

# Antiangiogenic Effect of Deguelin on Choroidal Neovascularization

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## ABSTRACT

Age-related macular degeneration is the leading cause of blindness in the elderly. Choroidal neovascularization (CNV) leads to severe vision loss in patients of age-related macular degeneration. Previously, we have demonstrated that deguelin, isolated from plants in the *Mundulea sericea* family, is a chemopreventive agent. This study evaluates the antiangiogenic effect of deguelin on CNV. The toxicity of deguelin was evaluated through 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay in human umbilical vein endothelial cells (HUVECs) as well as histological examination and terminal deoxynucleotidyl transferase dUTP nick-end labeling staining in the deguelin-injected retina. Antiangiogenic activity of deguelin was evaluated by in vitro tube formation assay of HUVECs and

in vivo angiogenesis of chick chorioallantoic membrane (CAM). In C57BL/6 mice with laser-induced CNV, deguelin or phosphate-buffered saline was injected intravitreally. CNV lesions were examined by fluorescence angiography and vessel counting in cross-sections. Deguelin showed no effect on cell viability of HUVECs and no retinal toxicity in a concentration range of 0.01 to 1  $\mu$ M. Deguelin effectively inhibited in vitro tube formation of HUVECs and in vivo angiogenesis of CAM. Interestingly, deguelin significantly reduced CNV and its leakage in mouse model of laser photocoagulation-induced CNV. Our data suggests that deguelin is a potent inhibitor of CNV and may be applied in the treatment of other vasoproliferative retinopathies such as retinopathy of prematurity and diabetic retinopathy.

Angiogenesis occurs with the imbalance between positive and negative factors that keep angiogenesis. Under certain conditions such as hypoxia or inflammation that activate angiogenic factors, the balance may shift to angiogenesis (Folkman, 2006). Stimulation of endothelial cells with angiogenic factors, such as vascular endothelial growth factor (VEGF) and fibroblast growth factor induce endothelial cells to produce and export a plethora of proteases, such as matrix metalloproteases and plasminogen activators to digest the

basement membrane and to allow endothelial cells to invade the surrounding tissue. Endothelial cells proliferate and migrate to form a sprout (Risau, 2003). Pathological angiogenesis in the eye is the most common cause of blindness in all age groups (Aiello et al., 1994). Particularly, age-related macular degeneration (AMD) is the leading cause of blindness over the age of 55 years in developed countries (Klein et al., 1995). Although the detailed mechanism was not elucidated as yet, choroidal neovascularization (CNV) leads to severe vision loss in patients of AMD (Macular Photocoagulation Group, 1991). CNV invades the subretinal space by the vessel proliferation from the choroidal vessels following the rupture of Bruch's membrane. The new vessels are fragile and leaky, inducing hemorrhages or exudation damaging to photoreceptor cell function. On the other hand, these proliferating vessels induce the formation of fibrovascular scar, which results in irreversible damage to the retinal function and likely

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**ABBREVIATIONS:** VEGF, vascular endothelial growth factor; AMD, age-related macular degeneration; CNV, choroidal neovascularization; PI3K, phosphatidylinositol 3-kinase; HUVEC, human umbilical vein endothelial cell; CAM, chick chorioallantoic membrane; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labeling; PBS, phosphate-buffered saline; HIF, hypoxia-inducible factor.

blindness (Gehrs et al., 2006). Because excessive proliferation of vascular cells is the essential mechanism of CNV, it is reasonable to directly ameliorate the proliferating vascular endothelial cells. The most common model of CNV is created by the laser photocoagulation-induced rupture of Bruch's membrane, which stimulates proliferation of vascular endothelial cells in pre-existing choroidal capillary networks (Miller et al., 1986).

Rotenoids, compounds from the flavonoid family, have chemopreventive activity by inhibiting NADH:ubiquinone oxidoreductase activity and by suppressing steady-state mRNA levels and enzymatic activity of 12-*O*-tetradecanoylphorbol 13-acetate-induced ornithine decarboxylase (Gerhäuser et al., 1995; Fang and Casida, 1998). The rotenoid, deguelin, was found in several plant species, including *Mundulea sericea* (Leguminosae) (Gerhäuser et al., 1997). We have demonstrated that deguelin inhibits cyclooxygenase-2 expression and phosphatidylinositol 3-kinase (PI3K)/Akt-mediated signaling pathways and that contributes to its antiproliferative effects (Chun et al., 2003; Lee, 2004; Lee et al., 2004, 2005). However, we also found that deguelin reduced the expression of Hsp90-binding proteins, including hypoxia-inducible factor (HIF)-1 $\alpha$  protein, and induced the degradation of HIF-1 $\alpha$  protein independent of the reactive oxygen species and PI3K-Akt pathways (Oh et al., 2007).

In the present study, we demonstrate that deguelin inhibits *in vitro* angiogenesis of human umbilical vein endothelial cells (HUVECs) and *in vivo* angiogenesis of chick chorioallantoic membrane (CAM) without cytotoxic effect and significantly reduces laser-induced CNV in a mouse model of AMD without significant retinal toxicity. In addition to the antiproliferative activity of deguelin to cancer cells (Chun et al., 2003; Lee, 2004; Lee et al., 2004, 2005; Oh et al., 2007), we herein suggest that deguelin, a new angiogenesis inhibitor, may have a therapeutic potential in the treatment of CNV of AMD as well as in other vasoproliferative retinopathies such as retinopathy of prematurity and diabetic retinopathy.

## Materials and Methods

**Animals.** C57BL/6 mice were purchased from Samtako (Kyungki-do, Korea). Care, use, and treatment of all animals in this study were in strict agreement with the Association for Research in Vision and Ophthalmology statement for the Use of Animals in Ophthalmic and Vision Research.

**Cell Culture.** HUVECs were maintained in a gelatin-coated dish at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>-95% air in M199 medium with 20% fetal bovine serum, 100 units/ml penicillin, 100 g/ml streptomycin, 3 ng/ml basic fibroblast growth factor, and 1 ml of heparin. HUVECs used in this study were taken from passages 6 to 7.

**Preparation of Deguelin.** Deguelin was manufactured from the natural product rotenone (Sigma-Aldrich, Milwaukee, WI) via four steps, as described previously (Anzenveno, 1979). The final product was more than 98% pure. Deguelin was stored at a stock concentration of 1 mM in a nitrogen tank.

**Cell Viability Assay.** Cell viability was evaluated with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. HUVECs ( $1 \times 10^5$  cells) were plated in 96-well plates and cultured overnight. Cells were treated with deguelin (0.01~10  $\mu$ M) for 48 h. The medium was then replaced with fresh medium containing 0.5 mg/ml MTT for 4 h. After incubation, the medium was carefully removed from the plate, and DMSO was added to solubilize formazan produced from

MTT by the viable cells. Absorbance was measured at 540 nm using a microplate reader (Molecular Devices, Sunnyvale, CA).

**Terminal Deoxynucleotidyl Transferase dUTP Nick-End Labeling Assay.** Deguelin was intravitreally injected to 7- to 8-week-old female C57BL/6J mice. The mice were sacrificed at 3 days after 0.1  $\mu$ M deguelin injection, and eyes were enucleated. Enucleated globes were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer for 24 h and embedded in paraffin. Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) staining was performed with a kit (ApopTag Fluorescein Green; Intergen, Purchase, NY), according to the manufacturer's instructions. TUNEL-positive cells were evaluated in randomly selected fields at 400 $\times$  magnification viewed under fluorescence microscopy (BX50; Olympus, Tokyo, Japan).

**Tube Formation Assay.** Tube formation was assayed as described previously (Min et al., 2007). HUVECs ( $1 \times 10^5$  cells) were inoculated on the surface of the Matrigel and treated with 0.1  $\mu$ M deguelin or VEGF (20 ng/ml) for 18 h. The morphological changes of the cells and tubes formed were observed under a microscope and photographed at 200 $\times$  magnification. Tube formation was quantified by counting the number of connected cells in randomly selected fields at 200 $\times$  magnification (Carl Zeiss, Chester, VA) and dividing that number by the total number of cells in the field.

**CAM Assay.** Three-day-old fertilized eggs were incubated at 37°C, and a window was made after the extraction of 3 to approximately 4 ml of ovalbumin. After 2 days of incubation, a thermanox coverslip (Nalge Nunc International, Naperville, IL) covered with deguelin (0.1  $\mu$ M) was applied on the CAM of individual embryos. After 48 h, the intralipose was injected into the chorioallantois of the embryos, and CAMs were evaluated (Carl Zeiss).

**Western Blotting.** Cell extracts were prepared, and aliquots (10–30- $\mu$ g protein) were loaded onto 9% gel and transferred to protein nitrocellulose membrane. The membrane was incubated in blocking buffer (5% skim milk in 0.1% Tween 20 in PBS) at room temperature. Then the filter was incubated with anti-VEGF antibody (1:2000; Santa Cruz Biochemicals, Santa Cruz, CA) at 4°C for overnight and washed with 0.1% Tween 20 in PBS three times every 10 min, followed by incubation with anti-rabbit polyclonal antibody at room temperature for 1 h. Immunoreactive bands were visualized using chemiluminescent reagent.

**Laser Photocoagulation-Induced CNV.** Seven to 8-week-old female C57BL/6J mice were anesthetized, and the pupils were dilated with 1% tropicamide (Alcon Laboratories Inc., Forth Worth, TX). Three burns of 831-nm diode laser photocoagulation (75- $\mu$ m spot size, 0.1-s duration, 120 mW) were delivered to each 3, 6, 9, and 12 o'clock position of two disc diameters from optic disc by using indirect head set delivery system of a photocoagulator (OcuLight; Iridex, Mountain View, CA) and a handheld +78 diopter lens. The bubbling or pop sensing with laser photocoagulation was considered as the successful rupture of Bruch's membrane.

To assess the antiangiogenic activity of deguelin, the mice were injected intravitreally with 0.1  $\mu$ M deguelin in 1  $\mu$ l of PBS on the 10th day after laser photocoagulation, when maximum CNV began. These experiments were repeated at least 25 times.

**Qualitative Assessment of CNV by Fluorescein Angiography.** At 14 days after laser photocoagulation, deeply anesthetized mice were perfused through the tail vein with high-molecular mass (500,000) fluorescein-conjugated dextran (Sigma-Aldrich Ltd.) dissolved in PBS. After 1 h of perfusion, the eyes were enucleated and fixed in 4% paraformaldehyde for 4 h. The eyeballs were dissected, flat-mounted in Dako mounting medium (DakoCytomation, Glostrup, Denmark), and viewed by fluorescein microscopy (BX50; Olympus) at a 100 $\times$  magnification.

**Quantitative Assessment of CNV by Counting Vessels from Subretinal Fibrovascular Membrane.** At 14 days after laser photocoagulation, the eyes were removed, fixed in 4% paraformaldehyde in 0.1 M phosphate buffer for 24 h, and embedded in paraffin. Sagittal sections of 4 to 5  $\mu$ m, each 10  $\mu$ m apart, were cut through the center of the laser photocoagulation site. The sections were

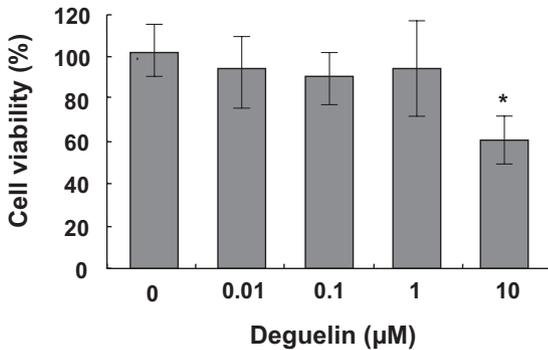
stained with hematoxylin and eosin to assess CNV via light microscopy (Carl Zeiss). Any vessels from subretinal fibrovascular membrane were counted in five sections from each laser photocoagulation site by two independent observers blind to treatment (J.Hy.K. and Y.S.Y.). The average was calculated for 100 sites of each group. There were at least 25 animals in each group.

**Statistical Analysis.** Statistical differences between groups were evaluated with the Student's unpaired *t* test (two-tailed). Mean ± S.D. is shown. *P* ≤ 0.05 was considered significant.

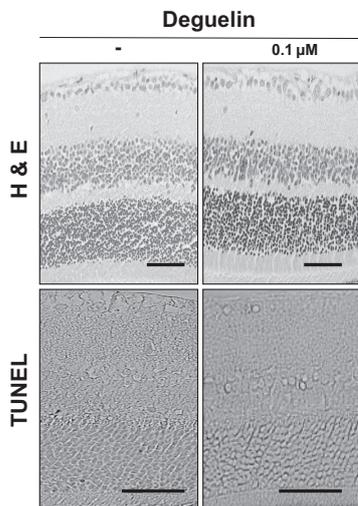
**Results**

**Effect of Deguelin on the Viability of HUVECs.** To investigate cytotoxic effect of deguelin on HUVECs, MTT assay was carried out in various concentrations of deguelin (0–10 μM). The viability of HUVECs was not affected by up to 1 μM deguelin (Fig. 1). Deguelin (0.1 μM), effective therapeutic concentration to inhibit VEGF expression in our previous report (Oh et al., 2007), did not affect the viability of HUVECs.

**Retinal Toxicity of Deguelin.** Retinal toxicity to intravitreal injection of 1 μM deguelin (10 times of effective therapeutic concentration of deguelin) was evaluated through histo-



**Fig. 1.** Effect of deguelin on the viability of HUVECs. Various concentrations of deguelin (0–10 μM) were treated on HUVECs, and cells were incubated for 2 days. Cell viability was measured by MTT assay. Each value represents means ± S.E. from three independent experiments (\*, *P* < 0.05).



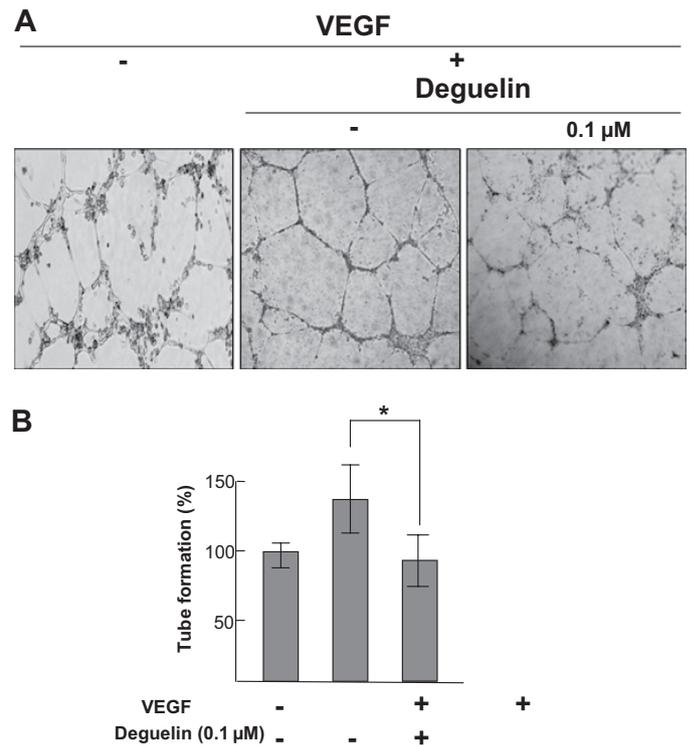
**Fig. 2.** Retinal toxicity of deguelin. Deguelin (1 μM) was intravitreally injected, and the globes were enucleated 3 days after treatment. The retina was normal without any inflammatory cells in the vitreous, retina, or choroid. Scale bars, 50 μm.

logical examination and TUNEL assay. As demonstrated in Fig. 2, the retina was of normal thickness, and all retinal layers were clear without any cell inflammation in the vitreous, retina, or choroid. Compared with control, TUNEL-positive cells were not increased with deguelin injection.

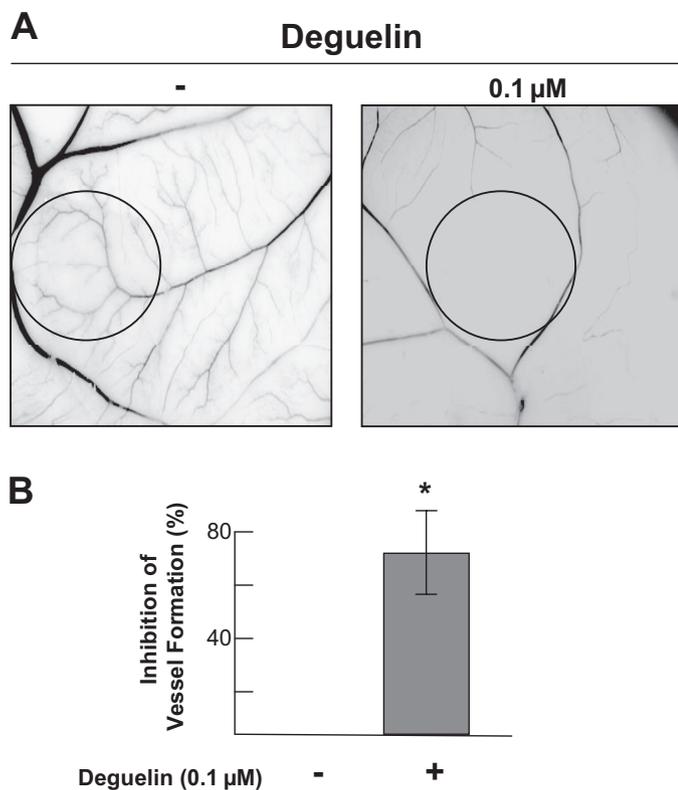
**Effect of Deguelin on Tube Formation of HUVECs.** To investigate the effect of deguelin on the angiogenic phenotype of tube formation in vitro assay, VEGF was used as an angiogenic factor. VEGF induced the formation of extensive capillary-like networks of HUVECs cultured on two-dimensional Matrigel matrix, and this effect was almost completely inhibited by co-treatment with deguelin (Fig. 3A). VEGF-stimulated tube formation of HUVECs approximately 1.3-fold, and this effect was abolished by deguelin (Fig. 3B).

**Effect of Deguelin on in Vivo Angiogenesis of CAM.** Consistent with inhibition of tube formation, in vivo angiogenesis of CAM was blocked by deguelin (Fig. 4A). Deguelin inhibited in vivo angiogenesis to approximately 70% (Fig. 4B).

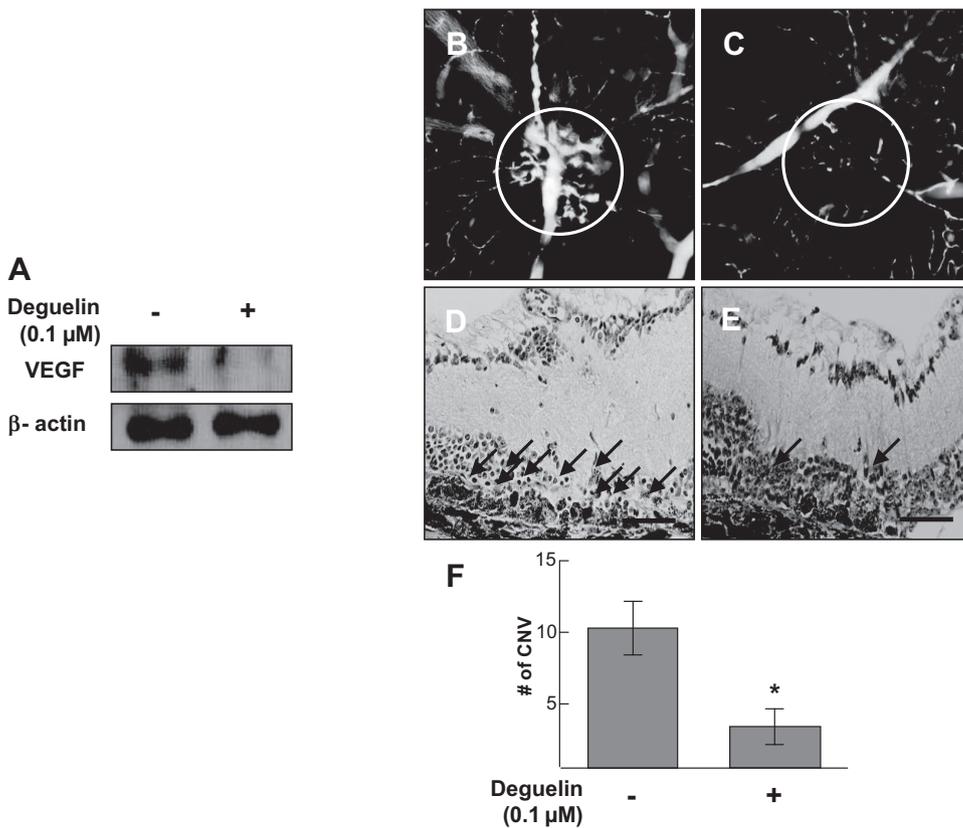
**Effect of Deguelin on VEGF Expression in HUVECs and Laser Photocoagulation-Induced CNV.** Based on our previous data of inhibition of VEGF expression (Oh et al., 2007), we injected deguelin intravitreally with 0.1 μM deguelin in 1 μl of PBS on 10 days after laser photocoagulation when active neovascularization occurs. We confirmed that 0.1 μM deguelin dramatically reduces VEGF expression in HUVECs (Fig. 5A). To visualize CNV, fluorescein angiography using fluorescein-conjugated dextran was performed. Retinas from control mice subjected to laser photocoagulation showed CNVs with diffuse leakage on the laser photocoagulation site (Fig. 5B). In contrast, retinas from deguelin-



**Fig. 3.** Effect of deguelin on VEGF-induced tube formation of HUVECs. A, each figure is representative of three independent experiments. B, basal tube formation of HUVECs that were left in serum-free media was normalized to 100%, respectively. Each value represents means ± S.E. from three independent experiments (\*, *P* < 0.05).



**Fig. 4.** Effect of deguelin on in vivo angiogenesis of CAM. A, each figure is representative of three independent experiments. Sector form indicates the position of thermanox with PBS or 0.1 μM deguelin. B, each value to indicate inhibition of capillary formation represents means ± S.E. from three independent experiments (\*,  $P < 0.05$ ).



**Fig. 5.** Effect of deguelin on VEGF expression in HUVECs and laser photocoagulation-induced CNV. A, deguelin (0.1 μM) dramatically reduces VEGF expression in HUVECs. Whole-mount preparation from control (B) and 0.1 μM deguelin-treated (C) mice subjected to laser photocoagulation-induced CNV was performed after 1-h perfusion of fluorescein-conjugated dextran, respectively. Circles, CNVs in the laser photocoagulation site. Hematoxylin-stained cross-sections prepared from control (D) and 0.1 μM deguelin-treated (E) mice subjected to CNV, respectively. Arrows, new vessels growing from choroidal vessels. F, data in each column are the mean ± S.D. values from 100 sites of 25 mice (\*,  $P < 0.05$ ). Scale bars, 50 μm (D and E).

treated mice showed significantly reduced CNV and its leakage (Fig. 5C). To quantify CNV, vessels from subretinal fibrovascular membrane were counted in a masked fashion. The vessels were evaluated as the mean number per section found in five sections per laser photocoagulation site. Retinas from control mice demonstrated multiple CNVs (Fig. 5D), whereas retinas from deguelin-treated mice showed significantly fewer neovascular lumens (Fig. 5E). We found that deguelin-injected groups had significant decrease of CNV compared with controls (Fig. 5F).

**Discussion**

We have demonstrated that deguelin has antiproliferative activity to cancer cells via regulation of cyclooxygenase-2 expression and PI3K/Akt-mediated signaling pathways (Chun et al., 2003; Lee, 2004; Lee et al., 2004, 2005). Recently, we also found out that deguelin reduces VEGF expression through the destabilization of HIF-1α protein. Interestingly, this antiangiogenic activity of deguelin was independent on reactive oxygen species and PI3K-Akt pathways (Oh et al., 2007).

Because deguelin is derived from rotenone, which can inhibit NADH:ubiquinone oxidoreductase in mitochondrial oxidative phosphorylation (Fang and Casida, 1998), it is possible that deguelin induces side effects in cardiovascular, respiratory, and nervous system. However, we never observed major toxicity in the mice at the therapeutically effective dose (Lee et al., 2005; Oh et al., 2007). In terms of the mechanism of action, deguelin may be different from that of rotenone to inhibit tubulin polymerization (Marshall and Himes, 1978; Srivastava and Panda, 2007), which could be one reason that deguelin is safer. However, it is also possible

that the reason of no grave toxicity in deguelin may be in degree, which means that deguelin may be a weaker inhibitor. In this study, deguelin showed no effect on the viability of HUVECs and no retinal toxicity up to 1  $\mu\text{M}$ , which is equivalent to 10 times the effective dose (0.1  $\mu\text{M}$ ) to CNV.

The blood vessel growth in CNV correlates with the expression of VEGF, basic fibroblast growth factor, and their receptors (Edelman and Castro, 2000). At the concentration to effectively reduce VEGF expression in cancer cells and inhibit tumor growth (Oh et al., 2007), deguelin reduces VEGF expression in HUVECs (Fig. 5A) and inhibits in vitro tube formation (Fig. 3) and in vivo chorioallantoic membrane of chick embryo (Fig. 4). Choroidal neovascularization and vascular leakage are two important factors that induce serious visual loss in AMD. In the present study, we presented the inhibitory effect of deguelin on choroidal neovascularization. Deguelin reduced the incidence of clinically significant vascular leakage in an experimental model of CNV. This was consistent with histologic findings of significantly less number of CNV in the deguelin-treated group. Based on these results, it is possible that deguelin may attenuate laser photocoagulation-induced CNV through a direct antiangiogenic effect without cytotoxic effect in the therapeutic range.

Given the well documented antiproliferative effect on HUVECs and antiangiogenic effect on CAM and CNV in the present study, we suggest that deguelin could be a new antiangiogenic agent to CNV, which is from rotenone. Furthermore, deguelin may be also applied to other vasoproliferative retinopathies such as retinopathy of prematurity and diabetic retinopathy.

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