

Effects of GSK2606414 on cell proliferation and endoplasmic reticulum stress-associated gene expression in retinal pigment epithelial cells

XINTONG JIANG*, YANTAO WEI*, TING ZHANG, ZHAOTIAN ZHANG,
SUO QIU, XUEZHI ZHOU and SHAOCHONG ZHANG

State Key Laboratory of Ophthalmology, Zhongshan Ophthalmic Center,
Sun Yat-sen University, Guangzhou, Guangdong 510060, P.R. China

Received January 13, 2016; Accepted January 17, 2017

DOI: 10.3892/mmr.2017.6418

Abstract. GSK2606414 is a novel, highly selective inhibitor of protein kinase R-like endoplasmic reticulum kinase (PERK). GSK2606414 and its analogues have recently been demonstrated to delay tumor growth and prevent neurodegeneration. The present study investigated the effects of GSK2606414 on proliferation, apoptosis, and the expression of activating transcription factor 4 (ATF4), CCAAT/enhancer-binding protein homologous protein (CHOP) and vascular endothelial growth factor (VEGF) in human retinal pigment epithelial (RPE) cells under endoplasmic reticulum (ER) stress. ARPE-19 human RPE cells were treated with 0.01-50 μ M GSK2606414, and ER stress was induced by thapsigargin (TG) treatment. Cell proliferation was assessed using the Cell Counting kit-8 cell viability assay. Apoptosis was detected by Annexin-V/propidium iodide double staining using flow cytometry. Western blot analysis was used to measure eukaryotic initiation factor 2 α (eIF2 α) phosphorylation levels. ATF4, CHOP and VEGF mRNA expression levels were assessed using reverse transcription-quantitative polymerase chain reaction. GSK2606414 treatment inhibited RPE cell proliferation in a dose-dependent manner, however it did not induce apoptosis. In addition, GSK2606414 treatment inhibited eIF2 α phosphorylation and reduced CHOP and VEGF mRNA expression levels in RPE cells under TG-induced ER stress. To the best of our knowledge, the present study is the first to demonstrate

that GSK2606414 has a potential antiproliferative effect in RPE cells *in vitro*. This effect appeared to be achieved via inhibition of the PERK/ATF4/CHOP signaling pathway and suppression of VEGF expression levels.

Introduction

Age-related macular degeneration (AMD) is a retinal degenerative disease, which causes progressive loss of central vision in the elderly (1). Clinically, AMD is comprised of two forms: Dry and wet. Dry AMD is characterized by drusen formation and geographical atrophy. Wet AMD is characterized by choroidal neovascularization (CNV) and the subsequent development of hemorrhage, exudation, scarring or retinal detachment. The retinal pigment epithelium (RPE) is the primary target of AMD (2,3). During AMD progression, the RPE is damaged, accompanied by a disruption of the choroidal blood-eye barrier and degeneration of photoreceptors. In addition, RPE cells proliferate and secrete various proangiogenic factors, including vascular endothelial growth factor (VEGF), which serves an important role in AMD-associated CNV (4).

Although the precise underlying mechanisms of AMD are not fully understood, numerous lines of evidence have indicated that endoplasmic reticulum (ER) stress contributes to the etiology of RPE cell damage and neovascularization formation (5-7). Several types of cellular stress, including hypoxia (8), infection (9), nutrient deprivation (10), oxidative stress (11) and dysfunctional calcium homeostasis (12), may induce accumulation of unfolded proteins in the ER lumen. This process activates protein kinase R (PKR)-like endoplasmic reticulum kinase (PERK), inositol-requiring kinase 1 and activating transcription factor (ATF) 6, and initiates unfolded protein response (UPR) signaling pathways (13,14). As one of three primary UPR effectors, PERK directly phosphorylates eukaryotic initiation factor 2 α (eIF2 α), which consequently inhibits initiation of general translation and reduces ER burden (15). However, eIF2 α phosphorylation leads to selective translation of numerous mRNAs, including ATF4. ATF4 induces expression of the proapoptotic transcription factor CCAAT/enhancer-binding protein homologous protein (CHOP), which mediates PERK-induced apoptosis (16).

Correspondence to: Professor Shaochong Zhang, State Key Laboratory of Ophthalmology, Zhongshan Ophthalmic Center, Sun Yat-sen University, 54 South Xianlie Road, Guangzhou, Guangdong 510060, P.R. China
E-mail: zhshaochong@outlook.com

*Contributed equally

Key words: GSK2606414, protein kinase R-like endoplasmic reticulum kinase, eukaryotic initiation factor 2 α , age-related macular degeneration, ARPE-19

Furthermore, ATF4 serves an important role in VEGF expression under hypoxia or chemical stress (17,18). Activation of the PERK/eIF2 α /ATF4 signaling pathway has been reported in numerous retinal degenerative diseases including AMD (16), glaucomatous retinopathy (19) and diabetic retinopathy (20). Disruption of PERK activity has been demonstrated to reduce hydroquinone-induced apoptosis and hypoxia-induced VEGF expression in human RPE cells *in vitro* (5,21). These findings suggested that inhibition of the PERK/eIF2 α /ATF4 signaling pathway may be a novel therapeutic strategy for the treatment of AMD.

Screening for PERK inhibitors has led to the identification of a family of highly selective and potent molecules, including GSK2606414 (molecular formula, C₂₄H₂₀F₃N₅O). GSK2606414 is an adenosine triphosphate-competitive inhibitor of PERK with a half maximal inhibitory concentration (IC₅₀) of 0.4 nM (22). GSK2606414 and its analogues have previously been demonstrated to inhibit ER stress, delay tumor growth and prevent neurodegeneration (23-25). To the best of our knowledge, the effects of GSK2606414 on cell proliferation and PERK signaling in cultured RPE cells under ER stress have yet to be reported.

The present study investigated the effects of GSK2606414 on PERK signaling and VEGF expression levels in RPE cells under ER stress, and examined the potential underlying mechanisms of GSK2606414 in RPE proliferation. The results of the present study provided valuable information for drug development or potential novel strategies for the treatment of AMD.

Materials and methods

Cell culture. ARPE-19 human RPE cells were purchased from the American Type Culture Collection (Manassas, VA, USA). Cells were cultured in a 1:1 mix of Dulbecco's modified Eagle's medium and F-12 medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.). Cells were cultured at 37°C in a humidified atmosphere containing 5% CO₂.

Reagents. GSK2606414 (EMD Millipore, Billerica, MA, USA) and thapsigargin (TG; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) were dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich; Merck KGaA). The concentration of DMSO was <0.1% for all experiments to avoid cytotoxicity.

Cell Counting kit-8 (CCK8) cell viability assay. Cell viability was assessed using a CCK8 assay (Dojindo Molecular Technologies, Inc., Kumamoto, Japan). ARPE-19 cells were seeded into a 96-well plate at a density of 2x10³ cells/well and incubated with 0.01, 0.05, 0.1, 0.5, 1, 5, 10 or 50 μ M GSK2606414 for 24, 48 or 72 h at 37°C. Subsequently, the medium was replaced with 100 μ l of fresh medium and CCK8 reagent (10 μ l) was added to each well, and cells were incubated for 2 h at 37°C. Absorbance (optical density) was measured at a wavelength of 450 nm.

Cell morphology assay. ARPE-19 cells (2x10⁵ cells/well) were plated on 6-well plates and incubated at 37°C for 24 h. Cells were

subsequently treated with or without 5 μ M GSK2606414 for 24 h at 37°C. Cell morphology was observed by phase-contrast microscopy using an Axiovert 200 inverted microscope (Carl Zeiss AG, Oberkochen, Germany).

Quantitative detection of apoptosis by flow cytometry. Cell apoptosis was measured using the Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) Apoptosis Detection kit (eBioscience, Inc., San Diego, CA, USA) according to the manufacturer's protocol. ARPE-19 cells were seeded into a 25 cm² plate at a density of 3x10⁶ cells/well for 24 h, and were subsequently treated with various concentrations of GSK2606414 (0.5-5 μ M). Cells treated with 200 μ M H₂O₂ served as a positive control. After 24 h treatment, cells were washed once with phosphate-buffered saline (PBS) and resuspended in 1X binding buffer at a concentration of 1x10⁶ cells/ml. Annexin V-FITC (5 μ l) was added to 100 μ l cell suspensions, and the cells were incubated at room temperature for 15 min in the dark. Cells were subsequently washed in 1X binding buffer and resuspended in 200 μ l binding buffer, followed by the addition of 5 μ l PI. Annexin V- and PI-stained cells were analyzed using a Cytomics FC500 flow cytometer (Beckman Coulter, Inc., Brea, CA, USA) and CXP Analysis Software version 2.2 (Beckman Coulter, Inc.).

Western blotting. ARPE-19 cells were treated with various concentrations of GSK2606414 (0.5-1 μ M) for 1 h, followed by stimulation with TG (1 μ M) for 2 h at 37°C. For the extraction of total cellular protein, cells were washed twice in cold PBS and homogenized in radioimmunoprecipitation assay lysis buffer (Cell Signaling Technology, Inc., Danvers, MA, USA) containing PhosSTOP™ and protease inhibitors (Roche Diagnostics, Indianapolis, IN, USA). Lysates were centrifuged at 13,000 x g for 15 min at 4°C. Protein concentrations were measured using a Bicinchoninic Acid protein assay kit (Pierce; Thermo Fisher Scientific, Inc.). Samples (40 μ g) were separated by 10% (w/v) SDS-PAGE and subsequently transferred onto polyvinylidene difluoride membranes (Merck KGaA). Membranes were blocked in 5% bovine serum albumin (Sigma-Aldrich; Merck KGaA) for 2 h at room temperature and incubated overnight at 4°C with the following primary antibodies: Anti-eIF2 α (1:1,000; Cell Signaling Technology, Inc.; catalog no. 5324), anti-phosphorylated (p)-eIF2 α (1:1,000; Cell Signaling Technology, Inc.; catalog no. 3398), and anti- β -actin (1:10,000; Sigma-Aldrich; Merck KGaA; catalog no. A2228). Membranes were incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody (1:5,000; Abcam, Cambridge, UK; catalog no. ab6721) or (HRP)-conjugated goat anti-mouse secondary antibody (1:5,000; Abcam; catalog no. ab6789) for 1 h at room temperature, and the immunoreactive bands were visualized by an Enhanced Chemiluminescence detection system (Pierce; Thermo Fisher Scientific, Inc.). Band densitometry was quantified using Quantity One image analysis software version 4.62 (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

RNA isolation and reverse transcription-quantitative polymerase chain reaction (qPCR). ARPE-19 cells were treated with various concentrations of GSK2606414 (0.5-5 μ M) for 1 h, followed by TG stimulation (1 μ M) for 24 h at 37°C. Total RNA

was isolated using a PureLink RNA Mini kit (Invitrogen; Thermo Fisher Scientific, Inc.) and quantified by ultraviolet spectrometry at a wavelength of 260 nm. RNA was reverse transcribed into cDNA in a total reaction volume of 10 μ l using PrimeScript RT Master Mix Perfect Real Time kit (Takara Bio, Inc., Otsu, Japan). The reaction system contained 200 ng/ μ l total RNA (2 μ l), 5X PrimeScript Buffer (2 μ l) and RNase-free distilled water (6 μ l); the reactions were incubated at 37°C for 15 min, followed by 85°C for 5 sec. qPCR reactions were conducted in a 20 μ l reaction volume containing SYBR Green Real-Time PCR Master Mix (10 μ l; Roche Diagnostics), diluted cDNA (2 μ l), 10 μ M forward primer (1 μ l), 10 μ M reverse primer (1 μ l) and 6 μ l distilled water. The primers used were as follows: ATF4, forward 5'-CCCTTCACCTTCTTACAACCTC-3', reverse 3'-GTCTGGCTTCCTATCTCCTTCA-5'; CHOP, forward 5'-ATGAACGGCTCAAGCAGGAA-3', reverse 3'-TGTGGGATTGAGGGTCACATC-5'; VEGF, forward 5'-TCACAGGTACAGGGATGAGGACAC-3', reverse 3'-TCCTGGGCAACTCAGAAGCA-5'; and β -actin, forward 5'-ACAATGTGGCCGAGGACTTT-3', reverse 3'-TGTGTGGACTTGGGAGAGGA-5'. Samples were analyzed in triplicate in a LightCycler 480 Instrument (Roche Diagnostics GmbH, Mannheim, Germany). The reaction conditions were as follows: Initial denaturation step at 95°C for 5 min, followed by 50 cycles at 95°C for 10 sec, 60°C for 10 sec, 72°C for 10 sec and a final elongation step at 72°C for 10 min. The $2^{-\Delta\Delta C_q}$ method was applied to estimate relative transcription levels (26), and the results were normalized to β -actin. ATF4, CHOP and VEGF mRNA expression levels are expressed relative to the control group.

Statistical analysis. Data were analyzed with SPSS 19.0 (IBM SPSS, Armonk, NY, USA). Data are expressed as the mean \pm standard deviation. Unpaired Student's t-test was used to compare differences between two groups. One-way analysis of variance was used to compare differences between three or more groups, followed by Bonferroni's post hoc test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

GSK2606414 suppresses proliferation of human RPE cells. To assess the effects of GSK2606414 on RPE cell proliferation, ARPE-19 cells were treated with 0.01-50 μ M GSK2606414 for 24, 48 or 72 h. Cell proliferation was quantified by CCK8 assay. Cell viability was inhibited by GSK2606414 in a dose- and time-dependent manner (Fig. 1A). At increased concentrations (0.5-50 μ M), GSK2606414 significantly inhibited cell proliferation compared with the control group ($P < 0.05$). The IC_{50} of GSK2606414 was 1.7 μ M in ARPE-19 cells treated with GSK2606414 for 72 h. The results were confirmed by morphological observation (Fig. 1B), which revealed that GSK2606414 (5 μ M) did not cause changes in ARPE-19 cell morphology compared with control group, but it did significantly reduced cell growth *in vivo*.

GSK2606414 does not induce apoptosis in human RPE cells. The effects of GSK2606414 on apoptosis were examined in ARPE-19 cells using an Annexin V-FITC/PI Apoptosis Detection kit. Treatment with 0.5-5 μ M GSK2606414 for 24 h did not significantly induce apoptosis in ARPE-19 cells

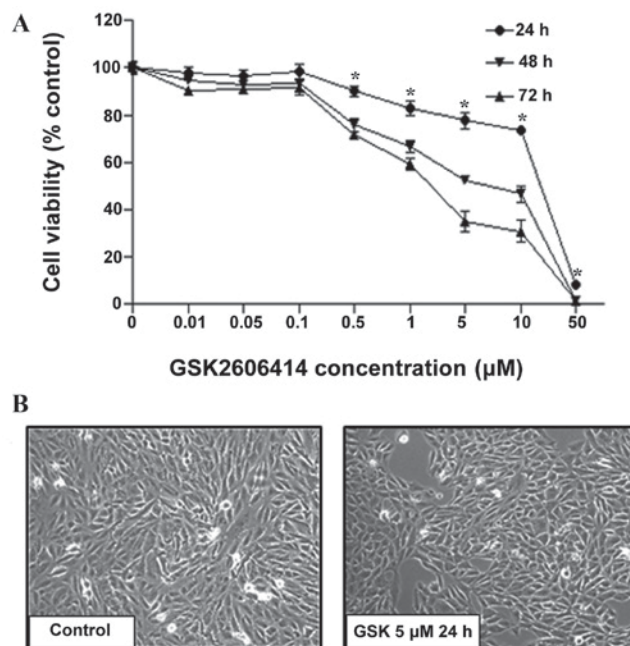


Figure 1. Effects of GSK2606414 on ARPE-19 cell proliferation. (A) ARPE-19 cells were treated with various concentrations of GSK2606414 (0-50 μ M) for 24, 48 or 72 h, and cell viability was detected by Cell Counting kit-8 assay. Data are presented as the mean \pm standard deviation (n=3). * $P < 0.05$ vs. control. (B) Cell morphology analysis; phase contrast images of ARPE-19 cells untreated and treated with GSK2606414 (5 μ M) for 24 h; magnification, x100. GSK, GSK2606414.

(Fig. 2A). However, apoptosis was significantly increased in ARPE-19 cells under 200 μ M H_2O_2 -induced oxidative conditions (Fig. 2B) compared with in 5 μ M GSK2606414-treated cells (Fig. 2C).

GSK2606414 inhibits eIF2 α phosphorylation in ARPE-19 cells. PERK serves an important role in the UPR by phosphorylating the translation initiation factor, eIF2 α . To determine the effects of GSK2606414 on eIF2 α phosphorylation, ARPE-19 cells were pretreated with 0.05-1 μ M GSK2606414 for 1 h followed by treatment with the ER stress inducer, TG, for 2 h. Protein expression levels of p-eIF2 α were reduced compared with eIF2 α (Fig. 3A). GSK2606414 treatment dose-dependently inhibited TG-induced eIF2 α phosphorylation (Fig. 3B).

GSK2606414 inhibits ER stress-induced ATF4, CHOP and VEGF expression in ARPE-19 cells. ATF4 and CHOP are primary transcription factors involved in the PERK signaling pathway, which serve important roles in regulating cellular fate and angiogenesis under stress conditions (15,27). The effects of GSK2606414 on ER stress-induced ATF4, CHOP and VEGF mRNA expression levels were examined in ARPE-19 cells. ATF4 (Fig. 4A), CHOP (Fig. 4B) and VEGF (Fig. 4C) mRNA expression levels were significantly increased in ARPE-19 cells following TG-induced ER stress. ATF4, CHOP and VEGF mRNA expression levels were increased in cells treated with low concentrations of GSK2606414 (0.05-0.1 μ M) compared with cells treated with TG alone. CHOP and VEGF mRNA expression levels were significantly reduced in cells treated with increased concentrations of GSK2606414 (0.5-5 μ M) compared with cells treated with TG alone.

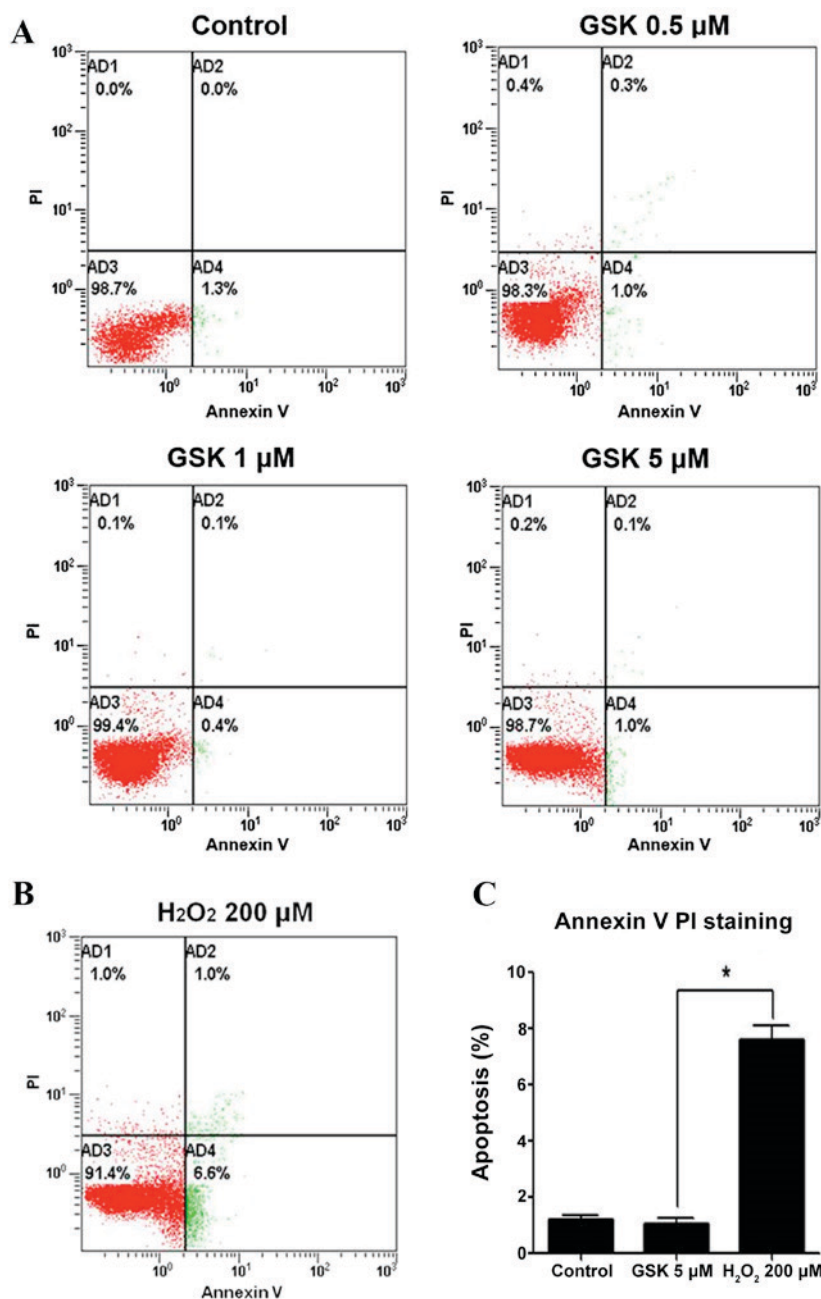


Figure 2. Effects of GSK2606414 on ARPE-19 cell apoptosis. ARPE-19 cells were incubated with various concentrations of GSK2606414 (0.5-5 μ M) for 24 h. (A) Apoptosis was measured using an Annexin V/PI detection kit. (B) ARPE-19 cells treated with 200 μ M H₂O₂ served as a positive control. Cells in the early stage of apoptosis are Annexin V⁺ and PI⁻, and cells that are dead or are in the late stage of apoptosis are Annexin V⁺ and PI⁺. (C) Apoptotic rates were analyzed by flow cytometry. Data are presented as the mean \pm standard deviation (n=3). *P<0.05. GSK, GSK2606414; PI, propidium iodide.

Discussion

The present study investigated the effects of GSK2606414 on cell proliferation, apoptosis, and ATF4, CHOP and VEGF expression levels in human RPE cells under TG-induced ER stress. GSK2606414 was originally synthesized by GlaxoSmithKline as a potent and selective inhibitor of PERK, with IC₅₀ values in the low nanomolar range (22,28). GSK2606414 and its analogue GSK2656157 have previously been demonstrated to inhibit tumor growth in a murine xenograft model (21,22). RPE cell proliferation is involved in late AMD and proliferative retinopathy (4,29-31). In the present study, GSK2606414 was revealed to inhibit RPE cell

proliferation in a dose- and time-dependent manner. The inhibitory effects of GSK2606414 on cell proliferation were more potent at concentrations >0.5 μ M. GSK2606414 did not induce RPE cell apoptosis. Furthermore, GSK2606414 was demonstrated to inhibit eIF2 α phosphorylation in ARPE-19 cells, suggesting that RPE cell proliferation may be associated with PERK-eIF2 α phosphorylation.

eIF2 α is believed to serve a fundamental role in integrating stress response and cell survival. eIF2 α may be phosphorylated by four kinases including PERK, PKR, general control non-repressible 2 and heme-regulated inhibitor in response to various cellular stressors, including misfolded proteins, oxidative stress, viral infection and nutrient deprivation (32).

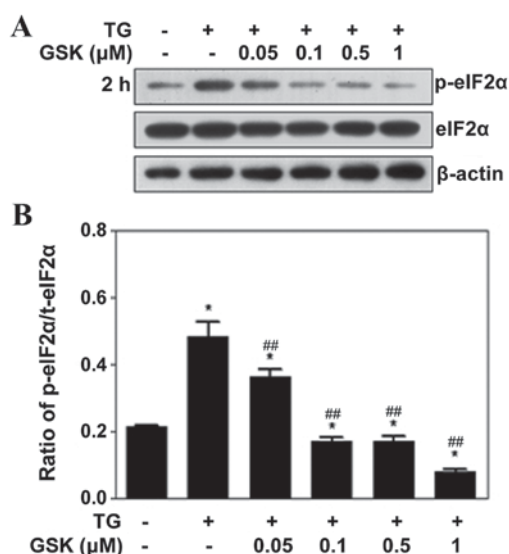


Figure 3. Effects of GSK2606414 on eIF2 α phosphorylation in ARPE-19 cells treated with TG. ARPE-19 cells were pretreated with various concentrations of GSK2606414 (0-1 μ M) for 1 h and then exposed to 1 μ M TG for 2 h. (A) Protein expression levels of t-eIF2 α and p-eIF2 α were measured by western blot analysis. β -actin served as a loading control. (B) Densitometry ratio of protein expression levels in ARPE-19 cells. Data are presented as the mean \pm standard deviation (n=3). *P<0.05 vs. control; **P<0.05 vs. TG-treated cells. TG, thapsigargin; p, phosphorylated; t, total; eIF2 α , eukaryotic initiation factor 2 α ; GSK, GSK2606414.

PERK is primarily activated by accumulation of misfolded proteins under ER stress conditions. eIF2 α phosphorylation by PERK may block protein synthesis (32,33). Axten *et al* (22) reported that 0.03 μ M GSK2606414 adequately inhibited PERK activity in A549 lung adenocarcinoma cells. The present study demonstrated that the ER stress inducer TG increased eIF2 α phosphorylation in RPE cells, and eIF2 α phosphorylation was inhibited by GSK2606414 at concentrations >0.1 μ M. These results suggested that the novel PERK inhibitor may inhibit protein synthesis by inhibiting eIF2 α phosphorylation.

eIF2 α phosphorylation has been reported to increase translation of basic leucine zipper transcription factors, such as ATF4 (32). The present study examined the effects of GSK2606414 on the expression levels of numerous ATF4-associated genes in ARPE-19 cells under ER stress. Notably, the inhibitory effect of GSK2606414 on gene expression did not correspond to its inhibitory effect on eIF2 α phosphorylation. GSK2606414 at 0.05 and 0.1 μ M inhibited eIF2 α phosphorylation; however, GSK2606414 at these doses upregulated ATF4, CHOP and VEGF mRNA expression levels. At increased concentrations (>0.5 μ M), GSK2606414 consistently inhibited eIF2 α phosphorylation and CHOP and VEGF mRNA expression levels. These results suggested that a concentration >0.5 μ M may be required for GSK2606414 to inhibit ATF4 signaling. CHOP, which is a proapoptotic transcription factor, is a key downstream target of ATF4 (13,34). Downregulation of CHOP has been reported to serve a neuroprotective role against stress-induced injury (13,35). The present study demonstrated that increased concentrations of GSK2606414 inhibited CHOP expression without significantly inhibiting ATF4 expression in RPE cells. This result may explain why GSK2606414 did not induce apoptosis. Therefore, GSK2606414 may be useful in preventing

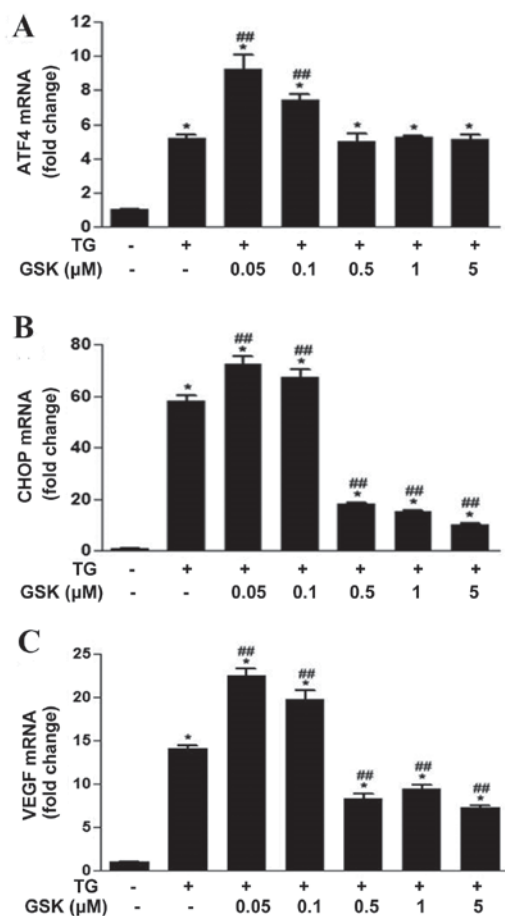


Figure 4. Effects of GSK2606414 on ATF4, CHOP and VEGF mRNA expression in ARPE-19 cells following TG treatment. ARPE-19 cells were pretreated with 0-5 μ M GSK2606414 for 1 h followed by treatment with 1 μ M TG for 24 h. (A) ATF4, (B) CHOP and (C) VEGF mRNA expression levels were measured by reverse transcription-quantitative polymerase chain reaction. Data are presented as the mean \pm standard deviation (n=3). *P<0.05 vs. control; **P<0.01 vs. TG-treated cells. ATF4, activating transcription factor 4; CHOP, CCAAT/enhancer-binding protein homologous protein; VEGF, vascular endothelial growth factor; TG, thapsigargin; GSK, GSK2606414.

ER stress-induced apoptosis in RPE cells. In addition, VEGF secretion by RPE cells is thought to be a key factor responsible for ocular neovascularization. Subretinal injection of adeno-associated virus-VEGF was reported to induce subretinal neovascularization and RPE cell proliferation in rats (36). Conversely, knockdown of VEGF by various pharmacologic agents effectively inhibited neovascularization in animal models of CNV (37,38). PERK/eIF2 α /ATF4 signaling has previously been reported to regulate VEGF expression (6,17). The present study demonstrated that VEGF mRNA expression levels were decreased in RPE cells treated with GSK2606414. This result may explain, at least in part, why GSK2606414 inhibited RPE cell proliferation without inducing apoptosis. These findings additionally indicated that GSK2606414 may serve as a relatively safe antiproliferative and antiangiogenic drug for the treatment of AMD and numerous retinal proliferative diseases. The *in vivo* effects of GSK2606414 on the treatment of AMD require further examination in animal models in future studies.

In conclusion, the results of the present study demonstrated that GSK2606414 inhibits eIF2 α phosphorylation, and

downregulates the expression levels of CHOP and VEGF in RPE cells, which serves an important role in AMD. These findings suggested that GSK2606414 may function as a potential neuroprotective and antiangiogenic drug for the treatment of AMD.

Acknowledgements

The present study was supported by grants from the National Natural Science Foundation of China (grant no. 81441025) and the Guangdong Science and Technology Plan Project (grant no. 2012B031800380).

References

- Bressler NM: Age-related macular degeneration is the leading cause of blindness. *JAMA* 291: 1900-1901, 2004.
- Ramkumar HL, Zhang J and Chan CC: Retinal ultrastructure of murine models of dry age-related macular degeneration (AMD). *Prog Retin Eye Res* 29: 169-190, 2010.
- Ambati J and Fowler BJ: Mechanisms of age-related macular degeneration. *Neuron* 75: 26-39, 2012.
- Jager RD, Mieler WF and Miller JW: Age-related macular degeneration. *N Engl J Med* 358: 2606-2617, 2008.
- Chen C, Cano M, Wang JJ, Li J, Huang C, Yu Q, Herbert TP, Handa JT and Zhang SX: Role of unfolded protein response dysregulation in oxidative injury of retinal pigment epithelial cells. *Antioxid Redox Signal* 20: 2091-2106, 2014.
- Salminen A, Kauppinen A, Hyttinen JM, Toropainen E and Kaarniranta K: Endoplasmic reticulum stress in age-related macular degeneration: Trigger for neovascularization. *Mol Med* 16: 535-542, 2010.
- Libby RT and Gould DB: Endoplasmic reticulum stress as a primary pathogenic mechanism leading to age-related macular degeneration. *Adv Exp Med Biol* 664: 403-409, 2010.
- Bi M, Naczki C, Koritzinsky M, Fels D, Blais J, Hu N, Harding H, Novoa I, Varia M, Raleigh J, *et al*: ER stress-regulated translation increases tolerance to extreme hypoxia and promotes tumor growth. *EMBO J* 24: 3470-3481, 2005.
- Häcker G: ER-stress and apoptosis: Molecular mechanisms and potential relevance in infection. *Microbes Infect* 16: 805-810, 2014.
- Mei Y, Thompson MD, Cohen RA and Tong X: Endoplasmic reticulum stress and related pathological processes. *J Pharmacol Biomed Anal* 1: 1000107, 2013.
- Dandekar A, Mendez R and Zhang K: Cross talk between ER stress, oxidative stress, and inflammation in health and disease. *Methods Mol Biol* 1292: 205-214, 2015.
- Rizzuto R, Pinton P, Carrington W, Fay FS, Fogarty KE, Lifshitz LM, Tuft RA and Pozzan T: Close contacts with the endoplasmic reticulum as determinants of mitochondrial Ca²⁺ responses. *Science* 280: 1763-1766, 1998.
- Hiramatsu N, Chiang WC, Kurt TD, Sigurdson CJ and Lin JH: Multiple mechanisms of unfolded protein response-induced cell death. *Am J Pathol* 185: 1800-1808, 2015.
- Wu H, Ng BS and Thibault G: Endoplasmic reticulum stress response in yeast and humans. *Biosci Rep* 34: pii: e00118, 2014.
- Liu Z, Lv Y, Zhao N, Guan G and Wang J: Protein kinase R-like ER kinase and its role in endoplasmic reticulum stress-decided cell fate. *Cell Death Dis* 6: e1822, 2015.
- Jing G, Wang JJ and Zhang SX: ER stress and apoptosis: A new mechanism for retinal cell death. *Exp Diabetes Res* 2012: 589589, 2012.
- Pollreis A, Afonyushkin T, Oskolkova OV, Gruber F, Bochkov VN and Schmidt-Erfurth U: Retinal pigment epithelium cells produce VEGF in response to oxidized phospholipids through mechanisms involving ATF4 and protein kinase CK2. *Exp Eye Res* 116: 177-184, 2013.
- Wang Y, Alam GN, Ning Y, Visioli F, Dong Z, Nör JE and Polverini PJ: The unfolded protein response induces the angiogenic switch in human tumor cells through the PERK/ATF4 pathway. *Cancer Res* 72: 5396-5406, 2012.
- Doh SH, Kim JH, Lee KM, Park HY and Park CK: Retinal ganglion cell death induced by endoplasmic reticulum stress in a chronic glaucoma model. *Brain Res* 1308: 158-166, 2010.
- Miranda S, González-Rodríguez Á, García-Ramírez M, Revuelta-Cervantes J, Hernández C, Simó R and Valverde AM: Beneficial effects of fenofibrate in retinal pigment epithelium by the modulation of stress and survival signaling under diabetic conditions. *J Cell Physiol* 227: 2352-2362, 2012.
- Atkins C, Liu Q, Minthorn E, Zhang SY, Figueroa DJ, Moss K, Stanley TB, Sanders B, Goetz A, Gaul N, *et al*: Characterization of a novel PERK kinase inhibitor with antitumor and antiangiogenic activity. *Cancer Res* 73: 1993-2002, 2013.
- Axten JM, Medina JR, Feng Y, Shu A, Romeril SP, Grant SW, Li WH, Heerding DA, Minthorn E, Mencken T, *et al*: Discovery of 7-methyl-5-(1-([3-(trifluoromethyl)phenyl]acetyl)-2,3-dihydro-1H-indol-5-yl)-7H-pyrrolo [2,3-d]pyrimidin-4-amine (GSK2606414), a potent and selective first-in-class inhibitor of protein kinase R (PKR)-like endoplasmic reticulum kinase (PERK). *J Med Chem* 55: 7193-7207, 2012.
- Moreno JA, Halliday M, Molloy C, Radford H, Verity N, Axten JM, Ortori CA, Willis AE, Fischer PM, Barrett DA and Mallucci GR: Oral treatment targeting the unfolded protein response prevents neurodegeneration and clinical disease in prion-infected mice. *Sci Transl Med* 5: 206ra138, 2013.
- Krishnamoorthy J, Rajesh K, Mirzajani F, Kesoglidou P, Papadakis AI and Koromilas AE: Evidence for eIF2 α phosphorylation-independent effects of GSK2656157, a novel catalytic inhibitor of PERK with clinical implications. *Cell Cycle* 13: 801-806, 2014.
- Axten JM, Romeril SP, Shu A, Ralph J, Medina JR, Feng Y, Li WH, Grant SW, Heerding DA, Minthorn E, *et al*: Discovery of GSK2656157: An optimized PERK inhibitor selected for preclinical development. *ACS Med Chem Lett* 4: 964-968, 2013.
- Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 25: 402-408, 2001.
- Pereira ER, Liao N, Neale GA and Hendershot LM: Transcriptional and post-transcriptional regulation of proangiogenic factors by the unfolded protein response. *PLoS One* 5: pii: e12521, 2010.
- Wang H, Blais J, Ron D and Cardozo T: Structural determinants of PERK inhibitor potency and selectivity. *Chem Biol Drug Des* 76: 480-495, 2010.
- Pereira ER, Frudd K, Awad W and Hendershot LM: Endoplasmic reticulum (ER) stress and hypoxia response pathways interact to potentiate hypoxia-inducible factor 1 (HIF-1) transcriptional activity on targets like vascular endothelial growth factor (VEGF). *J Biol Chem* 289: 3352-3364, 2014.
- Liegl R, Koenig S, Siedlecki J, Haritoglou C, Kampik A and Kernt M: Temsirolimus inhibits proliferation and migration in retinal pigment epithelial and endothelial cells via mTOR inhibition and decreases VEGF and PDGF expression. *PLoS One* 9: e88203, 2014.
- Campochiaro PA: Ocular neovascularization. *J Mol Med (Berl)* 91: 311-321, 2013.
- Donnelly N, Gorman AM, Gupta S and Samali A: The eIF2 α kinases: Their structures and functions. *Cell Mol Life Sci* 70: 3493-3511, 2013.
- Szegezdi E, Logue SE, Gorman AM and Samali A: Mediators of endoplasmic reticulum stress-induced apoptosis. *EMBO Rep* 7: 880-885, 2006.
- Zinszner H, Kuroda M, Wang X, Batchvarova N, Lightfoot RT, Remotti H, Stevens JL and Ron D: CHOP is implicated in programmed cell death in response to impaired function of the endoplasmic reticulum. *Genes Dev* 12: 982-995, 1998.
- Zhang SX, Sanders E, Fliesler SJ and Wang JJ: Endoplasmic reticulum stress and the unfolded protein responses in retinal degeneration. *Exp Eye Res* 125: 30-40, 2014.
- Wang F, Rendahl KG, Manning WC, Quiroz D, Coyne M and Miller SS: AAV-mediated expression of vascular endothelial growth factor induces choroidal neovascularization in rat. *Invest Ophthalmol Vis Sci* 44: 781-790, 2003.
- Cao J, Zhao L, Li Y, Liu Y, Xiao W, Song Y, Luo L, Huang D, Yancopoulos GD, Wiegand SJ and Wen R: A subretinal matrigel rat choroidal neovascularization (CNV) model and inhibition of CNV and associated inflammation and fibrosis by VEGF trap. *Invest Ophthalmol Vis Sci* 51: 6009-6017, 2010.
- Saishin Y, Saishin Y, Takahashi K, Lima e Silva R, Hylton D, Rudge JS, Wiegand SJ and Campochiaro PA: VEGF-TRAP(R1R2) suppresses choroidal neovascularization and VEGF-induced breakdown of the blood-retinal barrier. *J Cell Physiol* 195: 241-248, 2003.