의학석사 학위논문

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이 논문을 의학석사 학위논문으로 제출함

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김 고 은

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2013년 2월

위 원 장 _____ (인)

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위 원 _____ (인)

Abstract

Purpose: Anti-cancer, anti-viral, anti-bacterial, and anti-inflammatory effect of MB660 (3,4-dihydro-2,2-dimethyl-2H-naphtho-[1,2-b]pyran-5,6-dione) has been well known. We aimed to demonstrate the anti-angiogenic effect of MB660 on the retinal neovascularization via HIF-1α degradation.

Methods: The toxicity of MB660 was evaluated by modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay in human retinal microvascular endothelial cells (HRMECs) as well as histologic examination and terminal deoxynucleotidyl transferase biotin-dUTP nick-end labeling staining in the MB660-injected retina of C57BL/6J mice. Retinal neovascularization was examined by fluorescence angiography and vessel counting in cross sections after intravitreal injection of MB660 in the oxygen induced retinopathy (OIR) mouse model. Western blot analysis was used to assess inhibition of hypoxia inducible factor (HIF)-1α by MB660 at a protein level under hypoxic condition induced by $CoCl_2$.

Results: MB660 did not show any toxicity on HRMECs and did not induce any structural or inflammatory changes in normal retina layers up to 1 μM. Intravitreal injection of MB660 effectively reduced anomalous retinal angiogenesis, shown as decreased neovascular tufts on fluorescein angiography and decreased vascular lumens in cross section in the OIR mouse model and also attenuated $CoCl_2$ induced HIF-1α production.

Conclusions: Our data suggest that MB660 may have anti-angiogenic activity on retinal neovascularization by suppressing hypoxia-induced vascular endothelial growth factor expression via attenuating HIF-1α without retinal toxicity.

Keywords: MB660, anti-angiogenesis, vascular endothelial growth factor, hypoxia inducible factor-1α

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Introduction

Angiogenesis is the process of the formation of vascular networks characterized by sprouting, branching, and regression of new blood vessels.(1, 2) Pathologic angiogenesis plays an important role in the occurrence and progression of many ophthalmologic diseases, such as diabetic retinopathy, exudative age-related macular degeneration, and retinopathy of prematurity (ROP).(3) In the process of pathologic angiogenesis, vascular endothelial growth factor (VEGF) and associated signaling pathway plays a major role.(4) Inhibition of pathologic angiogenesis targeting VEGF signaling pathway would be the main therapy in preventing patients with such diseases from going blind. Thus, ongoing efforts have been done to develop anti-angiogenic drugs which can selectively target abnormal vessels, without affecting homeostasis and normal vessel development.

MB660 (3,4-dihydro-2,2-dimethyl-2H-naphtho-[1,2-b]pyran-5,6-dione) is an ortho naphthoquinone, originally isolated from the lapacho tree, has been proven to have anti-cancer, anti-fungal, anti-bacterial, and anti-inflammatory effects.(5-8) It is an inhibitor of DNA-topoisomerase (I and II) and blocks the DNA repair process, thus sensitizing cells to DNA-damaging agents.(5, 6) Another proposed mechanism of cell death is via activation of a futile cycling of the drug by the cytoplasmic two-electron reductase NAD(P) H: quinone oxidoreductase, also known as NQO1, DTdiaphorase and Xip3.(6-10) At higher drug concentrations, the production of reactive oxygen species appears to be responsible for its anti-cancer

mechanism. Yet, detailed mechanism of apoptotic or necrotic cell death induced by MB660 is still under investigation.

In addition to its multipotent effects, Kung et al.(11) have investigated its apoptotic and anti-angiogenic effects on endothelial cells, suggesting that MB660 may have potential as an anti-angiogenic drug to block tumor growth. However, besides the cytotoxic effect of MB660 on human endothelial cells associated with the NO/cGMP signaling pathway, anti-angiogenic effect of MB660 was not clearly described in the study. Thus, we examined whether MB660 has an anti-angiogenic effect on retina related with VEGF signaling pathway.

In the current study, we investigated that intravitreal injection of MB660 could inhibit angiogenesis in human retinal microvascular endothelial cells (HRMECs) and the retinal neovascularization in oxygen induced retinopathy (OIR) animal model, possibly through attenuating hypoxia inducible factor (HIF)-1α production. We demonstrate that MB660 induces a degradation of HIF-1α in the process of anti-angiogenesis.

Materials and Methods

Animals

C57BL/6J mice were purchased from Samtako (Seoul, Korea) and they were kept in standard 12-hour dark-light cycle and approximately 23℃ room temperature. Care, use, and treatment of all animals in this study were in strict agreement with the ARVO statement for the Use of Animals in Ophthalmic and Vision Research.

Cell cultures

HRMECs purchased from the Applied Cell Biology Research Institute (Kirkland, WA, USA) were used in the study. Cells were isolated and maintained in M199 culture medium (Gibco BRL, Carlsbad, CA, USA) containing 20% fetal bovine serum (Gibco BRL, Carlsbad, CA, USA), 20 mmol/L HEPES (pH 7.4), 3 ng/ml recombinant fibroblast growth factor (Millipore, Bedford, MA, USA) and 10 U/ml heparin (Sigma, St.Louis, MO, USA) and antibiotics on plates coated with 0.3% gelatin. HRMECs used in this study were taken from passages 4 to 9. Human brain astrocytes were purchased from the Applied Cell Biology Research Institue (Kirkland, WA, USA) and were grown in DMEM (Thermo scientific Hyclone, Logan, UT, USA) supplemented with 20% fetal bovine serum (Gibco BRL, Carlsbad, CA, USA), and N-2 supplement (Gibco BRL, Carlsbad, CA, USA). For hypoxic condition, human brain astrocytes were incubated at 5% CO_2 and 1% O_2, balanced with N_2 at 37℃ in hypoxic chamber and they were also treated with

cobalt chloride ($CoCl_2$).

Cell viability assay

Cell viability was evaluated with 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assays. HRMECs were seeded into each well of 96-well plates at a concentration of 4×10^3 cells/well and human brain astrocytes were seeded into each well of 96-well plates at a concentration of 5×10^3 cells/well. After incubation for 24 hours, cells were treated with either dimethyl sulfoxide (DMSO) as a control or various concentration of MB660 (0.01-10 μM) for 48 hours. The medium was exchanged with fresh media containing MTT solution (0.5 mg/mL). After incubation at 37℃ for 2 hours, the medium was carefully removed from the plate and 200 μL DMSO was added to solubilize formazan produced from MTT by the viable cells. Absorption at 540 nm was measured using a microplate spectrophotometer (Molecular Devices, Sunnyvale, CA, USA). Three independent experiments were performed for each experimental condition.

Histology

We intravitreally injected 1 μL MB660 of 1 μM, to 8-week-old C57BL/6J mice. The mice were sacrificed 7 days after the treatment and enucleation was performed. Enucleated eyes were fixed in 4% paraformaldehyde for 24 hours, and embedded in paraffin for histological analysis and cross section. From paraffin blocks, 4-μm-thick serial sections were prepared. Sections were deparaffinized and hydrated by

sequential immersion in xylene and graded alcohol solutions. Then sections were stained with hematoxylin and eosin (H&E) for histological examination. To asses changes in the retinal layers, the ratio of A (retinal thickness from the internal limiting layer to the innernuclear layer) to B (retinal thickness from the internal limiting layer to the outer nuclear layer) was measured in all sections via the light microscope (Carl Zeiss, Chester, VA, USA).

Terminal deoxynucleotidyl transferase biotin-dUTP nick-end labeling (TUNEL) Assay

MB660 was injected intravitreally to 7- to 8-week-old C57BL/6J mice. The mice were sacrificed at 3 days after 0.1 μM MB660 injection, and eyes were enucleated.(12) Enucleated eyes were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer for 24 hours and embedded in paraffin. TUNEL assay 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 400x magnification viewed under fluorescein microscopy (BX50, Olympus, Tokyo, Japan).

Oxygen induced retinopathy (OIR)

OIR was induced as the method described by Smith et al.(13) with some modification.(14) Newborn mice were randomly assigned to either control or experimental groups. At postnatal day (P)7, pups in the experimental group were

exposed to hyperoxia (75 ± 0.5% O_2) for 5 days (P7 to P12) and then returned to normoxia (room air) for another 5 days. Neovascularization occurred on returning to normoxia and peaked at P17. To evaluate the anti-angiogenic effect of MB660, we injected MB660 of 0.1 μM in 1 μL PBS intravitreally at P14, when retinal neovascularization began.

Qualitative assessment of retinal neovascularization by fluorescein angiography

At P17, deeply anesthetized mice were perfused through the tail vein with high molecular weight (MW=500,000) fluorescein conjugated dextran dissolved in PBS. After 1 hour of perfusion, the eyes were enucleated and fixed in 4% paraformaldehyde for 4 hours. The retinas were dissected, flat-mounted in Dako mounting medium (DakoCytomation, Glostrup, Denmark) and viewed by fluorescein microscopy (BX50, Olympus, Tokyo, Japan) at a magnification of 4x.

Quantitative assessment of retinal neovascularization by counting vascular lumens

At P17, the eyes were removed from OIR model, fixed in 4% paraformaldehyde in 0.1 M phosphate buffer for 24 hours, and embedded in paraffin. Sagittal sections of 4 μm, each 30 μm apart, were cut through the cornea parallel to the optic nerve. The sections were stained with H&E to assess retinal vasculature via light microscopy (Carl Zeiss, Chester, VA, USA). Any vascular lumens between

posterior lens capsule and the vitreal side of the inner limiting membrane were counted. The neovascular lumens were defined as the mean number per section found in at least 10 sections (at least five on each side of the optic nerve) per eye. The average intravitreal vessels per section were calculated for each group.

Western blot

Western blotting was performed using standard western blotting methods. The protein concentration was measured using a BCA protein assay kit (Pierce, Rockford, IL, USA). Equal amounts of protein from the supernatant were separated by electrophoresis on SDS-PAGE and transferred to nitrocellulose membrane (Amersham Hybond ECL, GE Healthcare, Cardiff, Wales, United Kingdom). The membranes were incubated overnight at 4℃ with anti-HIF-1α and anti-β-actin monoclonal antibody (Cell Signaling Technology, Beverly, MA). After the incubation with primary antibodies, the membranes were washed and incubated with peroxidase-conjugated secondary antibodies (Cell Signaling Technology). Then the membranes were treated with Amersham ECL western blot detection reagent (GE ECL, GE Healthcare). The blots were scanned using a scanner and the band intensity was analyzed using ImageJ 1.40 software (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis

Differences between groups were evaluated with the Mann-Whitney U-test.

Statistical analyses were performed using SPSS software version 18.0 (SPSS Inc., Chicago, IL, USA). P values less than 0.05 were considered to be statistically significant.

Results

Effect of MB660 on the viability of HRMECs and retina

MTT assay was performed in various concentrations (0.01-10 μM) of MB660 to investigate cytotoxic effect of MB660 on HRMECs. MB660 up to 1 μM did not affect the viability of HRMECs, but significant reduction of cell viability was observed in 10 μM of MB660 (Figure 1). Retinal toxicity of MB660 was evaluated by histologic examination with H&E stain and TUNEL assay after 1 μL intravitreal injection of 1 μM MB660 in C57BL/6J mice. As shown in figure 2, the retina was normal without any structural changes and no inflammatory cells were found in all retinal layers. Compared to the control, TUNEL-positive cells were not also increased after the treatment. Therefore, MB660 up to 1 μM never showed cytotoxicity on HRMECs or retinal toxicity in C57BL/6J mice.

Anti-angiogenic effect of MB660 on retinal neovascularization in OIR

To determine the anti-angiogenic effect of MB660, 1 μL intravitreal injection of 1μM MB660 was done on P14 of OIR mouse model. Retinal neovascularization was analyzed qualitatively with fluorescein angiography. Figure 3A showed neovascular tufts with fluorescein leakage at the junction of vascular and avascular retina. In contrast, the neovascular tufts with fluorescein leakage were significantly decreased after the treatment (Figure 3B and 3C). Quantitative analysis of anti-angiogenesis was done by counting vascular lumens. Many vascular lumens were observed in control mice (Figure 3D), whereas notably decreased vascular lumens

were counted in MB660-treated mice (Figure 3E). Significant difference in the number of vascular lumen was noticed between control (19.1 ± 3.24) and MB660-treated mice (10.68 ± 3.06, $p<0.05$).

Effect of MB660 on cobalt chloride induced HIF-1α expression in human brain astrocytes

Astrocyte is one of the major sources of VEGF expression in the retina under hypoxia, so we used human brain astrocyte to elucidate the down regulation of HIF-1α expression by MB660. Under hypoxic condition stimulated by $CoCl_2$, western blot analysis showed a significant decrease in HIF-1α protein levels in MB660-treated cells (Figure 4).

Figure 1. MB660 did not induce retinal toxicity on HRMECs and human brain astrocytes up to 1 μM. Cell viability was evaluated with MTT assay (*P < 0.05). (A) Various concentrations of MB660 (0.01 to 10 μM) were treated on HRMECs and cells were incubated for 48 hrs. (B) Human brain astrocytes were also treated with various concentrations (0.01 to 10 μM) of MB660 and incubated for 48 hrs.

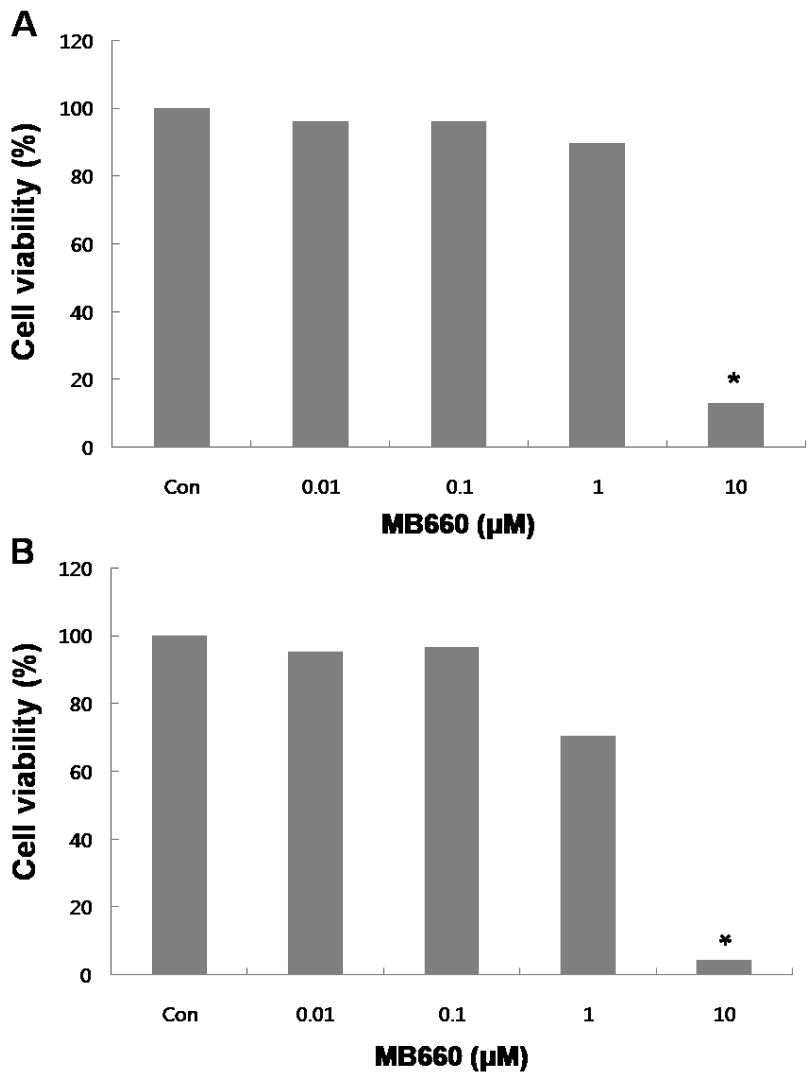


Figure 2. MB660 showed no toxic effect on retina in histologic analysis and TUNEL assay. MB660 1 μL (1 μM) was intravitreally injected in the C57BL/6J mice and the globes were enucleated 7 days after the treatment. Hematoxylin & eosin staining for histological examination and TUNEL assay for apoptotic cell counts were performed. **(A)** For the evaluation of changes in the retinal layers, the ratio of A (retinal thickness from the internal limiting layer to the inner nuclear layer) to B (retina thickness from the internal limiting layer to the outer nuclear layer) was measured in all sections**. (B)** For the evaluation of apoptotic cell death in the retinal layers, the apoptotic cells in sagittal sections from 12 o'clock to 6 o'clock through the cornea parallel to the optic nerve were assessed through TUNEL assay. TUNEL-positive cells were randomly measured on randomly selected 10 fields in each slide at x400 magnification. Arrows indicated TUNEL-positive cells. Figures were selected as representative data from 3 independent experiments. Data were the mean ± SD of the 3 independent experiments (**P > 0.05). GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer.

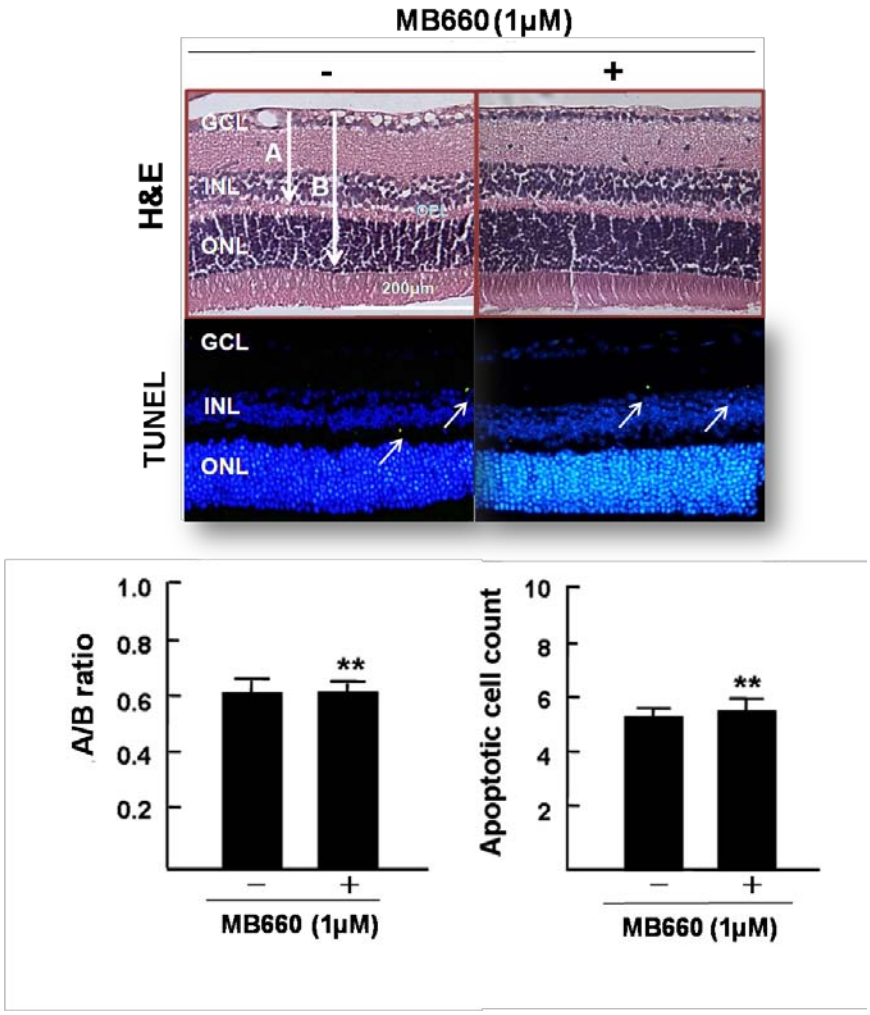

MB660 (1μM)
-
+
H&E
GCL
A
INL
B
ONL
200μm
TUNEL
GCL
INL
ONL
A/B ratio
1.0
0.8
0.6
0.4
0.2
**
−
+
MB660 (1μM)
Apoptotic cell count
10
8
6
4
2
**
−
+
MB660 (1μM)

Figure 3. MB660 inhibited retinal neovascularization when we intravitreally injected 1 μM of MB660 in 1μL PBS at P14 of OIR mouse model. For qualitative analysis of retinal neovascularization, control **(A)** and experimental **(B, C)** mice with different concentrations (0.1 and 1 μM) of MB660 were perfused with fluorescein-conjugated dextran (molecular weight = 500,000) through the tail vein at P17. After the perfusion of 1hour, enucleation was performed, and the retina was dissected, flat-mounted, and viewed under the fluorescein microscope. White arrows indicated neovascular tufts at the border of vascularized and nonvascularized retina. Figures were representative of 3 independent experiments. **(D, E)** For quantitative analysis, hematoxylin and eosin staining was performed for vessel counting. Black arrows indicate the vascular lumens of new vessel from the enucleated eyes of P17 control **(D)** and 1 μM MB660-injected OIR model **(E)**. Figures were representative of 3 independent experiments. **(F)** Each value indicated the mean ± SD of the 3 independent experiments (*$P < 0.05$).

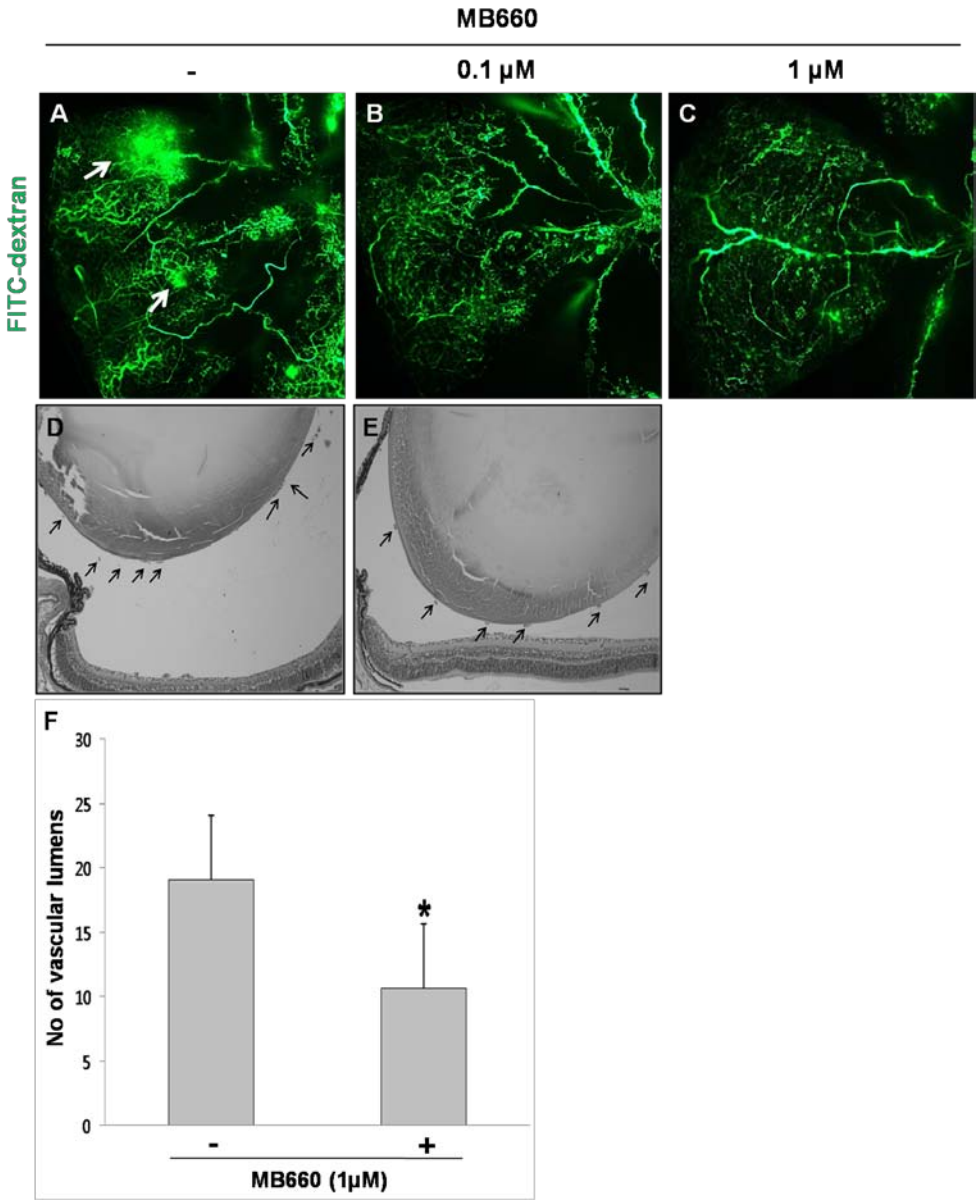
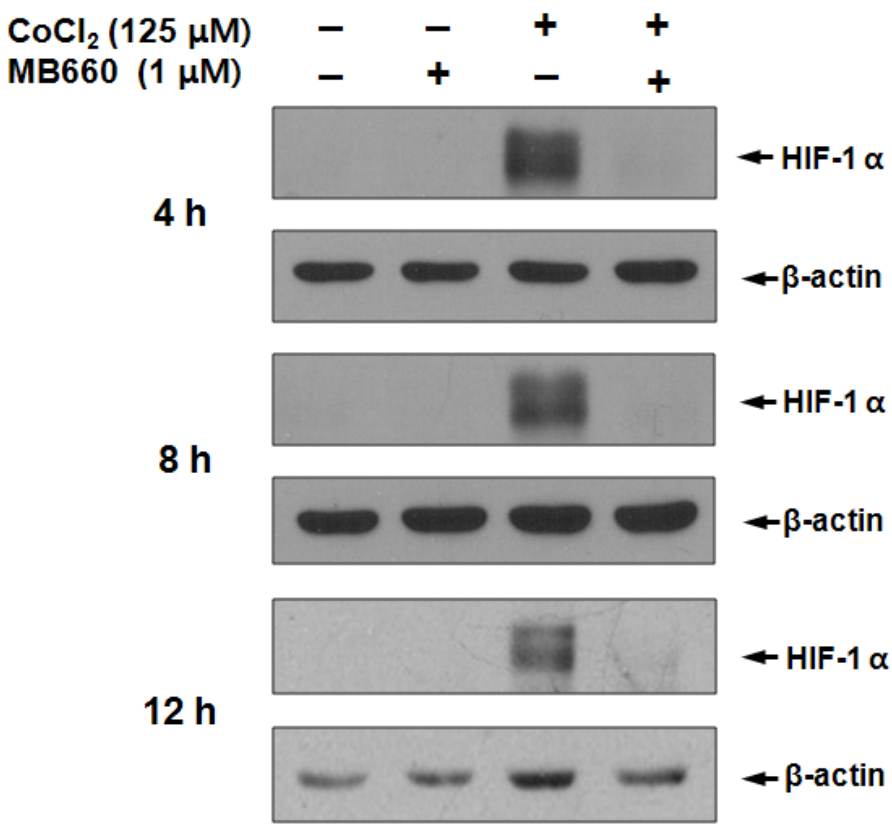
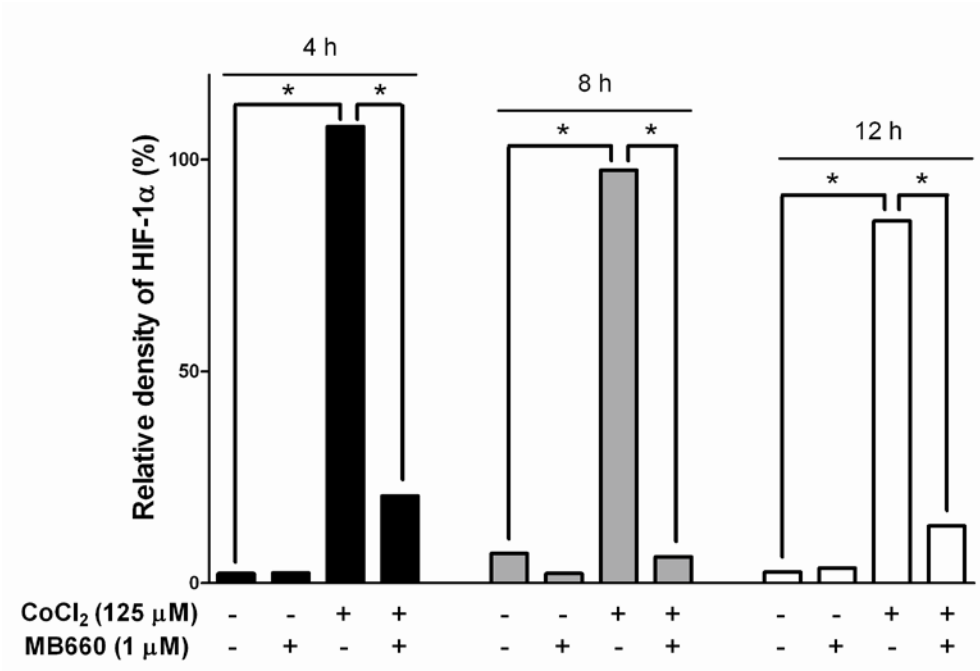

MB660
-
0.1 μM
1 μM
FITC-dextran
A
B
C
D
E
F
No of vascular lumens
30
25
20
15
10
5
0
*
-
+
MB660 (1μM)

Figure 4. Suppression of HIF-1α in human brain astrocytes by MB660. Under hypoxic condition induced by $CoCl_2$, human brain astrocytes were treated with 1 μM of MB660. Western blot analysis for HIF-1α was performed and β-actin was served as the loading control (*P < 0.05).




4 h
8 h
12 h
*
Relative density of HIF-1α (%)
100
50
0
CoCl2 (125 μM) - - + + - - + + - - + +
MB660 (1 μM) - + - + - + - + - + - +

Discussion

In the present study, we demonstrated that MB660 successfully inhibited angiogenesis in OIR animal model and HRMECs via attenuating HIF-1α expression. Most of all, MB660 did not affect the cellular viability of HRMECs and the histologic examination and TUNEL assay showed that intravitreal injection would not induce toxicity to retina as well. The results from in vitro and in vivo experiments suggest that MB660 could be a possible candidate for the treatment of VEGF associated pathologic retinal neovascularization.

The VEGF is a major angiogenesis inducer, which is involved in regulating endothelial cell proliferation and blood vessel formation and its transcription is regulated by HIF-1α in response to hypoxia.(15, 16) Hypoxia increases HIF-1 levels in cells by strongly stabilizing HIF-1α through the inhibition of its rapid degradation by proteasome.(17) The angiogenic properties of VEGF are important but degradating HIF-1 can have more benefit in shutting down many genes related to angiogenesis, such as VEGF, VEGFR2, PAI-1, MMPs, EPO and IGF-2. Drugs not only targeting VEGF, but also HIF-1 are currently under developments.(17, 18)

Kung et al.(11) noted that administration of MB660 to human vascular endothelial cells decreased the VEGF secretion from endothelial cells. Their results from the in vitro tubular formation assay demonstrated that MB660 inhibited the formation of a tubule-like structure, suggesting the possibility of the tubule de-formation by MB660 due to decrease in the VEGF levels. Based on their results, on

the assumption that mechanism of anti-angiogenic activity of MB660 would be related to VEGF and HIF-1α signaling pathway, we also performed western blotting to find out the reduction of $CoCl_2$ induced HIF-1α expression in human brain astrocytes. $CoCl_2$ has been widely used as hypoxia mimics in cell culture and it is known to activate hypoxic signaling by stabilizing the HIF-1α. $CoCl_2$ administration caused a dramatic expression of HIF-1α protein, but after the treatment with MB660, HIF-1α expression was significantly reduced. This indicates that suppression of HIF-1α expression may sequentially reduce VEGF related angiogenesis.

We can presume that the mechanism of MB660 suppressing HIF-1α expression can be related with its own anti-cancer effect. Several studies have demonstrated that the anticancer agents inhibiting topoisomerase I, II have been shown to reduce HIF-1α expression or transcriptional activity; however the specific mechanism of action is still unestablished.(18-22) MB660 is also known to inhibit DNA-topoisomerase and that this might be related to its activity of attenuating HIF-1α expression, but further analysis should be done to evaluate the actual relationship.

Taken together, our data indicate that MB660 inhibited VEGF induced retinal neovascularization by attenuating HIF-1α expression, but further experiments should be performed to find out the more specific mechanism of MB660 in anti-angiogenesis. Also, to determine the effect of MB660 in vitro angiogenesis, VEGF induced wound migration and tube formation assay in

HRMECs should be added.

In conclusion, our results revealed that MB660 could effectively inhibit retinal neovascularization in vivo and in vitro via down-regulating HIF-1α expression and MB660 could be safely applied to retina without any toxicity. We propose that MB660 has a potential to be applicated as an anti-angiogenic drug for the treatment of neovascular eye diseases.

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초 록

목적: MB660 의 항암, 항균, 항염증 효과는 널리 알려져 있다. 최근 MB660 의 신생혈관효과에 대한 의견이 제기되고 있으나 아직 활발한 연구가 이뤄지지 않은 실정이다. 이에 저자는 본 연구를 통해 MB660 이 HIF(hypoxia inducible factor)-1α 의 분해를 저해함으로써 망막신생혈관의 형성을 억제하는 효과를 알아보고자 하였다.

방법: 수정된 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide 분석방법을 이용하여 MB660 의 인간망막모세혈관내피세포(Human retinal microvascular endothelial cells)에 대한 세포독성 정도를 확인하였다. 또한 C57BL/6 mouse 의 유리체강내로 MB660 을 주사한 후 조직학적 검사 및 terminal deoxynucleotidyl transferace dUPT nick-end labeling 염색을 통해 망막에 미치는 독성 효과를 알아보았다. 망막신생혈관에 대한 억제효과를 정량적으로 분석하기 위해 MB660 을 유리체강내로 주사한 mouse oxygen induced retinopathy (OIR)모델에서 적출 및 절단한 눈의 단면적에서 관찰되는 혈관 내강의 수를 세었다. $CoCl_2$ 에 의해 유발된 저산소 환경에서의 MB660 의 HIF-1α에 대한 억제효과를 확인하고자 western blot 방법을 사용하였다.

결과: MB660 은 1 μM 의 농도까지는 인간망막모세혈관내피세포에 대해 독성이 없었으며, 정상 망막조직에 대해서 어떠한 구조적 변화도 유발하지 않음을 확인하였다. 유리체강내로 MB660 을 주사했을 경우, mouse OIR 모델에서의 망막신생혈관이 유의하게 감소함을 확인하였고, $CoCl_2$ 로 유발한 저산소 환경에서의 HIF-1α 생성 역시 효과적으로 억제됨을 확인할 수 있었다.

결론: MB660 은 망막에 독성을 미치지 않는 농도내에서 HIF-1α의 생성을 저해하는 기전을 통해 VEGF (vascular endothelial growth factor)에 의한 혈관신생을 유의하게 억제함으로써 신생혈관형성 억제 치료에 쓰일 수 있는 가능성을 보여주었다.

주요어: MB660, 혈관신생, VEGF, HIF-1α

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