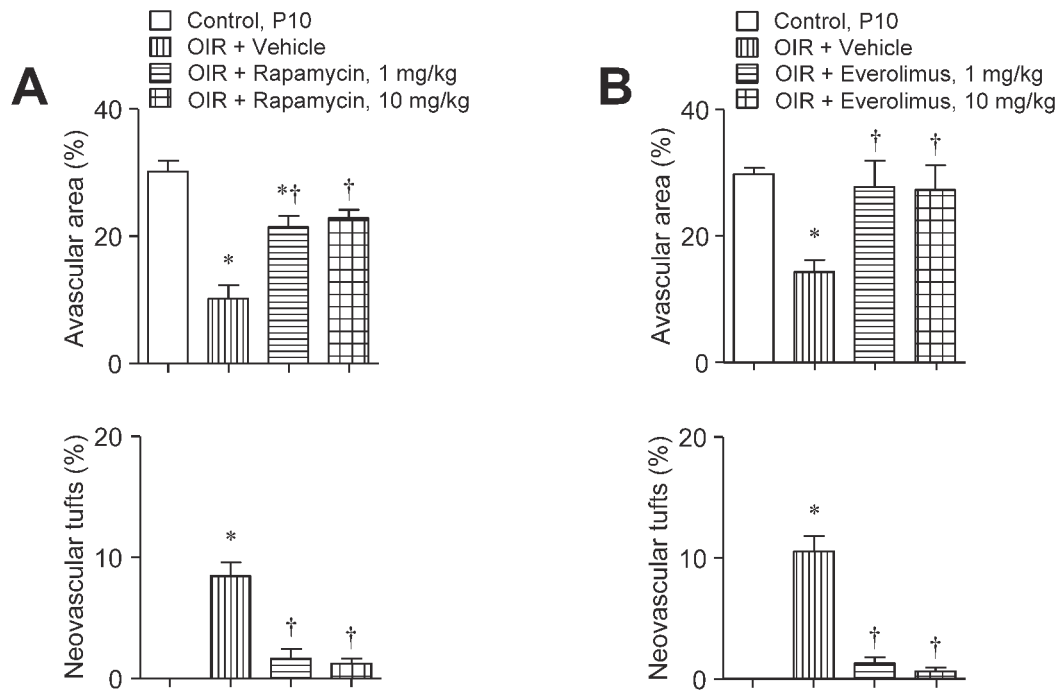


Fig. 3. Effects of Rapamycin (A) and Everolimus (B) on Retinal Neovascularization in OIR Mice

Quantitative analyses revealed that treatment with rapamycin or everolimus significantly increased the avascular area in retinas and reduced retinal neovascular tufts compared with vehicle-treated OIR mice. Each column with a vertical bar represents the mean \pm S.E. from 5–14 animals. * $p < 0.05$ versus Control; † $p < 0.05$ versus vehicle.

RESULTS

Figure 2 shows the vascular pattern of flat-mounts stained with anti-CD31 antibodies in 15-d-old OIR mice treated with either vehicle (Fig. 2A) or rapamycin (Fig. 2B). In the retinas of vehicle-treated mice, many blood vessels were observed growing toward the central avascular area from surviving vessels near the optic nerve head. Neovascular tufts were found on superficial blood vessels at the border between the vascularized and the central avascular regions.

Many pS6-positive cells were detected in the retinas of vehicle-treated mice, and pS6 staining was more intense at the transition zone between the vascular and avascular retina. However, pS6 staining was not observed in a subset of CD31-positive endothelial cells. Non-vascular cells in the retinal parenchyma also exhibited pS6 immunoreactivity. High-magnification images revealed strong pS6 staining in some neovascular tufts, but not in others (Fig. 2A). The co-localization of pS6 immunoreactivity with CD31-positive endothelial cells was also examined by a confocal microscopy (Supplemental figure).

The avascular retina area increased and the formation of neovascular tufts and the immunoreactivity for pS6 were markedly diminished in rapamycin-treated mice compared to vehicle-treated mice (Fig. 2B). Similar observations were made in mice treated with everolimus instead of rapamycin.

The quantitative data are summarized in Fig. 3. In 15-d-old vehicle-treated OIR mice, the decreased avascular area and the increased neovascular tufts were observed. These OIR-induced vascular changes were significantly attenuated by rapamycin or everolimus.

DISCUSSION

The present study demonstrates that mTOR inhibitors, rapamycin and everolimus, prevent retinal pathogenic angiogenesis in mice with OIR, an animal model of proliferative ischemic retinopathy. The inhibitory effects of rapamycin on retinal neovascularization were consistent with previous findings.¹³ In addition, our study revealed intense staining for pS6, a downstream indicator of mTOR activity, in neovascular tufts and growing endothelial cells at the transition zone between the vascular and avascular retina, which was markedly diminished by mTOR inhibition. These results suggest that the mTOR pathway is activated in proliferating endothelial cells, and mTOR inhibitors target these cells.

The vascular pathology of OIR is associated with increased levels of VEGF.¹⁷ Therefore, the anti-angiogenic action of mTOR inhibitors on retinal pathologic angiogenesis in OIR mice might be mediated by decreasing VEGF production^{9,11} and/or by attenuating the response of endothelial cells to VEGF.⁹ However, it was reported that VEGF levels were not reduced in the retinas of OIR mice treated with rapamycin. Rather, VEGF levels increased, even though the extent of neovascularization was significantly reduced.¹³ Thus, diminished VEGF expression is unlikely to play a major role in the anti-angiogenic effects of rapamycin in OIR mice. Alternatively, increased VEGF seems to stimulate endothelial cell growth toward the hypoxic avascular area *via* activation of the mTOR pathway. In fact, mTOR inhibitors have been shown to prevent VEGF-driven retinal angiogenesis.¹²

Other systems may also activate the mTOR pathway. For example, apelin and its receptor, APJ, play an important role in endothelial cell proliferation in the retina.^{18,19} Retinal vascular phenotypes of apelin-deficient mice resembled those of mice treated with rapamycin, and rapamycin did not show any

significant effects on parameters related to retinal angiogenesis, such as radial vascular growth and branching, in apelin-deficient mice.¹⁹ Therefore, mTOR appears to act downstream of the apelin-APJ system in endothelial cells on the vascular front.¹⁹ Moreover, apelin expression dramatically increased during the hypoxic phase in OIR mice, and APJ was colocalized in proliferative endothelial cells.²⁰

Inhibition of VEGF and its receptors represent a promising therapeutic strategy^{2–6}; however, adverse effects associated with VEGF inhibition, including impairments of normal retinal vascular growth and retinal function, have been reported.^{21,22} The present results suggest that the mTOR pathway is activated in proliferating endothelial cells within retinal vasculature, and mTOR inhibitors display a narrow-spectrum effect on endothelial cells in a region- and status-dependent manner. The ability of these inhibitors to target endothelial cells in a proliferative state is a favorable trait for anti-angiogenic therapy in vasoproliferative retinal diseases. However, the mTOR pathway is apparently activated in several non-vascular cell types; therefore, it is important to determine effects of mTOR inhibitors on such non-vascular cells in future studies.

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