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Structure based modification of chalcone analogue activates Nrf2 in the human retinal pigment epithelial cell line ARPE-19

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Abstract

Oxidative stress-induced degeneration of retinal pigment epithelial (RPE) cells is known to be a key contributor to the development of age-related macular degeneration (AMD). Activation of the nuclear factor-(erythroid-derived 2)-related factor-2 (Nrf2)-mediated cellular defense system is believed to be a valid therapeutic approach. In the present study, we designed and synthesized a novel chalcone analogue, 1-(2,3,4-trimethoxyphenyl)-2-(3,4,5-trimethoxyphenyl)-acrylketone (Tak), as a Nrf2 activator. The potency of Tak was measured in RPE cells by the induction of the Nrf2-dependent antioxidant genes HO-1, NQO-1, GCLc, and GCLm, which were regulated through the Erk pathway. We also showed that Tak could protect RPE cells against oxidative stress-induced cell death and mitochondrial dysfunction. Furthermore, by modifying the α , β unsaturated carbonyl entity in Tak, we showed that the induction of antioxidant genes was abolished, indicating that this unique feature in Tak was responsible for the Nrf2 activation. These results suggest that Tak is a potential candidate for clinical application against AMD.

Key words: Nrf2, oxidative stress, mitochondrial dysfunction, acrolein, macular degeneration

Introduction

Age-related macular degeneration (AMD) is an eye disease characterized by the presence of extracellular depositions, known as drusen, accumulating between Bruch's membrane and the neural retina [1]. AMD affects the macula, a highly specialized region of the central retina responsible for fine and color vision, and has been recognized as a leading cause of vision loss that affects millions of individuals over the age of 65 years, especially in the United States and Europe [2]. The current incidence of AMD has reached 8.7% worldwide and is projected to be 196 million by 2020 and 288 million by 2040 [3]. In the past few decades, dramatic progress has been made in understanding the mechanisms of AMD progression, and multiple risk factors have been identified that are useful to the public for knowledge of AMD prevention. However, there is neither definitive treatment for the disease nor intervention to slow the progression of AMD, and targeted drug and nutrient screening remains of popular interest in the study of AMD [4].

It is commonly accepted that oxidative stress plays a pivotal role in AMD pathogenesis, and many risk factors, such as aging, smoking, UV and blue light exposure, chronic inflammation, and improper diet, have been identified as related to oxidative stress [5-9]. Retinal pigment epithelial (RPE) cells are highly specialized epithelial cells with an apical side in contact with photoreceptor segments. RPE cells maintain the health and regular activity of these photoreceptors [10] and function in the outer blood-retinal barrier to provide selective entry and removal of metabolites and molecules [11]. Given the remarkable and diverse functions of RPE cells, it has been well demonstrated that impairing RPE cell function is a major contributor to AMD progression [12], and it has been suggested that oxidative stress is the major factor for the protein damage and aggregation that ultimately leads to the degeneration of the RPE cells [13].

Previous studies have shown that the endogenous antioxidant defense system is activated in response to oxidative stress, which is regulated by nuclear

factor-(erythroid-derived 2)-related factor-2, also known as Nrf2. Nrf2 is a major regulator that binds to antioxidant response elements (AREs) to induce the expression of antioxidant enzymes, also known as phase II enzymes, which has been suggested to play vital roles in protecting RPE cells [14-18]. Under resting conditions, Nrf2 is trapped in the cytoplasm by Keap1 protein, thereby promoting Nrf2 ubiquitination and subsequent degradation by the proteasome [19, 20]; however, under stress conditions, Keap1 serves as the primary sensor of the stress signals and undergoes conformational changes due to protein modification, thereby inhibiting Nrf2 ubiquitination [21]. Nrf2 can translocate to the nucleus, where it binds to a small Maf protein and activates the transcription of target genes, such as heme oxygenase-1 (HO-1) [22], NAD (P) H:quinone oxidoreductase 1 (NQO-1) [23] and γ -glutamyl-cysteine ligase (GCL) [24]. GCL is the rate-limiting enzyme for the synthesis of glutathione (GSH), which is one of the major antioxidants produced in vivo [25], and Nrf2 can regulate GSH levels by modulating the expression of the GCL catalytic (GCLc) and modifier (GCLm) subunits. Consistently, Nrf2 mRNA and protein levels have been shown to decline with aging, which renders aging RPE cells vulnerable to oxidative damage [18, 26]. Therefore, it has been suggested that Nrf2 signaling is a promising target for novel pharmacological and genetic therapeutic strategies [27].

Many reports have demonstrated that dietary polyphenols beneficially contribute to enhanced human health, such as by improving vision. In recent years, several polyphenolic compounds that target Nrf2, such as quercetin, resveratrol, curcumin, and chlorogenic acid, have been identified as protectors of RPE cells [4]. Chalcones, which constitute a subclass of the flavonoid family, are the precursors of the flavones that participate in the biosynthesis of flavonoids and have been reported to exhibit a broad spectrum of pharmacological properties such that they have continuously attracted increased attention [28]. Chalcones often exhibit low bioavailability and poor absorption, leading to limited efficacy *in vivo* [29]. Studies have been dedicated to improving the chalcone derivatives that can induce Nrf2-mediated HO-1 expression and GSH synthesis [30]. A recent report suggested that the ability of chalcone

derivatives to increase intracellular GSH levels was drastically improved with the addition of three methoxyl and/or hydroxyl groups on phenyl ring A [31]. Here, we synthesized a new chalcone analogue, 1-(2,3,4-trimethoxyphenyl)-2-(3,4,5-trimethoxyphenyl)-acrylketone (Tak), which has three methoxyl groups on both phenyl ring A and phenyl ring B. It is tempting to speculate that Tak activates the phase II detoxifying enzyme system to create pronounced antioxidant capacity. We also designed another two compounds with modified carbonyl entities between the phenyl rings to compare to Tak and thus confirm the key structure responsible for Nrf2-induced activity.

Material and Method

Chemicals and reagents. LY294002, SB203580, SP600125, U0126, JC-1, and ATP mixes were obtained from Sigma (St. Louis, MO, USA). Antibodies against p-Akt, Akt, p-Erk1/2, Erk1/2, p-p38, p38, JNK, p-JNK and β-actin were purchased from Cell Signaling Technology (Danvers, MA, USA). Antibodies against PGC-1, TFAM, OPA1, Mfn1, Mfn2, Drp1, Fis1, HO-1 and NQO-1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against complexes I, II, and III were purchased from Life Technologies (San Diego, CA, USA). The cell culture medium, Mito-Tracker Green/Red and MitoSOX Red were purchased from Life Technologies (San Diego, CA, USA). The synthesis scheme and compound structure are provided in Figure 1. The reactions were monitored by thin-layer chromatography (TLC) and visualized under UV light. The ¹H NMR and ¹³C NMR spectra were measured on a Bruker Advance III 400 MHz instrument (Supplementary method). The purity (≥95%) of each compound was verified by HPLC (Fig. S1-3).

Cell culture. The human ARPE-19 cell line was obtained from the ATCC (Manassas, VA, USA) and was cultured in DMEM-F12 supplemented with 10% fetal bovine serum, 0.348% sodium bicarbonate, 2 mM L-glutamine, 100 U/ml penicillin and 100 μ g/ml streptomycin. The cell cultures were maintained at 37 °C in a humidified atmosphere of 95% air and 5% CO₂. The cell medium was changed every two days.

ARPE-19 cells were harvested within the first 10 passages.

Cell viability assay. Cell viability was analyzed using the MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide) assay. The cells were seeded at a density of 3.5×10^4 cells/ml in 96-well plates. After incubation, 200 µL of MTT- medium solution (1:9) was added to each well. The plates were further incubated for 4 h and then 200 µL of DMSO was added to each well. The absorbance was measured at 490 nm using a microplate fluorometer (Fluoroskan Ascent; Thermo Fisher Scientific, Inc.). The bar graph was generated by the GraphPad Prism. Three independent experiments with six repeats in each group were performed for the assay.

JC-1 assay for the MMP. Cells were cultured at a density of 3.5×10^4 cells/ml in 96-well plates. After treatment, the mitochondrial membrane potential (MMP) was measured using the lipophilic cationic probe 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl-imidacarbocyanine iodide (JC-1). The cells were washed with PBS once and scanned with a microplate fluorometer (Fluoroskan Ascent; Thermo Fisher Scientific, Inc.) at 488 nm excitation and 538 and 590 nm emission wavelengths to measure green and red JC-1 fluorescence, respectively. The red/green fluorescence intensity ratio reflects the MMP. The bar graph was generated by the GraphPad Prism. Three independent experiments with six repeats in each group were performed for the assay.

Intracellular ATP levels. Cells were cultured in six-well plates. After treatment, the cells were lysed using 0.5% Triton X-100 in 100 mM glycine buffer at pH 7.4. To determine intracellular ATP levels, an ATP bioluminescent assay kit (Sigma, St. Louis, MO, USA) was used. The assay was performed following manufacture instruction. The ATP was consumed, and light was emitted when the firefly luciferase catalyzed the oxidation of D-luciferin [32]. The bar graph was generated by the GraphPad Prism. Three independent experiments with three repeats in each group were performed for the assay.

Mitochondrial superoxide measurement. Cells were cultured at a density of 5×10^4 cells/ml in 24-well plates. After treatment, the generation of mitochondrial superoxides was observed by an MitoSOXTM Red Mitochondrial Superoxide Indicator, and the mitochondria were assessed with Mito-Tracker Green FM. Briefly, cells were stained with Mito-Tracker Green FM at 500 nM in serum-free medium for 30 min, followed by 10 μ M MitoSOXTM Red incubation in serum-free medium for 10 min. After washing with PBS, the cells were visualized by laser scanning confocal microscopy (Zeiss, Jena, Germany). Similar procedure was used to observe mitochondria distribution with Mitotracker Red.

Real-time PCR. Total RNA was extracted using TRIzol (Invitrogen, San Diego, CA, USA) following the manufacturer's protocol. Reverse transcription was performed using a PrimeScript RT-PCR kit (Otsu, Shiga, Japan) and was followed by semiquantitative real-time PCR using specific primers. Data were normalized to the mRNA levels of beta-actin, which was used as a housekeeping gene and were analyzed by the $2^{-\Delta\Delta CT}$ method. The final results are presented as percentages of the control. The primer sequences are presented in detail in Table S1. The bar graph was generated by the GraphPad Prism. Three independent experiments with three repeats in each group were performed for the assay.

Western blot analysis. Cells were lysed with western and IP lysis buffers (Beyotime, Nanjing, China). The lysates were centrifuged at 13,000 g for 6 min at 4° C. The supernatants were collected, and the protein concentration was determined with a BCA protein assay kit (Pierce, San Diego, CA, USA). Equal aliquots of protein samples were loaded onto 10% SDS-PAGE gels and transferred to nitrocellulose membranes. After blocking with 5% nonfat milk for 1 h, the membranes were incubated with primary antibodies overnight at 4° C, and then, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. Western blots were developed using an enhanced

chemiluminescence (ECL) western blot detection kit (Pierce, Rockford, IL) and quantified by scanning densitometry.

Statistical analysis. Data are presented as the mean \pm standard error of the mean (SEM) from at least three independent experiments and were analyzed using GraphPad Prism-5 software. The statistical significance was evaluated using one-way analysis of variance (ANOVA) followed by post hoc comparisons with Tukey's HSD test. The differences between two groups were analyzed using Student's *t*-test, and the level of significance was set at a value of P<0.05.

Results

Effects of Tak, Tap, and Tbp on cell viability and MMP. The chalcone analogue Tak was synthesized from 1,2,3-trimethoxybenzene with three methoxyl groups in both phenyl ring A and phenyl ring B and an α , β unsaturated carbonyl entity between the two phenyl rings. The compounds Tap and Tbp have the same phenyl ring structure as Tak, although Tab has two carbonyl units and Tbp has one carbonyl group between the phenyl rings (Fig. 1). The three compounds were tested in ARPE-19 cells with an acrolein challenge, which is a well-established cellular model that mimics smoking-promoted RPE cell death during AMD progression [33]. As shown in Fig. 2A, Tak at 5 and 10 µM could sufficiently inhibit acrolein-induced loss of cell viability, while neither Tap nor Tbp had a protective effect (Fig. 2B, C). The mitochondrial membrane potential (MMP) is a key factor in maintaining normal mitochondrial function and cellular activity. Following a cell viability assay, we further tested the protective effects of three compounds on the MMP. Consistently, Tak presented significant protection against acrolein-induced MMP loss, while Tap and Tbp had no effect (Fig. 2E, F). In addition, we also applied Tak to a t-BHP-challenged cellular model. As expected, Tak at 10 µM significantly protected both cell viability and MMP against *t*-BHP-induced damage (Fig. 3A, B).

Effects of Tak on mitochondrial superoxide. It is estimated that 1-3% of the

mitochondrial oxygen consumed during the process of oxidative phosphorylation is incompletely reduced, which results in the formation of superoxide anions, a predominant form of ROS produced in mitochondria, and its accumulation could induce oxidative stress to suppress mitochondria activity [34]. Since MMP was significantly decreased after acrolein treatment, we analyzed the extent of mitochondrial superoxide with confocal microscopy and found that the acrolein challenge dramatically increased superoxide levels in the RPE cells and that this increase was significantly prevented by Tak pretreatment (Fig. 3C).

Effects of Tak on mitochondrial dynamic markers. To explore the mechanism by which Tak confers its protective effect, we first analyzed mitochondrial dynamic markers, including mitochondrial biogenesis, fusion and fission related proteins. After treating the RPE cells with 1 μ M and 10 μ M Tak for 24 h, we analyzed the protein levels of PGC-1, TFAM, complexes I, II, and III and found no significant changes (Fig. 4A). Consistently, the mRNA levels of the mitochondrial complex subunits were not affected (Fig. 4B). Considering these results, together with the unchanged mitochondrial copy number (Fig. 4E), we speculated that Tak had no effect on mitochondrial biogenesis. Furthermore, fusion- and fission-related proteins were also tested, and no significant changes were observed (Fig. 4C). Meanwhile, Mitotracker Red staining of mitochondria showed no significant changes on general morphology and cellular localization (Fig. 4D). In addition, the cellular ATP level was not affected by Tak treatment (Fig. 4F). Taken together, these data suggested that Tak had no significant effect on mitochondrial number, cellular localization, and general fusion/fission related proteins.

Effects of Tak on Nrf2-regulated phase II enzymes. Since the Nrf2/Keap1 complex is the regulator of endogenous antioxidative enzymes, also known as phase II enzymes, we found that the mRNA levels of both Nrf2 and Keap1 were not changed after treatment with each of the three compounds (Fig. 5A, B). Interestingly, further analysis of phase II enzymes revealed that Tak could dramatically induce the

expression of HO-1, NQO-1, GCLc and GCLm, indicating that Tak could activate Nrf2 for the induction of phase II enzymes (Fig. 5C-F). Consistent with the observations from the MTT and MMP assays, neither Tap nor Tbp had significant effects on phase II enzyme levels (Fig. 5C-F). In addition, the changes in mRNA levels were further confirmed by measuring the protein levels of Nrf2, HO-1 and NQO-1, and the findings showed unique induction of phase II enzymes only with the Tak treatment (Fig. 6). Since total Nrf2 protein level was not affected by Tak, we further analyzed nuclear level of Nrf2 and found that Tak treatment at 6 h could sufficiently promote Nrf2 nucleus translocation (Fig. 6D, E). Taken together, these data suggested that Tak could activate Nrf2 through promoting its nuclear translocation for the induction of phase II enzymes, which was the key factor responsible for the protection conferred by Tak.

Tak activates phase II enzymes through Erk signaling. To further explore the upstream pathway that regulates phase II enzymes by Tak, we treated RPE cells in a time-dependent manner and found that Tak could efficiently increase the levels of p-Akt and p-Erk without affecting other MAP kinases (Fig. 7A). Employment of pathway inhibitors showed that only inhibition of the Erk pathway by U0126[35] could significantly abolish the induction of phase II enzymes, which included HO-1, NQO-1, GCLc and GCLm (Fig. 7B-E), while inhibition of Akt by LY294002[36], inhibition of JNK by SP600125[37], and inhibition of p38 by SB203580 [38] had no significant effects on Tak-induced phase II enzyme expression (Fig. 7B-E). These data suggested that Tak could induce antioxidant enzyme expression through activation of the Erk pathway, which is consistent with previous reports showing that Erk activation is required for Nrf2 nuclear localization in HepG2 cells [39, 40].

Discussion

AMD is a multifactorial disease with no definitive treatment. Nutritional and chemical interventions targeting Nrf2-mediated phase II enzymes are acknowledged

and popular strategies for disease prevention. Even though chalcones have reportedly exhibited multiple benefits, their bioactivities in RPE cells have not been evaluated. Since the low bioavailability and poor absorption of chalcone limit its efficacy *in vivo*, recent studies have been dedicated to developing functional chalcone derivatives. In the present study, we designed and synthesized a series of chalcone analogues and showed that the chalcone analogue Tak could dramatically induce Nrf2-mediated phase II enzymes through activation of the Erk pathway and thus protect RPE cells from oxidative stress-induced mitochondrial dysfunction and cell death. We also confirmed that the α , β unsaturated carbonyl entity in Tak is the key structural feature that mediates Nrf2 activation.

Accumulating evidence has now been consolidated to show a role for oxidative stress in aging and many diseases, and retinal diseases are no exception to this pattern. As a result, targeting oxidative stress has been extensively studied in disease pathogenesis and prevention, and increasing data have suggested a role for Nrf2 in these processes [41]. A decrease in Nrf2 DNA-binding activity was reported in the retina of patients with diabetic retinopathy, and a role for Nrf2 in the onset of AMD has been well documented [42, 43]. Data from Nrf2-knockout mice contribute to the rationale for the suggested Nrf2-mediated cellular protection. Even though Nrf2-knockout mice develop without critical deficits, the abnormal ocular phenotypes observed in these mice were exacerbated under oxidative stress, and age-dependent deterioration in RPE cells was also observed in the Nrf2-knockout mice [44, 45]. An increasing number of studies have proposed that activation of Nrf2 would be an ideal therapy for treating retinal diseases. We have previously reported that natural compounds such as hydroxytyrosol [46], zeaxanthin [17], and alpha-tocopherol [47] are all specific Nrf2 activators and each offer RPE cell protection. Through structural modification, we also designed curcumin analogues that had improved efficiency compared with curcumin in activating Nrf2 [48]. Therefore, targeting Nrf2 by natural compounds and synthetic chemicals can protect RPE cell function against oxidative damage. Since oxidative stress is the major risk factor for the early progression of AMD, also named "dry AMD", there's another aggressive type of AMD, named "wet

AMD", which presents unique angiogenesis. It's been proposed that chalcones may work as an inhibitor of angiogenesis in macular degeneration, which has not been extensively studied [49]. In the present study, we found that Tak had no significant effect on angiogenesis related gene expression in ARPE-19 cells (Fig. S4), future studies shall be dedicated to exploring whether Tak could prevent stress induced angiogenesis both *in vitro* and *in vivo*.

Previous studies have reported that chalcone derivatives induce Nrf2-mediated HO-1 expression and GSH synthesis [30], and this inducement was drastically enhanced with the addition of up to three methoxyl and/or hydroxyl groups on phenyl ring A [31]. Here, we synthesized a new chalcone analogue with three methoxyl groups on both phenyl ring A and phenyl ring B. We speculated that Tak activates the phase II detoxifying enzyme system and has pronounced antioxidant capacity. The results of the present study, obtained by measuring phase II enzyme expression, confirmed these roles of Tak. In addition, to further identify the key structure that modulates this Nrf2 activation, we synthesized another two analogues, Tap and Tbp, which had the same phenyl rings as Tak but different linkages between the rings. Interestingly, we found that removal of the α , β unsaturated ketone completely abolished the effect of Tak on Nrf2 activation, indicating the vital role of the α , β unsaturated carbonyl entity in regulating Nrf2 activity.

Nrf2 is the ubiquitously expressed transcription factor that regulates the expression of over 1000 genes [50]. Nrf2 can be regulated at the transcriptional level, posttranscriptional level or by other transcription factors [51]. Even though, the most efficient way of activating Nrf2 is to disrupt the binding between Nrf2 and Keap1, which locates in cytoplasm and target Nrf2 for ubiquitination and degradation. Known Nrf2 inducers such as ROS, sulforophane, tert-butyl hydroquinone can oxidize the active cysteine residues in Keap1 to induce conformational change and prevent Nrf2 ubiquitination [52]. Meanwhile, several kinases including the PI3K/Akt and the MAPK, including JNK, Erk, and p38 were also reported to regulate Nrf2 through phosphorylation of Keap1 [53-55]. We have previously found that natural compound hydroxytyrosol could promote Nrf2 nuclear translocation through JNK activation,

while zeaxanthin mediates Nrf2 activation through PI3/Akt pathway [17, 46]. Here we found that Tak could activate Nrf2 through Erk1/2 activation, further investigations should be explored for the mechanisms of Tak structure feature in Erk1/2 activation.

In conclusion, our study demonstrated that the newly synthesized chalcone analogue could induce Nrf2-mediated phase II enzyme expression to improve the cellular redox status and enhance mitochondrial function through activation of the Erk pathway. Such beneficial bioactivity could further protect RPE cells against oxidative stress-induced cell damage. We also confirmed that the α , β unsaturated carbonyl entity of the chalcone is the key structural feature that mediates Nrf2 activation. All together, these findings suggest that Tak may be an attractive agent for the prevention and treatment of retinal diseases.

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Author Contributions

Z.F. and J.L. conceived the study, analyzed the data. N.H., R.C., and L.M. synthesized the compounds. Y.X., Y.L., and Y.C. performed the biological experiments and analyzed the data. Y.L., Y.C., and Z.F. drafted the manuscript.

Y.X., Y.L., and Y.C. contributed equally to the study.

Competing interests

The authors declare that they have no competing interests.

Supporting information

List of primers for real-time PCR; HPLC analysis of compounds Tak, Tap, and Tbp was presented in supporting information.

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Figure Legends

Figure 1. Synthesis scheme and compounds structure.(A) Synthesis of1-(2,3,4-trimethoxyphenyl)-2-(3,4,5-trimethoxyphenyl)-acrylketone(Tak).(B)Synthesis of1-(2,3,4-trimethoxyphenyl)-2-(3,4,5-trimethoxyphenyl)-acetophenone(Tap).(C)Synthesisof(2,3,4-trimethoxyphenyl)-benzophenone(Tap).(C)(C)Synthesis

Figure 2. Effects of Tak, Tap, Tbp on cell viability and MMP. ARPE-19 cells were treated with compounds at indicated doses for 24 h, followed by 75 μ M acrolein for another 24 h, cell viability was assessed by MTT assay, MMP was measured with

JC-1 staining. (A) Effect of Tak on cell viability. (B) Effect of Tap on cell viability. (C) Effect of Tbp on cell viability. (D) Effect of Tak on MMP. (E) Effect of Tap on MMP. (F) Effect of Tbp on MMP. Values are means \pm S.E.M. from at least three independent experiments. *P < 0.05, **P < 0.01 vs. relative control.

Figure 3. Effect of Tak on mitochondrial superoxide. ARPE-19 cells were treated with Tak at indicated doses for 24 h, followed by 300 μ M *t*-BHP for another 6 h, cell viability (A) and MMP (B) was analyzed. (C) ARPE-19 cells were treated with 10 μ M Tak for 24 h, followed by 75 μ M acrolein for another 24 h, mitochondrial superoxide level was assessed by fluorescent confocal microscope. Values are means \pm S.E.M. from at least three independent experiments. *P < 0.05, **P < 0.01 vs. relative control.

Figure 4. Effect of Tak on mitochondrial dynamics. ARPE-19 cells were treated with Tak at indicated doses for 24 h, cells were collected for (A) mitochondrial biogenesis related proteins, (B) mitochondrial complex subunits mRNA level, (C) mitochondrial fusion and fission related proteins, (D) Mitotracker Red staining by fluorescent confocal microscope, (E) mtDNA copy number, (F) cellular ATP level. Values are means \pm S.E.M. from at least three independent experiments. *P < 0.05, **P < 0.01 vs. relative control.

Figure 5. Effect of compounds on phase II enzymes mRNA. ARPE-19 cells were treated with compounds at indicated doses for 6 h, mRNA levels of phase II enzymes were analyzed by qPCR. (A) mRNA level of Nrf2. (B) mRNA level of Keap1. (C) mRNA level of HO-1. (D) mRNA level of NQO-1. (E) mRNA level of GCLc. (F) mRNA level of GCLm. Values are means \pm S.E.M. from at least three independent experiments. *P < 0.05, **P < 0.01 vs. relative control.

Figure 6. Effects of Tak on phase II proteins. ARPE-19 cells were treated with Tak at indicated doses for 24 h, protein levels of HO-1 and NQO-1 were analyzed by Western blot. (A) Western blot image. (B) Statistical analysis of HO-1. (C) Statistical analysis of NQO-1. Cells were treated with Tak at indicated doses for 6 h, cytosolic and nuclear fractions were prepared for analyzing Nrf2 protein level. (D) Western blot image. (E) Statistical analysis of nuclear Nrf2. Values are means \pm S.E.M. from at least three independent experiments. *P < 0.05, **P < 0.01 vs. relative control.

Figure 7. Tak activates phase II enzymes through Erk pathway. ARPE-19 cells were treated with Tak at 5 and 10 μ M for indicated time, Akt and MAPK pathway activation was analyzed by western blot (A). ARPE-19 cells were treated with Akt and

MAPK pathway inhibitors for 1 h, followed by Tak treatment for another 6 h, mRNA levels of (B) HO-1, (C) NQO-1, (D) GCLc, and (E) GCLm were analyzed by qPCR. Values are means \pm S.E.M. from at least three independent experiments. *P < 0.05, **P < 0.01 vs. relative control.









Acrolein 75 µM



Figure.4







Figure.6



Figure.7









Tak

Tak

Highlights

- Modified chalcone analogue activates Nrf2 through Erk1/2 kinase
- The compound eliminates acrolein induced mitochondrial ROS overproduction
- The compound protects ARPE-19 cells against oxidative stress induced cell death
- α , β unsaturated carbonyl entity of the compound is responsible for Nrf2 • activation

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