·Basic Research·

Proliferation of retinal pigment epithelial cells induced by (R,R)-XY-10 and (S,S)-XY-10 and their action mechanisms

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Abstract

• AIM: To investigate the mechanism of proliferation effect induced by (R,R)-XY-10 and (S,S)-XY-10 on retinal pigmented epithelial cells (ARPE-19).

• METHODS: Human retinal pigmented epithelial cells (ARPE-19) and human umbilical vein endothelial cells (HUVECs) were used to investigate the effect of (R,R)-XY-10 and (S,S)-XY-10 on cell growth, and their mechanisms of proliferative action by using ERK, AKT, PI3K, Protein kinase C (PKC) and Nitric oxide synthase (NOS) inhibitors.

• RESULTS: (R,R)-XY-10 and (S,S)-XY-10 dose-dependently increased ARPE-19 cell proliferation, but not on HUVECs. When treated with proliferative inhibitors, H-7 (5μmol/L), hypericin (20μmol/L), PD98059 (2μmol/L), LY294002 (50μmol/L), SH-5 (10μmol/L) and L-NAME (100μmol/L), the proliferative effect was reduced by H-7, hypericin, PD98059 and LY294002, but not by SH-5 and L-NAME.

• CONCLUSION: (R,R)-XY-10 and (S,S)-XY-10 can induce cell proliferation through MAPK and PI3K dependent pathway.

• KEYWORDS: age-related macular degeneration; (R,R)-XY-10; (S,S)-XY-10; ARPE-19 cells; human umbilical vein endothelial cells; proliferation
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INTRODUCTION

The retinal pigmented epithelium (RPE) is a monolayer cell located between the retinal photoreceptors and the choroidal blood vessels [1,2] that plays a key role in the mechanical and metabolic support of the photoreceptors[3]. In normal eyes, there is very little RPE cell turnover, and most RPE cells survive for an individual's lifetime. RPE cell death is accompanied by underlying choriocapillaris atrophy and overlying retinal thinning, ultimately resulting in decreased visual acuity[4]. Therefore, RPE[5] cell is the main element of some ocular diseases, such as proliferative vitreoretinopathy (PVR), uvetitis and age-related macular degeneration (AMD)[6,7]. AMD exists in both non-exudative and exudative forms. The non-exudative form involves atrophy of the central macula with a slow and progressive loss of central vision. The exudative form is characterized by the growth of new blood vessels through Bruch's membrane into the subretinal space, and the development of choroidal neovascularization (CNV) through an angiogenic process. The progression of AMD is associated with retinal inflammation and drusogenesis [4,8]. Moreover, it has been shown that the RPE cells of atrophic (dry form) AMD patients are significantly higher on cell injury and death[9].

The Mitogen-activated protein kinases (MAPKs) are ubiquitous enzymes, which play a pivotal role in variety of cellular functions in many cell types [10,11]. Three major mammalian MAPK subfamilies have been described: ERK1 and 2, the c-Jun NH-2 terminal kinases (JNK), and the p38 kinases. Among these, ERK activation is typically associated with cell survival. Proliferation and differentiation [10,12]. Phosphatidylinositol 3-kinase (PI3K) is a family of enzymes that phosphorylate phospholipids which engage various other enzymes such as Akt (protein kinase B). It is well established that activation of PI3K plays an important role in promoting cell survival and proliferation in numerous cell systems [13]. Activation of PI3K results in recruitment of the serine-threonine kinase Akt, one of the downstream targets of PI3K, to the plasma membrane, where it is activated by the 3-phosphoinositide-dependent kinase PDK1.Activated Akt affects the activity or abundance of a number of transcription factors linked to cell survival and proliferation[13].

In this study, we want to investigate the effect of (R,R)

-XY-10 and (S,S)-XY-10 induced proliferation on ARPE-19 cells and the involvement of MAPK and PI3K and Akt pathway.

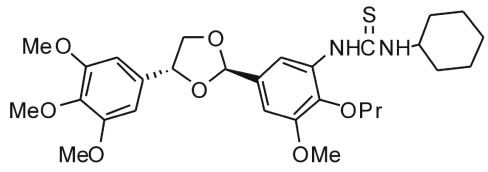
MATERIALS AND METHODS

Materials (R,R)-XY-10 and (S,S)-XY-10 were synthesized (Figure 1). H-7 (1-(5-Isoquinolinesulfonyl)-2-methylpiperazine, 2HCl), Hypericin ($C_3H_{16}O_8$), PD98059 ($C_{16}H_{13}NO_3$), LY294002(2-(4-Morpholinyl)-8-phenyl-4H-1-benzopyran-4-one, SH-5 ($C_{29}H_{59}O_{10}P$), MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide were purchased from Sigma-Aldrich (St. Louis, MO). The chemical structures of (R,R)-XY-10 and (S,S)-XY-10 are presented in Figure 1.

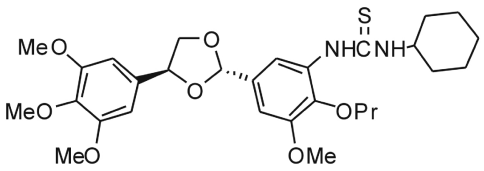
ARPE -19 Cell Culture The human REP cell line ARPE-19 obtained from American Type Culture Collections (ATCC; Manassas, VA) was cultured in Dulbecco's modified essential medium supplement with 100mL/L fetal bovine serum (FBS), 50kU/L penicillin/streptomycin and 2.5mmol/L glutamine at 37℃ incubator with 50mL/L CO_2. The cell line is not transformed and has structure and function properties characteristic of RPE *in vivo*. The cells were seeded into 96-well plates, and subconfluent cell monolayer was studied within three to ten passages. Before starting the experimental procedures, the medium was removed and replaced with phenol red-free low-glucose D-MEM supplemented with 1% calf serum, 0.6g/L glutamine, and 1% penicillin streptomycin.

HUVEC Culture HUVECs were purchased from ScienceCell (San Diego, CA) and cultured in low-glucose EBM-2 supplemented with 100mL/L calf serum, EBM-2 including EGM-2 SingleQuots(Clonetics),2mmol/L glutamine, 100kU/L penicillin, and 100g/L streptomycin in a humidified atmosphere of 50mL/L CO_2, 950mL/L air. The cell line is not transformed and has structure and function properties characteristic of HUVECs *in vivo* [14,15]. These cells were positive for the endothelial cell-specific von Willebrand factor and angiotensin-1-converting enzyme activity. The cells were seeded into 96-well plates, and subconfluent cell monolayer was studied within six to eight passages. Before starting the experimental procedures, the medium was removed and replaced with phenol red-free low-glucose D-MEM supplemented with 1% calf serum, 0.6g/L glutamine, and 1% penicillin streptomycin.

Cell Damage Assay Cultured RPE cells and HUVECs were seeded onto 96-well plates with a cell volume of 1×10^8 cells/L, and were grown to 80% confluence before treatment to prevent contact inhibition. The cells were exposed to the control or various concentrations of (R,R)-XY-10, (S,S)-XY-10 (1, 3, 10, 30, 100mg/L), the culture were then incubated at 37℃ for 24 or 48 hours. Cell viability was assessed by using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl



(R,R)-XY-10



(S,S)-XY-10

Figure 1 Chemical structures of (R,R)-XY-10 and (S,S)-XY-10

tetrazolium bromide (MTT) assay to determine the proportion of living cells in each culture (living cells are those with mitochondrial function of dehydrogenase)[16]. The 96 wells cultures, after exposure to the control or the test compounds for 24 hours, were incubated with 5g/L MTT at dilution of 1:10 base on the volume of culture medium for 3 hours at 37℃. At the end of incubation, the MTT solution was removed, and the cells were dissolved in 0.1mL/well DMSO. The proportion viable cells (those with mitochondria capable of cleaving the MTT molecule to produce the dark purple substance, formazan) was determined by measuring the absorbance (*A*) of each sample at 570nm using a SpectraCount plate reader (Packard BioScience, Meridan, CT). Exact cell number was determined using Trypan blue exclusion and a hemacytometer counting cells using trypan blue exclusion and simultaneously performing the MTT assay on cells seeded at identical densities established a standard curve of A570 *vs* cell number. The photographs were captured with Olympus DP Controller (Olympusoptical Co., LTD, Japan).

Effect of Inhibitors RPE cells were washed and changed with fresh DMEM-F12 medium were added proliferative inhibitors such as H-7 (PKC inhibitor), Hypericin (MAPK inhibitor), PD98059 (ERK inhibitor), LY294002 (PI3K inhibitor), SH-5 (Akt inhibitor) and L-NAME (NOS inhibitor) in medium containing various concentrations of (R,R)-XY-10 and (S,S)-XY-10 for 24 hours incubation at 37℃ incubator. Reactions were stopped by washing out the medium and 5g/L MTT at dilution of 1:10 base on the volume of culture medium was added for 3 hours at 37℃. At the end of incubation, the MTT solution was removed, and the cells were dissolved in 0.1mL/well DMSO. The proportion viable cells was determined by measuring the absorbance(A) of each sample at 570nm using a SpectraCount

plate reader (Packard BioScience, Meridan, CT).

Statistical Analysis All data were presented as mean ± standard errors (SEM). A nonpaired Student's *t* test was performed to analyze the significance between two means at a certain time point. The differences were considered significant if $P < 0.05$.

RESULTS

Effect of (R,R)-XY-10 and (S,S)-XY-10 on ARPE-19 Cells (R,R)-XY-10 and (S,S)-XY-10 concentration-dependently induced cell proliferation at 24 and 48 hours. The maximum increase of viability at 100mg/L was 127.0%± 2.2% (P <0.01) and 121.8%±2.2% (P<0.01), respectively with (R,R)-XY-10, and 115.9%±1.6% (P <0.01) and 87.9%± 5.8% , respectively with (S,S)-XY-10. The increasing potency of (R,R)-XY-10 was higher than that of (S,S) -XY-10 (Figure 2).

Effects of (R,R) -XY -10 and (S,S) -XY -10 on HUVECs (R,R)-XY-10 and (S,S)-XY-10 did not induce cell proliferation either at 24 or 48 hours (Figure 3). The viability at 100mg/L was 82.2%±0.6% and 82.5%±0.6%, respectively with (R,R)-XY-10, and 85.9% ±1.4% and 88.1%±0.7%, respectively with (S,S)-XY-10.

Effects of Inhibitors (R,R)-XY-10 and (S,S)-XY-10 dose-dependently increased cell proliferation in H_2O and DMSO treated ARPE-19 cells, H-7(PKC inhibitor), Hypericin (MAPK inhibitor), PD98059 (ERK inhibitor), LY294002 (PI3K inhibitor) significantly inhibited (R,R)-XY-10 induced cell proliferation especially at higher concentration of 100mg/L (Figure 4A). SH-5 and L-NAME did not affect the cell survival (Figure 4A). H-7 (PKC inhibitor), Hypericin (MAPK inhibitor) and LY294002 (PI3K inhibitor) significantly inhibited (R,R)-XY-10 induced cell proliferation especially at higher concentration of 100mg/L. PD98059, SH-5 and L-NAME did not affect the cell survival (Figure 4B).

DISCUSSION

It is known that deficiencies of the retinal epithelium are pivotal in development of certain eye diseases. This is in agreement with the concept that the loss of RPE cells is the main reason during the early phase of AMD [17]. Previous studies on transplantation of healthy isolated RPE cells in humans have shown to increase cell numbers and rescue photoreceptors or even prevent further visual loss [18,19]. Therefore, adequate proliferation of RPE cell is probably to increase wound healing of compromised retinal pigment epithelium in atrophic AMD [20]. In addition, replacing diseased RPE with healthier RPE can rescue photoreceptors, prevent further visual loss, or even promote visual improvement[19, 21, 22].

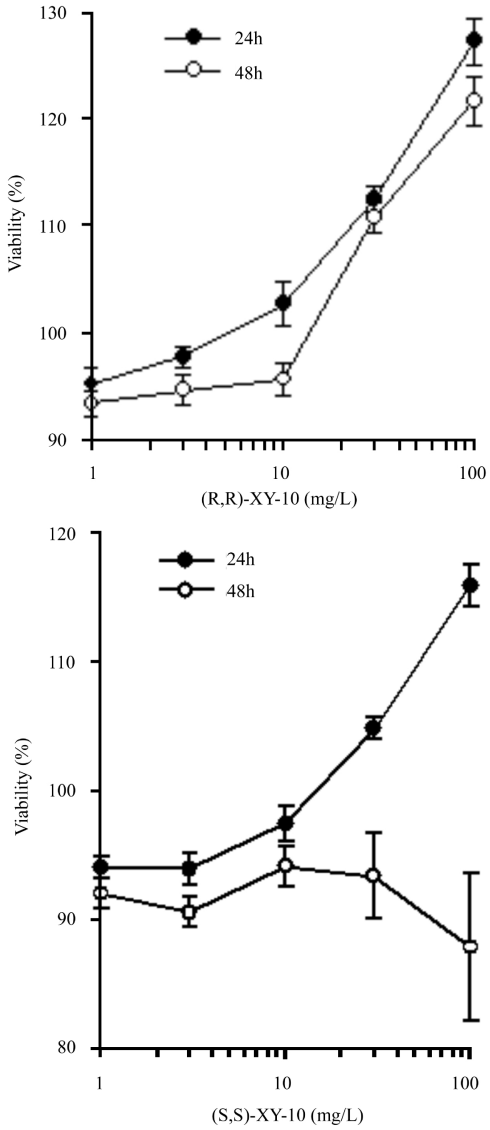


Figure 2 Effect of (R,R) -XY -10 and (S,S) -XY -10 on ARPE-19 cells (MTT assay, mean±SEM, n=6)

In our study, we found that (R,R)-XY-10 and (S,S)-XY-10 dose-dependently induced proliferation on ARPE-19 cells, but not HUVECs, indicating this effect was cell specific. When treated with proliferative inhibitors such as H-7 (PKC inhibitor), Hypericin (MAPK inhibitor), PD98059 (ERK inhibitor) and LY294002 (PI3K inhibitor), they significantly inhibited (R,R)-XY-10 induced cell proliferation, and (S,S)-XY-10 induced proliferation also reduced by H-7 (PKC inhibitor), Hypericin (MAPK inhibitor), and LY294002 (PI3K inhibitor), indicating the involvement of MAPK and PI3K signaling pathway.

In RPE cells, MAPK has been suggested to be associated with promotion of cell migration, ERK 1/2 activity is essential for RPE cell proliferation and involves the sequential phosphorylation of Ras/Raf/MEK/ERK 1/2 [23,24]. The reduction in proliferation coincided with reduced activation of ERK 1/2 and its upstream activator ERK, the

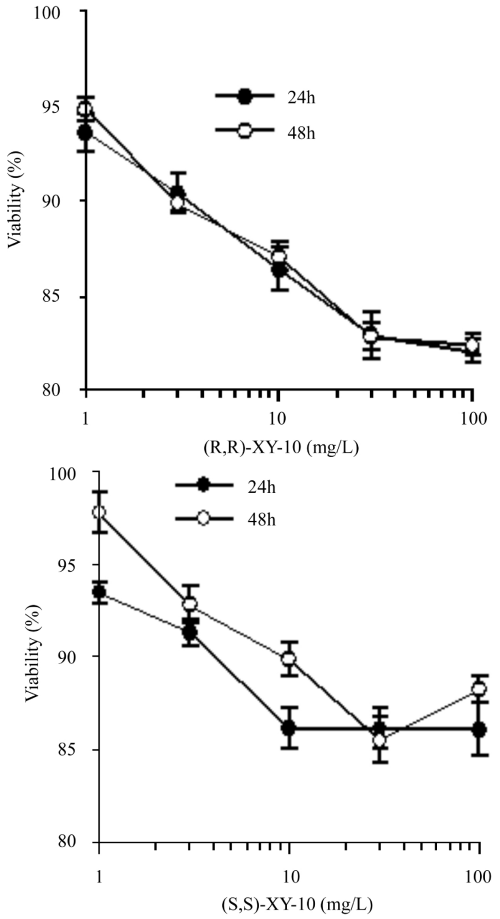


Figure 3 Effect of (R,R)-XY-10 and (S,S)-XY-10 on HUVEC (MTT assay, mean±SEM, n=6)

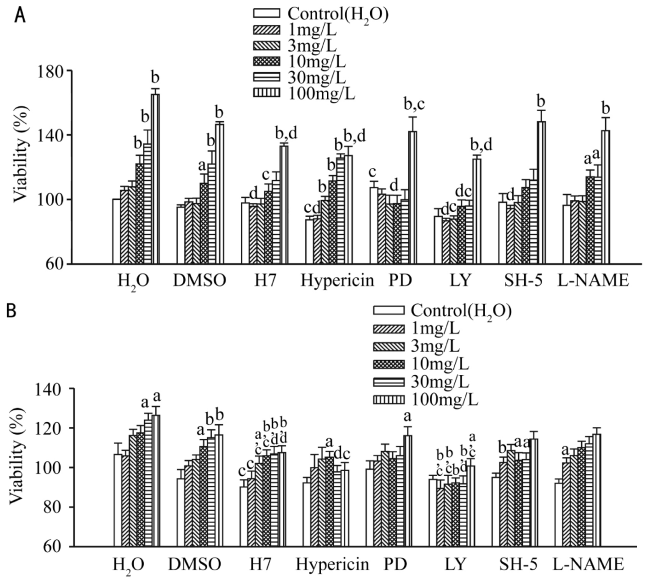


Figure 4 Effects of inhibitors on ARPE-19 cell proliferation A: (R,R)-XY-10; B: (S,S)-XY-10; (MTT assay, mean±SEM, n=6), ^{a}P<0.05, ^{b}P<0.01 *vs* control; ^{c}P< 0.05, ^{d}P <0.01 *vs* H_2O or DMSO control

inhibition effect of Hypericin in both (R,R)-XY-10 and (S,S)-XY-10, suggesting that activation of ERK involved in (R,R)-XY-10 and (S,S)-XY-10 induced cell proliferation. Moreover, the PI3K-Akt pathway is involved in all cell responses investigated (production of VEGF, cell migration, and proliferation). Inhibition of this pathway maybe a promising method to block RPE cell responses in proliferative retinopathies. Our data show that when pretreated with LY294002 (PI3K inhibitor) it also can inhibit both (R,R)-XY-10 and (S,S)-XY-10 induced cell proliferation, indicating the involvement of PI3K in this effect. On the other hand, not all proliferative effect on retinal cells are desired in physiological conditions, such as endothelial cell proliferation mediated angiogenesis is the initiating and leading to neovascularization. Our data suggested that (R,R)-XY-10 and (S,S)-XY-10 induced proliferation only on ARPE-19 cells but not on HUVECs.

Throughout lifetime, RPE cells are not only constantly under various burdens especially reactive-oxygen intermediates generated by phagocytosis of photoreceptors and the resultant lipid peroxidation of polyunsaturated fatty acid, but also have a very low turnover rate[25]. On this concept, we try to approach another method to improve the damaged RPE cell different from transplantation or surgery.

In conclusion, (R,R)-XY-10 and (S,S)-XY-10 can induce ARPE-19 cells proliferation on ARPE-19 cells but not HUVECs, and this effect might be through the MAPK and PI3K pathways. Although, the intracellular signaling involved in proliferative-mediated RPE cells survival is still poorly understood, however, searching the chemicals involved in either proliferative or recovery effect in RPE cells should allow the future development of therapeutic strategies against AMD. (R,R)-XY-10 and (S,S)-XY-10 have the potential on improving the RPE cells damaged through activated MAPK and PI3K pathway, and could be considered for the treatment of AMD and damaged RPE cells.

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