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Biapigenin, Candidate of an Agonist of Human Peroxisome Proliferator-Activated Receptor γ with Anticancer Activity

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Peroxisome proliferator-activated receptors (PPARs) are a subfamily of nuclear receptors (NRs). Human peroxisome proliferator-activated receptor gamma (hPPARγ) has been implicated in the pathology of numerous diseases, including obesity, diabetes, and cancer. ELISA-based hPPARγ activation assay showed that biapigenin increased the binding between hPPARγ and steroid receptor coactivator-1 (SRC-1) by approximately 3-fold. In order to confirm that biapigenin binds to hPPARγ, fluorescence quenching experiment was performed. The results showed that biapigenin has higher binding affinity to hPPARγ at nanomolar concentrations compared to indomethacin. Biapigenin showed anticancer activity against HeLa cells. Biapigenin was noncytotoxic against HaCa T cell. All these data implied that biapigenin may be a potent agonist of hPPARγ with anticancer activity. We will further investigate its anticancer effects against human cervical cancer.

Key Words : Biapigenin, PPARγ agonist, Biflavonoid, Anticancer agents

Introduction

Peroxisome proliferator-activated receptors (PPARs) are a subfamily of nuclear receptors (NRs). Nuclear receptors (NRs) are ligand activated transcription factors found in cells that are responsible for perceiving hormones and other molecules.[1,2] PPARs play central roles in regulating cellular differentiation, development, and tumorigenesis. Therefore, PPARs are noteworthy therapeutic targets for several metabolic disorders such as obesity, type 2 diabetes, and cancer.[3-6]

There are three types of PPARs (PPARγ, PPARγ, and PPARδ) in human. They share over 60% sequence homology in their ligand binding domains (LBDs) and DNA-binding domains.[7] PPARγ, the most studied PPAR, is present in adipocytes in high concentrations. Since it is highly expressed in adipocytes, for a long time, PPARγ was a typical therapeutic target for type 2 diabetes. PPARγ also regulates the proliferation and differentiation of cells and apoptosis. PPARγ's abilities of apoptosis and cell differentiation are advantageous in chemotherapy for various human cancers, including lung, breast, colon, cervix, and prostate cancers.[8-10] Activated PPARγ may acts on both a tumor suppressor and a tumor promoter.[11] A known mechanism of PPARγ in cancer is related to a tumor suppressor, phosphatase and tensin homolog (PTEN). As shown in Figure 1, activation of PPARγ causes an increase in PTEN protein levels or a decrease in transforming growth factor β1 (TGFβ1) levels, resulting in the induction of apoptosis and inhibition of cellular growth or cellular differentiation of cancer cells.[12-14]

The well known ligands of PPARγ are thiazolidinediones (TZDs), which are polyunsaturated fatty acids, and non-steroidal *anti*-inflammatory drugs (NSAIDs).[15,16] Indomethacin, a representative PPARγ ligand, inhibits the growth of human colorectal cancer cells by directly activating PPARγ.[17] Flavonoids are popular natural products with a broad range of medicinal activities, including antioxidant, antiviral, antibacterial, and anticancer activities.[18,19] Since they have low cytotoxicity in mammals, many flavonoids are used in therapeutic materials. Flavonoids are agonists of NRs, and they inhibit the growth of tumors in various cancer cell types.[20-22]

Biflavonoids are a class of flavonoids that form a homo- or hetero-dimer.[23] They are a minor class of flavonoids, distributed only in small plant families.[24] Biflavonoids are

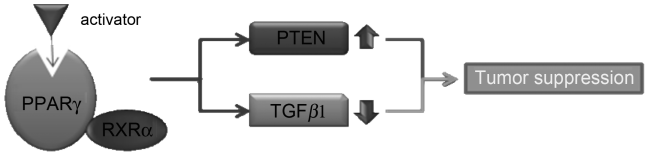

Figure 1. Schematic diagram of the mechanism of tumor suppression by PPARγ.

Abbreviations: hPPAR, Human peroxisome proliferator-activated receptor; LBD, ligand binding domain; NR, nuclear receptor; SRC-1, steroid receptor coactivator-1; RXRs, retinoid-X receptors; TGFβ, transforming growth factor; TZD, thiazolidinediones; NSAIDs, non-steroidal *anti*-inflammatory drugs; RT-PCR, reverse transcription-polymerase chain reaction; qPCR, real-time quantitative PCR.

formed by many different combinations of flavonoids, and over 200 biflavonoids with potential biological activities have been isolated.[25] Recently, oil extracts of flowering tops of Hypericum *richeri Vill* containing biapigenin showed *anti*-inflammatory and gastroprotective activities.[26] Crude extracts of *Selaginella tamariscina* including biapigenin are used as an oriental medicine, which has been reported to inhibit the production of proinflammatory cytokines and cause cell cycle arrest. It was reported that biapigenin blocked the transactivations of iNOS and COX-2 genes *via* the inactivation of nuclear factor-κB by preventing the nuclear translocation of p65.[27] Therefore, biapigenin can be a useful agent for cancer chemoprevention or for the treatment of inflammatory diseases. In this study, we demonstrated that biapigenin bound hPPARγ with high affinity. Also, we investigated that biapigenin can be a potent agonist of hPPARγ.

Methods

Extraction and Isolation of Biapigenin. The whole plant of *Selaginella tamariscina* (600 g) was extracted with MeOH at room temperature, yielding 50.54 g residue. The methanol extract was resuspended in water and partitioned sequentially with dichloromethane, ethyl acetate, and *n*-butanol. The EtOAc fraction (3.0 g) was placed in a silica gel (300 g, 4.8 × 45 cm) column and eluted using a $CHCl_3$-MeOH-H_2O (12:1:0.1 → 8:1:0.1 → 5:1:0.1 → 2:1:0.1 → 1:1:0.1 → MeOH only) gradient system. On the basis of their TLC pattern, the fractions were combined to yield subfractions, which were designated E1-10. Subfraction E4 (438.9 mg) was purified by column chromatography over a Sephadex LH 20 column and elution with MeOH-H_2O = 2:1 to give four subfractions (E41-E44). Subfraction E44 (196.5 mg) was finally purified by column chromatography over an MCI gel to afford biapigenin (25.0 mg). The physicochemical data, including ^{1}H NMR, ^{13}C NMR, and HSQC data, of biapigenin were identical with those reported in the literature (Markham *et al*., 1978; Silva *et al*., 1995). The structure of biapigenin is shown in Figure 2.

Expression and Purification of hPPARγ. A hexahistidine-tagged hPPARγ expression vector, pET-28a-hPPARγ-His, was constructed by cloning into the *Bam*HI/*Xho*I restriction sites, and transformed into the *Escherichia coli* strain BL21. To acquire the recombinant protein, transformed bacteria cultured in LB media were treated with 1 mM IPTG at OD_{600} 0.4-0.6 and induced overnight at 20 °C. After harvesting, the cells were resuspended in buffer comprising 20 mM HEPES (pH 7.4), 250 mM NaCl, and 50 mM imidazole. The cell lysate was centrifuged, and the supernatant was loaded onto a HiTrap chelating column (GE Healthcare) that had been pre-equilibrated with buffer A (20 mM HEPES (pH 7.4) and 10% glycerol). The column was washed with buffer A, and then, the bound material was eluted with a linear gradient of 0-500 mM imidazole. The hPPARγ-containing fractions were loaded onto a Superdex 75 column (GE Healthcare) pre-equilibrated with buffer B (10 mM Tris (pH 8.0), 0.5 mM EDTA, 50 mM NaCl, and 10 mM β-mercaptoethanol.

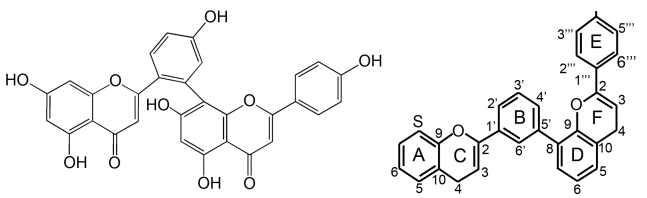

Figure 2. 2D structure of biapigenin.

Fluorescence-quenching Experiments. Experiments were performed at 25 ºC on an RF-5301PC spectrofluorophotometer (Shimadzu, Kyoto, Japan). hPPARγ (5 μM) was dissolved in buffer B. Biapigenin was titrated to give a protein:ligand molar ratio of 1:10. The sample was measured in a 2 mL thermostated cuvette with excitation and emission path lengths of 10 mm. Samples were excited at 280 nm, and the emission spectra were recorded for light-scattering effects from 260 nm to 600 nm. We estimated K_d according to the Stern-Volmer equation.[28]

A Simple Method to Screen PPARγ Ligands. In brief, each candidate ligand was diluted in a bacterial cell lysate containing His-tagged human PPARg and the mixture was added to 96-well plates pre-coated with SRC-1 recombinant protein. After incubation for 1 h, the wells were washed 3 times with phosphate-buffered saline containing 0.05% Tween 20 (PBST), before incubation with monoclonal *anti*-PPARγ antibody (Pγ48.34A) in 5% skim milk for 1 h. After washing 3 times with PBST, horseradish peroxidase (HRP)-conjugated *anti*-mouse IgG in 5% skim milk was added to the wells and further incubated for 1 h. After the plates were washed, SureBlue TMB Microwell Peroxidase Substrate (KPL, Inc. Gaithersburg, MD) was added, and the enzyme reaction was stopped by adding 2.5 N sulfuric acid. Enzyme activity was detected at 450 nm using an ELISA reader (Apollo LB 9110, Berthold Technologies GmbH, Germany).

Indomethacin, purchased from Sigma (St. Louis, MO), was used as a positive control and the *anti*-PPARγ monoclonal antibody (Pγ48.34A) was prepared as described previously.[29] Secondary antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). All the other reagents used in this study were of analytical grade and obtained commercially.

Determination of Anticancer Activity. Human breast cancer MCF-7 (KCLB30026) and MDA-MB-231 (KCLB30026) cells, human lung cancer A549 cells (KCLB10185), human cervical cancer HeLa cells (KCLB10002), and human prostate cancer PC3 cells (KCLB21435) were obtained from a Korean cell line bank (KCLB, Seoul, Korea). HeLa, MDA-MB-231, and PC3 cells were cultured at 37 °C in 5% CO_2 and Dulbecco's modified Eagle's medium (DMEM, Welgene Inc.) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin). The MCF-7 and A549 cells were cultured under the same conditions but in RPMI-1640 medium (Welgene Inc.) containing 10% FBS and 1% antibiotics. The cells were maintained in suspension or as

monolayer cultures and subcultured.

The anticancer activity of the biapigenin was evaluated with an MTT assay. For seeding, 100 μL of cell suspension was added to each well to afford a final seeding density of 2×10^4 cells/well before incubation at 37 °C in 5% CO_2 for 24 h. Various concentrations of biapigenin were then added to the wells, followed by incubation for an additional 24 h before adding 20 μL MTT solution to each well and substrate development for 4 h. The amount of resulting formazan was determined by measuring the absorbance at 570 nm, using a microplate reader.[30-32]

MTT Assay for Cytotoxicity. Human keratinocyte HaCaT cells (Heidelberg, Germany) were cultured at 37 °C in 5% CO_2 in DMEM medium (Welgene Inc., Daegu) supplemented with 10% heat-activated FBS and antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin). The cells were cultured in RPMI1640 supplemented with 10% FBS and antibiotic-antimycotic solution (100 U/mL penicillin, 100 g/mL streptomycin, and 25 g amphotericin B) in 5% CO_2 at 37 °C. The cultures were passaged every 3 to 5 days, and the cells were detached with brief trypsin treatment and visualized under an inverted microscope. The cells were maintained in suspension or as monolayer cultures and subcultured. Cytotoxicity of the compounds against mammalian cells was evaluated using an MTT assay. For seeding, 100 μL cell suspension was added to each well (2×10^4 cells/well) and then incubated at 37 °C in a 5% CO_2 for 24 h. Next, various concentrations of biapigenin were added to the plates, following which the plates were incubated for an additional 24 h. After the 24-h incubation, 20 μL MTT solution was added to each well, and the plates were incubated for 4 h. The amount of resulting formazan was determined by measuring the absorbance at 570 nm, using a microplate reader. Phase contrast microscopy with a Motic AE31 microscope equipped with a Moticam 2300 camera was used to examine cell morphology after the cells had been cultured for 24 h.

Results and Discussion

Binding Assay using Fluorescence Quenching. We determined the dissociation constant (K_d) of the biapigenin and indomethacin through fluorescence quenching experiments. Fluorescence curves of biapigenin in hPPARγ are depicted in Figure 3. Indomethacin had a dissociation constant of 10^{-7} and biapigenin had a dissociation constant of the 10^{-8} order. Biapigenin bound strongly to hPPARγ, with dissociation constant in nanomolar range. Its binding affinity was higher than that of the known agonist, indomethacin by over 1 order. The dissociation constants of the biapigenin and indomethacin are listed in Table 1.

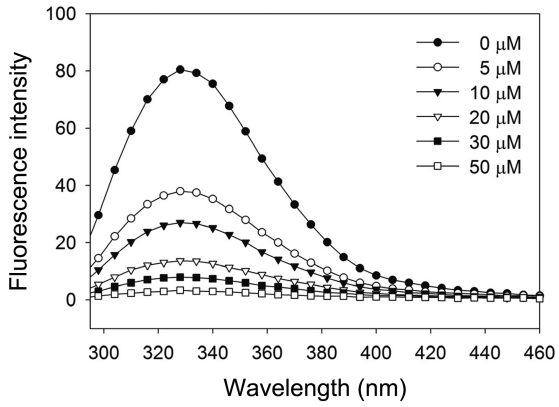

Figure 3. Fluorescence spectra of PPARγ with 0-50 μM of biapigenin.

PPARγ Agonist Activity. We used a simple ELISA-based ligand screening system and observed the binding between PPARγ and SRC-1 in the presence of biapigenin. The specific PPARγ ligands have high affinities to PPARγ and induce a conformation change of PPARγ protein. Ligand-bound PPARγ forms a heterodimer and it constructs the transcriptional machinery by recruiting transcriptional co-activators such as steroid receptor co-activator-1 (SRC-1). Therefore, if biapigenin was proper agonists of PPARγ, the expression of PPARγ would be stimulated and the binding between PPARγ and SRC-1 would increase. We used indomethacin as the positive control because of the high cost of TZD. As shown in Figure 4, biapigenin increased the binding between the two proteins approximately 3 fold compared to no ligand and up to 20% compared to indo-

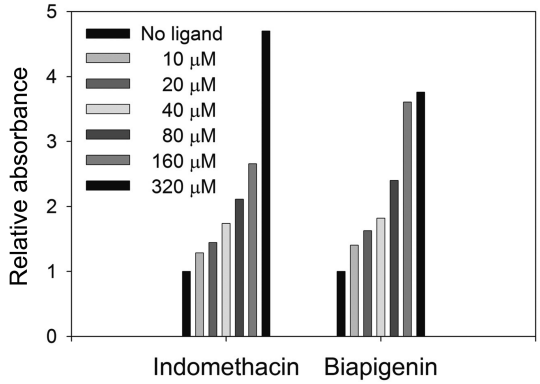

Figure 4. ELISA-based hPPARγ activation assay with biapigenin and a positive control, indomethacin. hPPARγ activation with ligand at a concentration range from 0 to 320 μM.

Table 1. Binding affinity and anticancer activity of hPPARγ agonist candidates

Compound	K_d (M)	Anticancer activities (IC_{50}, μM)				
		MCF-7	A549	HeLa	MDA-MB-231	PC3
Indomethacin	8.28×10^{-7}	> 100	50	25	> 100	> 100
Biapigenin	7.20×10^{-8}	> 100	> 100	67	> 100	> 100

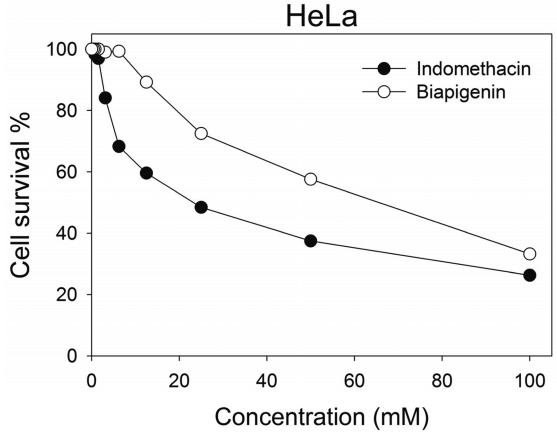

Figure 5. Anticancer activities of biapigenin and indomethacin against HeLa human cervical cancer cells.

methacin at 80 μM; binding increased in a concentration-dependent manner. At 160 μM, the agonist activity of biapigenin was significantly higher than that of indomethacin at the same concentration. However, at 320 μM, its activity increased less than 10%. At higher than 160 μM, indomethacin showed better activity than biapigenin. In conclusion, biapigenin increased binding between hPPARγ and SRC-1. Although compared with indomethacin, the biapigenin is not excellent for increasing the activation level of hPPARγ, they can be potent agonists of hPPARγ with high binding affinity.

Anticancer Activities of Biapigenin. The anticancer activities of biapigenin were estimated against five various human cancer cell lines (MCF-7, A549, HeLa, MDA-MB231, and PC3) using an MTT assay. The anticancer activities (IC_{50}) of the compounds are listed in Table 1, and the titration curves for the HeLa cell are presented in Figure 5. Indomethacin showed anticancer activity against HeLa and A549 celles, with an IC_{50} of 25 μM and 50 μM, and had no effect against the three other cancer cell lines. Biapigenin showed activity against HeLa cells with an IC_{50} of 67 μM. Therefore, biapigenin can be a potent agonist of hPPARγ with anticancer activities against human cervical cancers.

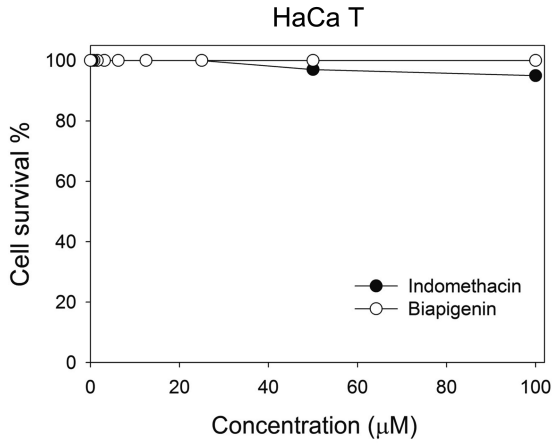

Figure 6. Cytotoxicity of biapigenin and indomethacin against normal cells (HaCa T).

Cytotoxicity. The cytotoxicity of biapigenin was investigated against a human keratinocyte cell line (HaCa T). Cell survival (%) of HaCa T cells *versus* the concentration of the compounds is depicted in Figure 6. We confirmed that the biapigenin did not exhibit cytotoxicity against HaCa T cells even at 100 μM, suggesting that biapigenin is a candidate for anticancer agent without toxicity.

Conclusion

In our previous report, we successfully identified a single flavonoid (3,6-dihydroxyflavone) as an agonist of hPPARγ and were confident that flavonoids can be potent agonists of PPARγ. In this paper, we confirmed the binding of biapigenin to hPPARγ with good binding affinity with K_d of 10^{-8} order.

Biapigenin increased binding between hPPARγ and SRC-1, implying its good activation activity for hPPARγ with high binding affinity in the nanomolar range. Further, biapigenin had anticancer activities against human cervical cancer cells (HeLa: IC_{50} = 67 μM). Biapigenin also showed almost no cytotoxicity against normal human keratinocyte cells (HaCa T), even at 100 μM. All these data implied that biapigenin may be a potent agonist of hPPARγ with anticancer activity. Further research will be carried out to investigate the anticancer mechanisms of compounds biapigenin.

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