# Anti-inflammatory effects of the NF-κB inhibitor dehydroxymethylepoxyquinomicin on ARPE-19 cells

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**Abstract.** The retinal pigment epithelium (RPE) is a polarized, monolayer of pigmented cells that forms the outer retinal layer. A key function of the RPE is to maintain the integrity of the photoreceptors mainly via phagocytosis and recycling of the digested photoreceptor outer segments. Moreover, RPE cells are a major source of inflammatory cytokines and chemokines, which play important roles in the activation of other immune cells under inflammatory conditions in the posterior segment of the eye. Dehydroxymethylepoxyquinomicin (DHMEQ) is a NF-κB inhibitor and its structure is related to that of epoxyquinomicin C, which is an antibiotic. The present study evaluated the anti-inflammatory effects of DHMEQ on a human retinal pigment epithelial cell line (ARPE-19). It was revealed that high concentrations of DHMEQ (100 $\mu$g/ml) induced apoptosis and necrosis of tumor necrosis factor (TNF)-α-stimulated ARPE-19 cells. Furthermore, the percentage of intercellular adhesion molecule 1 (ICAM-1)-positive TNF-α-stimulated cells was significantly reduced in the presence of DHMEQ (10 $\mu$g/ml), as determined by flow cytometry. It was also demonstrated that DHMEQ exposure significantly decreased the levels of interleukin (IL)-8 and monocyte chemoattractant protein-1 (MCP-1) in the supernatant of cultured ARPE-19 cells as determined by ELISA. Moreover, the protein expression levels of IL-8 and MCP-1 were significantly reduced in ARPE-19 cells exposed to DHMEQ compared with cells exposed to dexamethasone. PCR array analysis revealed that DHMEQ reduced the expression levels of MCP-1, ICAM-1, IL-6, Toll-like receptor (TLR)2, TLR3 and TLR4. Therefore, the present results indicated that DHMEQ has anti-inflammatory effects on TNF-α-stimulated ARPE-19 cells. Thus, DHMEQ may have therapeutic potential for TNF-α-mediated inflammatory disorders of the eye.

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## Introduction

The retinal pigment epithelium (RPE) is a polarized, monolayer of pigmented cells that forms the outer retinal layer, and maintains the integrity of the photoreceptors, primarily by phagocytosing and recycling the retinal photoreceptor outer segments (1). RPE cells are a major source of proinflammatory cytokines, including interleukin (IL)-6, and chemokines, such as monocyte chemotactic protein (MCP)-1 and IL-8 (1). RPE cells also secrete regulated on the activation of normal T-cell expressed and secreted (RANTES) and interferon (IFN)-γ induced protein (IP)-10 kDa (IP-10) (1-3). Furthermore, these cytokines and chemokines secreted by RPE cells play important roles in the activation of other immune cells under inflammatory conditions of the posterior segment of the eye (1).

Tumor necrosis factor-α (TNF-α) is an inflammatory cytokine that contributes to the progression of non-infectious uveitis, and it has been shown that blocking TNF-α is effective for treating refractory uveitis (4-8). Furthermore, TNF-α receptors activate the NF-κB signaling pathway (9). NF-κB, a member of a family of ubiquitously expressed proteins, is usually found in an inactive state in the cytoplasm, except during immune and inflammatory responses (9,10). The NF-κB family includes REL proto-oncogene (Rel)A, RelB, c-Rel, p50/p105 and p52/p100, all of which form homo- or heterodimers with each other (11). Inhibitors of NF-κB (IκB) proteins are phosphorylated and are degraded by proteasomes (12). Moreover, released NF-κB dimers translocate into the nucleus and bind to κB sites in the promoter and enhancer regions of targeted genes of various inflammatory cytokines and chemokines, including IL-1, IL-2, IL-6, TNF and macrophage inflammatory protein-1/2, and adhesion molecules, such as intercellular adhesion molecule-1 (ICAM-1) (11,13).

Dehydroxymethylepoxyquinomicin (DHMEQ) is a low molecular weight inhibitor of the NF-κB signaling pathway, and its structure is related to that of epoxyquinomicin C, which is an antibiotic (14,15). DHMEQ suppresses the TNF-α-induced nuclear translocation of NF-κB, but it does not prevent the phosphorylation and degradation of IκB (16).

A previous study also revealed that DHMEQ binds directly to the Rel-family proteins to prevent their DNA-binding activity (17). Furthermore, it has been revealed that DHMEQ is able to suppress inflammation and the progression of cancer in animal models without obvious adverse effects (18).

The cells of the human RPE cell line, ARPE-19, are frequently used in *in vitro* studies to investigate the mechanisms involved in posterior segment inflammatory disorders, including uveitis and age-related macular degeneration (19-21). Therefore, the aim of the present study was to determine whether DHMEQ has inhibitory effects on the expression of ICAM-1 in TNF-α-stimulated ARPE-19 cells. In addition, the present study examined whether DHMEQ can affect the production of TNF-α-stimulated ARPE-19 cells and the expression of NF-κB related-genes in TNF-α-stimulated ARPE-19 cells treated with DHMEQ.

## Materials and methods

*Materials*. DHMEQ was synthesized by Umezawa and Chaicharoenpong (16), and for the present study it was dissolved in 100% DMSO at a concentration of 10 mg/ml and stored at -30˚C (14,16). Before use in cell cultures, DHMEQ was diluted with the culture medium (DMEM/F-12; Invitrogen; Thermo Fisher Scientific, Inc.) to a final concentration of ≤0.1%. Dexamethasone was purchased from Sigma-Aldrich (Merck KGaA).

*Cell cultures*. ARPE-19 cells were purchased from the American Type Culture Collection and maintained in DMEM/F-12 supplemented with 10% FBS, 100 U/ml penicillin and 0.1 mg/ml streptomycin (all from Invitrogen; Thermo Fisher Scientific, Inc.) at 37˚C in 5% $CO_2$ in air. After reaching confluency, ARPE-19 cells were detached with a trypsin-EDTA solution (0.05%) (Thermo Fisher Scientific, Inc.) and plated for subcultures. Cells were passaged every 4-6 days, and those used in each experiment were confluent and exhibited no visible pigmentation. Moreover, cells were maintained for 3 weeks before the experimental procedures and were used at passages 4-6.

*Cell viability determined by MTT assay*. The effects of various concentrations of DHMEQ on ARPE-19 cells were evaluated by MTT. Cells were grown at 37˚C in 96-well plates at a density of $2x10^4$ cells/well for 24 h. Upon confluency, the medium was replaced with a serum-free medium, and ARPE-19 cells were cultured at 37˚C for 24 h with 0.1, 1.0, 5.0, 10.0, 50.0 or 100.0 μg/ml DHMEQ or without DHMEQ at 37˚C for 24 h. After 24 h, the assay was performed by adding 10 μl MTT solution to the wells (Biotium, Inc.). After incubation for 4 h at 37˚C, 200 μl DMSO was added to the cells. After incubation at room temperature for 5 min, the optical density (OD) at 570 nm (signal absorbance) and 630 nm (background absorbance) was measured using a microplate reader, and the normalized absorbance values (OD at 570 nm and OD at 630) were determined.

*Flow cytometric analyses*. ARPE-19 cells were seeded in 6-well plates at $2x10^5$ cells/well and cultured for 24 h. Upon confluency, the medium was replaced with serum-free medium, and cells were or were not exposed to 20 ng/ml TNF-α (R&D Systems, Inc.) with DHMEQ (1.0, 10.0 and 100.0 μg/ml) or without DHMEQ at 37˚C for 24 h. After exposure, cells were washed with phosphate-buffered saline (PBS, pH 7.4), detached by trypsin-EDTA (0.05%) and suspended in PBS. For staining of prepared cells, Annexin V-FITC solution (5 μl; Nacalai Tesque, Inc.) was added to 100 μl of cell suspension ($1x10^6$ cells), then <1% propidium iodide (PI) solution (5 μl; Nacalai Tesque, Inc.) was added to the cell suspension, according to the manufacturer's instructions (cat. no. 15342; Nacalai Tesque, Inc.). The cells were incubated at room temperature for 15 min and analyzed by flow cytometry (FACSCalibur) using CellQuest Pro software version 6.0 (both BD Biosciences).

ARPE-19 cells were seeded in 6-well plates at $2x10^5$ cells/well and cultured for 24 h. Upon confluency, the medium was replaced with serum-free medium, and cells were or were not exposed to 20 ng/ml TNF-α (R&D Systems, Inc.) with DMSO (0.1%) or DHMEQ (1.0 or 10.0 μg/ml) at 37˚C for 24 h. After exposure, cells were washed with PBS (pH 7.4), detached by trypsin-EDTA (0.05%) and suspended in PBS. The prepared ARPE-19 cells were incubated with phycoerythrin-conjugated monoclonal antibody to ICAM-1 (1:100; cat. no. 555511; BD Biosciences) at 4˚C for 20 min and analyzed by flow cytometry (FACSCalibur) using CellQuest Pro software version 6.0 (both BD Biosciences).

*Chemokine assay in culture supernatants*. ARPE-19 cells were seeded in 6-well plates at a density of $2x10^5$ cells/well and cultured for 24 h. Upon confluency (80-90% confluency), the medium was replaced with serum-free medium and cells were exposed to 20 ng/ml TNF-α and DMSO (0.1%), DHMEQ (1.0 μg/ml=4.0 μM, 10 μg/ml=40 μM) or dexamethasone (40 μM) at 37˚C for 24 h. After exposure, the supernatant was collected from each well, and the levels of IL-8 and MCP-1 in the supernatant were determined by Quantikine® Colorimetric Sandwich ELISA kits (R&D Systems, Inc.; IL-8. cat. no. D8000C; MCP-1, cat. no. DCP00).

In another experiment, ARPE-19 cells were exposed to 20 ng/ml TNF-α and DMSO (0.1%) or DHMEQ (10 μg/ml) at 37˚C for 6, 12 and 24 h. After exposure, the supernatant was collected from each well and the levels of IL-8 and MCP-1 in the supernatant was determined by the Quantikine® ELISA kits (R&D Systems, Inc.) as described above.

*NF-κB-associated gene expression level assay*. ARPE-19 cells were seeded in 6-well plates at a density of $2x10^5$ cells/well and cultured for 24 h. Upon confluency, the medium was replaced with serum-free medium and cells were exposed to 20 ng/ml TNF-α in the absence or presence of DHMEQ (10 μg/ml) at 37˚C for 24 h. After exposure, cells were washed with PBS, detached by trypsin-EDTA (0.05%) and suspended in PBS. Total RNA from the prepared ARPE-19 cells was extracted with ISOGEN (Nippon Gene Co., Ltd.) according to the manufacturer's instructions. Briefly, 10 μg total RNA from each sample was reverse-transcribed using the High Capacity RNA-to-cDNA kit (Applied Biosystems; Thermo Fisher Scientific, Inc.; reverse transcription conditions were as follows: Initial incubation at 37˚C for 60 min and 95˚C for 5 min), then loaded onto Human NFκB Pathway TaqMan®

Array plates (cat. no. 4414095; Applied Biosystems; Thermo Fisher Scientific, Inc.) for profiling of NF-κB-associated 92 genes expression levels. PCR was performed on a QuantStudio™ 12K Flex Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions. Raw cycle threshold (Cq) values were calculated with SDS software 1.2.3. (Applied Biosystems; Thermo Fisher Scientific, Inc.). The sequences of the forward and reverse primers are not commercially available. Data were analyzed according to the comparative Cq method, and the global median normalization method was used (22). The $2^{-\Delta\Delta Cq}$ method was performed to calculate the expression level of the fold change (23). The fold change in ARPE-19 cells exposed to 20 ng/ml TNF-α in the absence or presence of DHMEQ was calculated for each gene; genes with a 2-fold increase in this ratio were defined arbitrarily as upregulated in cells exposed to DHMEQ, whereas those with a 2-fold decrease were defined as downregulated genes.

*Quantitative PCR analysis*. In order to validate the expression levels of genes [lymphotoxin β receptor (LTBR), MCP-1, and Toll-like receptor 4 (TLR4)], which were either upregulated or downregulated in ARPE-19 cells treated with DHMEQ, quantitative PCR was carried out in duplicate using the TaqMan® Universal PCR Master Mix (Applied Biosystems; Thermo Fisher Scientific, Inc.) on a QuantStudio™ 12K Flex Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.), following the manufacturer's protocol. ARPE-19 cells were seeded in 6-well plates at a density of $2x10^5$ cells/well and cultured for 24 h. Upon confluency, the medium was replaced with serum-free medium and cells were exposed to 20 ng/ml TNF-α exposed to DMSO (0.1%) or DHMEQ (10 μg/ml) at 37˚C for 24 h. After exposure, cells were washed with PBS, detached by trypsin-EDTA (0.05%) and suspended in PBS. Total RNA from the prepared ARPE-19 cells was extracted with ISOGEN (Nippon Gene Co., Ltd.) as described above and reverse-transcribed using the High Capacity RNA-to-cDNA kit (Applied Biosystems; Thermo Fisher Scientific, Inc.) PCR conditions were as follows: Initial incubation at 50˚C for 2 min and 95˚C for 10 min, followed by 40 cycles two-step cycling (denaturing at 95˚C for 15 sec, annealing/extension at 60˚C for 60 sec). The TaqMan primers/probes pairs were obtained from Applied Biosystems (Thermo Fisher Scientific, Inc.) using inventoried TaqMan gene expression assays [LTBR assay ID, Hs01101194_m1; MCP-1 assay ID, Hs00234140_m1; TLR4 assay ID, Hs00152939_m1]. For an endogenous control mRNA, the β-actin (assay ID, Hs99999903_m1, Thermo Fisher Scientific, Inc.) was used for data normalization of the mRNA expression levels.

*Statistical analyses*. Data are presented as the mean ± SD. Statistical significance was evaluated with unpaired t-tests. To compare data among ≥3 groups, one-way ANOVA using the Bonferroni's multiple comparison tests was performed. $P<0.05$ was considered to indicate a statistically significant difference.

## Results

*Effect of DHMEQ on the viability of ARPE-19 cells*. Confluent ARPE-19 cells were exposed to various concentrations of DHMEQ, and it was revealed that DHMEQ at concentrations ≤10 μg/ml did not have toxic effects on cells. However, DHMEQ at concentrations of 50 and 100 μg/ml significantly inhibited the viability of ARPE-19 cells compared with the viability of cells cultured without DHMEQ (Fig. 1). Therefore, these results indicated that higher concentrations of DHMEQ, such as 50 and 100 μg/ml, reduce the viability of ARPE-19 cells.

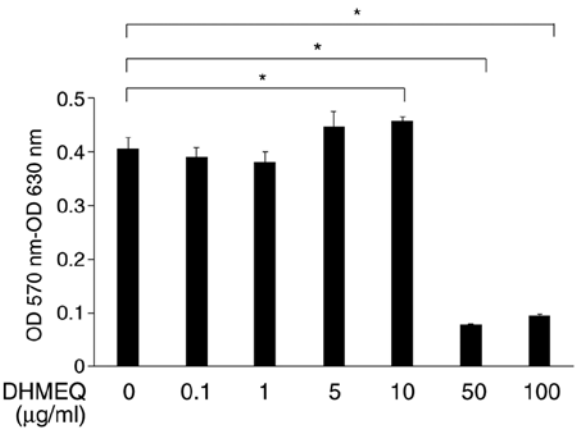


Figure 1. Viability of ARPE-19 cells incubated with DHMEQ. Cells were incubated with or without DHMEQ (0-100 μg/ml). After 24 h, cell viability was evaluated by MTT assay. Data are presented as the mean ± SD, n=4. *P<0.05. DHMEQ, dehydroxymethylepoxyquinomicin; OD, optical density.

*Effect of DHMEQ on the induction of apoptosis and necrosis of ARPE-19 cells*. To determine whether DHMEQ induces apoptosis or necrosis in ARPE-19 cells, Annexin-V and/or PI-positive cells were analyzed by flow cytometry. It was demonstrated that concentrations of 1.0 and 10 μg/ml DHMEQ did not alter the percentage of apoptotic cells (Annexin-V-positive and PI-negative; Fig. 2A and B). However, a dose of 100 μg/ml DHMEQ significantly increased the number of apoptotic cells compared with cells treated with TNF-α (20 ng/ml) without DHMEQ (Fig. 2A and B). In addition, concentrations of 100 μg/ml DHMEQ significantly increased the number of necrotic cells compared with cells treated with TNF-α (20 ng/ml) without DHMEQ (Annexin-V-positive and PI-positive; Fig. 2A and B). Collectively, the results indicated that a high concentration of DHMEQ, such as 100 μg/ml, promoted the induction of apoptosis and necrosis of ARPE-19 cells. Based on these findings, a concentration of DHMEQ <100 μg/ml was used in subsequent experiments.

*Suppression of ICAM-1 in ARPE-19 cells by DHMEQ*. Previous studies have revealed that TNF-α can enhance the protein expression of ICAM-1 synthesized by ARPE-19 cells (24). To assess these findings, the present study stimulated ARPE-19 cells with TNF-α (20 ng/ml) for 24 h and evaluated the protein expression of ICAM-1 by flow cytometry. The results revealed that the expression of ICAM-1 was increased 7-fold in cells stimulated by TNF-α compared with cells cultured without TNF-α (Fig. 3A and B).

The effects of DHMEQ on the protein expression level of ICAM-1 were also examined in ARPE-19 cells stimulated with

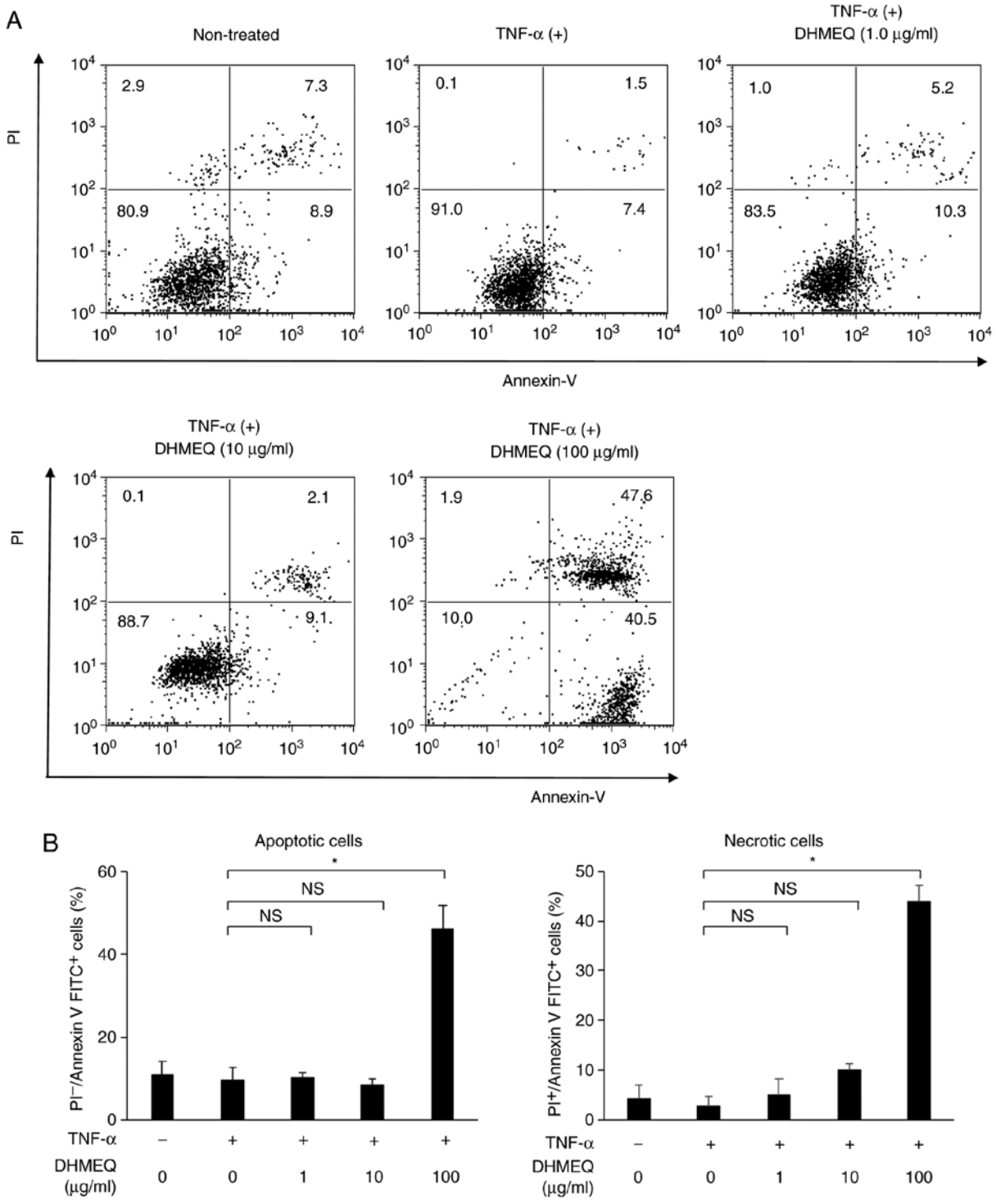


Figure 2. Effect of DHMEQ on apoptosis and necrosis of ARPE-19 cells. (A) Cells were cultured without TNF-α (non-treated) or with TNF-α (20 ng/ml) in the absence or presence of DHMEQ (1, 10 or 100 $\mu$g/ml) for 24 h. The percentage of apoptotic cells (PI-negative and Annexin-V FITC-positive) and necrotic cells (PI-positive and Annexin-V FITC-positive) was measured by flow cytometry. (B) Percentages of apoptotic and necrotic cells. Data are presented as the mean ± SD, n=3. *P<0.05. PI, propidium iodide; DHMEQ, dehydroxymethylepoxyquinomicin; TNF-α, tumor necrosis factor-α; NS, not significant.

TNF-α. It was revealed that DHMEQ (10 $\mu$g/ml) significantly reduced the expression of ICAM-1 in cells by ~50% compared with cells treated with DMSO (Fig. 3C-E). Thus, it was speculated that DHMEQ may be able to decrease TNF-α-induced ICAM-1 expression in ARPE-19 cells.

*Suppressive effect of DHMEQ on chemokine production in TNF-α-stimulated ARPE-19 cells*. MCP-1 and IL-8 have been revealed to be the major chemokines produced by TNF-α-stimulated ARPE-19 cells (25), and dexamethasone has been reported to have anti-inflammatory effects on ARPE-19 cells (20). Therefore, the present study investigated whether DHMEQ is able to decrease the protein expression levels of MCP-1 and IL-8 in ARPE-19 cells stimulated with TNF-α. Moreover, the anti-inflammatory effect of DHMEQ was compared with that of dexamethasone in cells. It was demonstrated that the

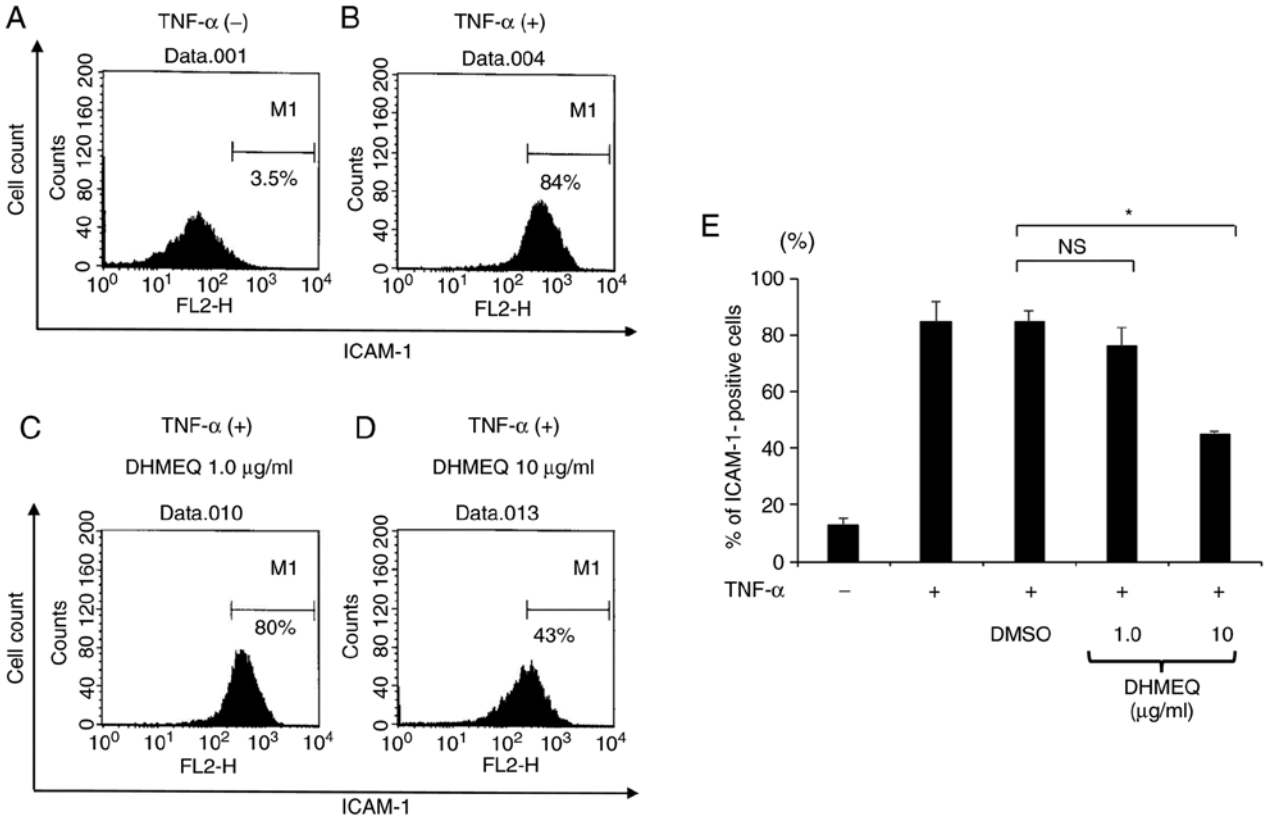


Figure 3. DHMEQ-mediated reduction of ICAM-1 expression in TNF-α-stimulated ARPE-19 cells. Cells were cultured (A) without TNF-α or (B) with TNF-α (20 ng/ml) in the presence of DMSO (0.1%) or (C) 1.0 or (D) 10 μg/ml DHMEQ for 24 h, and PE-labeled ICAM-1-positive cells were measured by flow cytometry. (E) Percentages of PE-labeled ICAM-1-positive cells. Data are presented as the mean ± SD, n=3. *P<0.05. NS, not significant; PE, phycoerythrin; ICAM-1, intercellular adhesion molecule 1; DHMEQ, dehydroxymethylepoxyquinomicin.

production of IL-8 and MCP-1 from cells treated with DHMEQ (40 μM=10 μg/ml) was significantly decreased compared with ARPE-19 cells treated with DMSO. In addition, there was a significant difference in the production of IL-8 and MCP-1 between cells treated with DHMEQ (40 μM) and those treated with dexamethasone (40 μM; Fig. 4A and B). These findings revealed that DHMEQ has strong suppressive effects on the production of MCP-1 and IL-8 by ARPE-19 cells compared with dexamethasone. Furthermore, the results indicated that DHMEQ significantly reduced the protein expression levels of MCP-1 and IL-8 at 6, 12 and 24 h (Fig. 4C and D). Therefore, DHMEQ may be able to decrease the TNF-α-induced chemokine production in ARPE-19 cells at several time-points after co-culturing.

*Suppression of NF-κB-related inflammatory gene expression levels of ARPE-19 cells by DHMEQ.* To determine the alterations of the expression levels of NF-κB-associated inflammatory genes in ARPE-19 cells exposed to DHMEQ, the present study compared RNA isolated from TNF-α-stimulated cells in the absence or presence of DHMEQ, using the Human NF-κB Pathway TaqMan® Array Plates that analyze 92 NF-κB-associated inflammatory genes. Moreover, summaries of the differentially expressed genes between the two cell populations are presented in Table I. A total of 19 genes were revealed to be upregulated and 25 genes were revealed to be downregulated in cells exposed to DHMEQ compared with those in the absence of DHMEQ. The differentially expressed genes are presented in Tables I and II. The gene expression levels of cytokines and chemokines, including MCP-1, ICAM-1, IL-6 and IL-8, and TLR2, TLR3 and TLR4, were downregulated in ARPE-19 cells treated with DHMEQ (Table I). In addition, DHMEQ suppressed TNF superfamily member 15 (TNFSF15) and TNF-α-induced protein 3 (TNFAIP3; Table I). However, it was revealed that DHMEQ increased the expression levels of numerous genes associated with the NF-κB signaling pathway, including prostaglandin E synthase (PTGES), mitogen-activated protein kinase 14 (MAP3K14), LTBR and TNFRSF1A associated via death domain (TRADD).

In addition, representative genes, LTBR, MCP-1 and TLR4, which were identified by NF-κB Pathway Array, were assessed by quantitative PCR analysis. Although the fold changes were not exactly the same between the two methods, the gene expression level of LTBR was significantly increased in the presence of DHMEQ compared to that in the presence of DMSO, whereas the gene expression levels of MCP-1 and TLR4 were significantly decreased in the presence of DHMEQ compared to that in the presence of DMSO (Fig. 5).

## Discussion

The present results indicated that DHMEQ significantly decreased the protein expression of TNF-α-induced ICAM-1 in ARPE-19 cells, and also decreased the production of IL-8 and MCP-1 by cells stimulated with TNF-α. In addition, it was determined that DHMEQ at higher concentrations

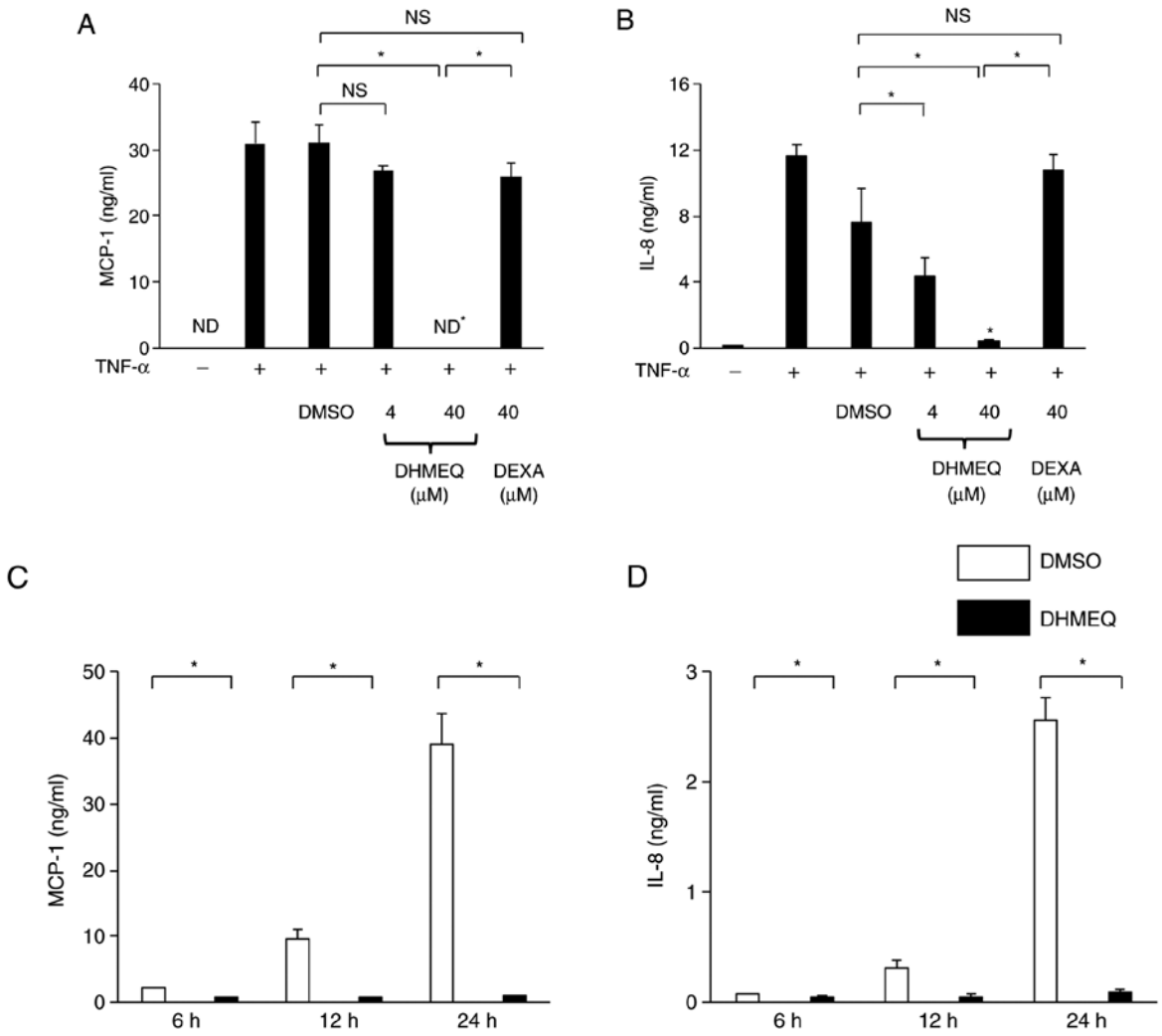


Figure 4. Effect of DHMEQ and DEXA on the levels of MCP-1 and IL-8 in ARPE-19 cells stimulated by TNF-α. Cells were cultured with TNF-α (20 ng/ml) in the presence of DMSO (0.1%), DHMEQ (1 $\mu$g/ml=4 $\mu$M, 10 $\mu$g/ml=40 $\mu$M) or DEXA (40 $\mu$M) for 24 h. The effect of DHMEQ on levels of (A) MCP-1 and (B) IL-8 in the culture supernatants of cells stimulated with TNF-α. ARPE-19 cells were cultured with TNF-α (20 ng/ml) in the presence of DMSO (0.1%) or DHMEQ (10 $\mu$g/ml) for 6, 12 and 24 h. The effect of DHMEQ on levels of (C) MCP-1 and (D) IL-8 in the culture supernatants of cells stimulated with TNF-α, as measured by ELISA. Data are presented as the mean ± SD, n=3. *P<0.05. ND, not detected; DEXA, dexamethasone; NS, not significant; TNF-α, tumor necrosis factor-α; MCP-1, monocyte chemoattractant protein-1; IL, interleukin; DHMEQ, dehydroxymethylepoxyquinomicin.

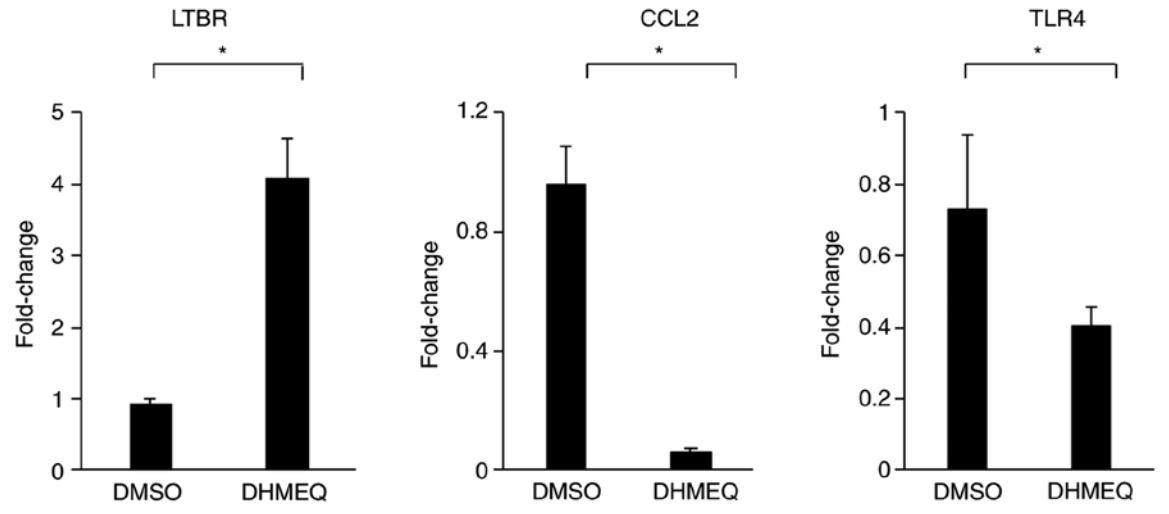


Figure 5. Validation of array data by quantitative PCR. mRNA expression levels of LTBR, MCP-1 and TLR4 of TNF-α-stimulated ARPE-19 cells treated with DMSO (0.1%) or DHMEQ (10 $\mu$g/ml) for 24 h were determined by quantitative PCR. Duplicate assays were performed for each gene. Gene expression levels were normalized to β-actin as endogenous control. Fold changes in mRNA expression from qPCR are presented as the mean ± SDs, n=4. *P<0.05. DHMEQ, dehydroxymethylepoxyquinomicin; TNF-α, tumor necrosis factor-α; TLR4, Toll-like receptor 4; LTBR, lymphotoxin β receptor.

Table I. Summary of downregulated genes in ARPE-19 cells stimulated with TNF-α in the presence of DHMEQ.

| Target gene | Probe ID | Fold change |
|---|---|---|
| BIRC5 | Hs00977611_g1 | 0.040 |
| TLR2 | Hs00152932_m1 | 0.053 |
| TRAF5 | Hs00182979_m1 | 0.082 |
| TNFSF15 | Hs00353710_s1 | 0.088 |
| BCL10 | Hs00184839_m1 | 0.127 |
| CHUK | Hs00989507_m1 | 0.130 |
| CSF2 | Hs00171266_m1 | 0.164 |
| BCL2 | Hs00608023_m1 | 0.228 |
| HPRT1 | Hs99999909_m1 | 0.236 |
| MCP-1 | Hs00234140_m1 | 0.238 |
| FADD | Hs00538709_m1 | 0.249 |
| TLR4 | Hs00152939_m1 | 0.274 |
| MALT1 | Hs00198984_m1 | 0.275 |
| EDARADD | Hs00369830_m1 | 0.281 |
| TNFAIP3 | Hs00234713_m1 | 0.388 |
| ICAM1 | Hs00164932_m1 | 0.389 |
| IRAK1BP1 | Hs00418138_m1 | 0.396 |
| CD83 | Hs00188486_m1 | 0.414 |
| TLR3 | Hs00152933_m1 | 0.416 |
| REL | Hs00968436_m1 | 0.437 |
| CSF1 | Hs00174164_m1 | 0.456 |
| CXCL1 | Hs00236937_m1 | 0.456 |
| ZNF675 | Hs00603247_m1 | 0.456 |
| IL6 | Hs00174131_m1 | 0.490 |
| RIPK1 | Hs00169407_m1 | 0.490 |

DHMEQ, dehydroxymethylepoxyquinomicin; TNF-α, tumor necrosis factor-α; TLR, Toll-like receptor; TNFSF15 TNF superfamily member 15; TNFAIP3, TNF-α-induced protein 3.

Table II. Summary of upregulated genes in ARPE-19 cells stimulated with TNF-α in the presence of DHMEQ.

| Target gene | Probe ID | Fold change |
|---|---|---|
| PTGES | Hs00610420_m1 | 18.850 |
| MAP3K14 | Hs00177695_m1 | 6.446 |
| LTBR | Hs00158922_m1 | 5.488 |
| TRADD | Hs00182558_m1 | 5.024 |
| BCL3 | Hs00180403_m1 | 4.300 |
| NKIRAS2 | Hs00383387_m1 | 3.855 |
| TNFRSF10A | Hs00269492_m1 | 3.773 |
| IKBKG | Hs00415849_m1 | 3.609 |
| MAP3K7IP1 | Hs00196143_m1 | 3.600 |
| MYC | Hs00153408_m1 | 3.194 |
| ZFP36 | Hs00185658_m1 | 3.091 |
| IRAK1 | Hs00155570_m1 | 3.065 |
| ENPP2 | Hs00196470_m1 | 2.653 |
| TRAF1 | Hs00194638_m1 | 2.614 |
| CARD10 | Hs00367225_m1 | 2.574 |
| NFKBIB | Hs00182115_m1 | 2.570 |
| IRAK2 | Hs00176394_m1 | 2.569 |
| RELA | Hs00153294_m1 | 2.446 |
| NFKB2 | Hs00174517_m1 | 2.341 |

DHMEQ, dehydroxymethylepoxyquinomicin; TNF-α, tumor necrosis factor-α; PTGES, prostaglandin E synthase; MAP3K14, mitogen-activated protein kinase 14; LTBR, lymphotoxin β receptor; TRADD, TNFRSF1A associated via death domain.

had increased anti-inflammatory effects on ARPE-19 cells compared with dexamethasone. The results also indicated that exposure to DHMEQ decreased the expression level of ICAM-1 in ARPE-19 cells. Moreover, the present results are consistent with those from a previous study, which reported that DHMEQ decreases the expression level of ICAM-1 in the retina of diabetic mice and that it reduces the number of retinal-adherent leukocytes (26). Furthermore, the expression of ICAM-1 in RPE cells has been revealed to be elevated under inflammatory conditions, leading to the enhancement of leukocyte-RPE cell interactions (27,28). Previous studies have also identified increased ICAM-1 expression levels in ocular tissues of patients with uveitis and revealed that antibody-based blockage of ICAM-1 led to a suppression of experimental autoimmune uveoretinitis (29-31). Collectively, both the present findings and previous results indicated that DHMEQ may be a potential anti-inflammatory compound for RPE cells due to its ability to reduce the expression of ICAM-1.

Elner *et al* (25) revealed that RPE cells produce several chemokines, including IL-8 and MCP-1. The present results revealed that DHMEQ inhibited the production of IL-8 and MCP-1 in TNF-α-stimulated ARPE-19 cells, although the effect of treatment with IL-8 on ARPE-19 cells in the presence or absence of DHMEQ was not examined in the present study. It has been demonstrated that MCP-1, IL-8 and RANTES are elevated in the ocular tissues of experimental autoimmune uveitis, which suggests that these upregulated chemokines are potent chemoattractants in the pathogenesis of uveitis (32-34). Wakamatsu *et al* (35) revealed that DHMEQ had a positive therapeutic effect on established murine arthritis, and Iwata *et al* (36) revealed that DHMEQ was able to ameliorate experimental autoimmune uveoretinitis. Our previous study revealed that DHMEQ has anti-inflammatory effects on ocular inflammation induced by lipopolysaccharide via the inhibition of TNF-α and IL-6 expression levels in the aqueous humor, which indicated that DHMEQ may be a potential candidate to treat intraocular inflammatory diseases (37).

Local and systemic corticosteroids have been used to control ocular inflammation in patients with uveitis; however, long-term corticosteroid treatment can lead to adverse local and systemic side effects (38). The present results indicated that TNF-α-stimulated ARPE-19 cells were resistant to dexamethasone in relation to the protein expression levels of IL-8 and MCP-1, but DHMEQ significantly decreased the production of IL-8 and MCP-1 of TNF-α-stimulated cells treated with DHMEQ. Furthermore, previous studies have revealed that TNF signaling can suppress the action of the glucocorticoid

receptor by interfering with the transactivation function of glucocorticoid (GC) (39), which contributes to tissue resistance to GCs in several pathologic inflammatory states (39). These findings indicate the possibility that DHMEQ may have anti-inflammatory properties, even in inflammatory conditions with glucocorticoid resistance.

In the present study, NF-κB-associated gene array analysis identified that the gene expression levels of cytokines and chemokines, including MCP-1, ICAM-1, IL-6, TNFSF15 and TNFAIP3, and TLR2, TLR3 and TLR4 were downregulated in ARPE-19 cells treated with DHMEQ, which was further demonstrated by quantitative PCR analysis. Moreover, it was revealed that DHMEQ increased the expression levels of several genes related to the NF-κB signaling pathway, including PTGES, MAP3K14, LTBR and TRADD. However, the present study did not examine the protein expression levels of the gene products either upregulated or downregulated in ARPE-19 cells by DHMEQ. In addition, the translocation of p65-NF-κB into the nucleus in the presence of DHMEQ in TNF-α-stimulated cells was not investigated. Therefore, future studies are required to assess post-transcriptional regulation by DHMEQ with western blotting and to examine the translocation of p65-NF-κB in the presence of DHMEQ with electrophoretic mobility shift assay.

The present results demonstrated that 50 and 100 $\mu$g/ml DHMEQ had severe cytotoxic effects on cultured ARPE-19 cells, and that high concentrations of DHMEQ (100 $\mu$g/ml) induced apoptosis and necrosis in TNF-α-stimulated cells. NF-κB is known to play important roles in protecting cells from apoptosis (40,41). Furthermore, previous studies have revealed that DHMEQ is able to induce apoptosis of cancer cells (42,43). Although the present study used an RPE cell line, it remains to be determined whether high concentrations of DHMEQ have cytotoxic or apoptotic effects on primary cultured RPE cells, and healthy and inflamed RPE cells *in vivo*. Thus, the relationship between the anti-inflammatory potential of DHMEQ and the induction of apoptosis by DHMEQ in healthy or inflamed RPE cells requires further examination.

In conclusion, the present results indicated that DHMEQ may have an anti-inflammatory effect on TNF-α-stimulated ARPE-19 cells. However, it is not fully understood whether DHMEQ has a suppressive effect on the expression of ICAM-1 and chemokine production in primary cultured human RPE cells and *in vivo* RPE monolayers, thus further studies are required to assess the anti-inflammatory effects and safety of DHMEQ on human RPE cells.

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**Availability of data and materials**

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

**Authors' contributions**

YA, HK and YS performed the experiments. YA and HK designed the experiments. AK contributed to the design of the methodology. YA, HK, TW, AH and AAO analyzed the results. YA and HK wrote the paper. KU prepared DHMEQ and analyzed the results. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

**Ethics approval and consent to participate**

Not applicable.

**Patient consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

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