http://dx.doi.org/10.5012/bkcs.2012.33.7.2219

Cytotoxic Activities of Amentoflavone against Human Breast and Cervical Cancers are Mediated by Increasing of PTEN Expression Levels due to Peroxisome Proliferator-Activated Receptor γ Activation

Eunjung Lee,[†] Soyoung Shin,[†] Jee-Young Lee,[†,‡] Sojung Lee,[†] Jin-Kyoung Kim,[†] Do-Young Yoon,[†] Eun-Rhan Woo,[§] and Yangmee Kim[†,*]

[†]*Department of Bioscience and Biotechnology, Bio/Molecular Informatics Center, Konkuk University, Seoul 143-701, Korea*
[*]*E-mail: ymkim@konkuk.ac.kr*
[‡]*Drug Discovery Team, Bioinformatics & Molecular Design Research Center, Seoul 120-749, Korea*
[§]*College of Pharmacy, Chosun University, Gwangju 501-759, Korea*
Received March 20, 2012, Accepted March 30, 2012

Human peroxisome proliferator-activated receptor gamma (hPPARγ) has been implicated in numerous pathologies, including obesity, diabetes, and cancer. Previously, we verified that amentoflavone is an activator of hPPARγ and probed the molecular basis of its action. In this study, we investigated the mechanism of action of amentoflavone in cancer cells and demonstrated that amentoflavone showed strong cytotoxicity against MCF-7 and HeLa cancer cell lines. We showed that hPPARγ expression in MCF-7 and HeLa cells is specifically stimulated by amentoflavone, and suggested that amentoflavone-induced cytotoxic activities are mediated by activation of hPPARγ in these two cancer cell lines. Moreover, amentoflavone increased PTEN levels in these two cancer cell lines, indicating that the cytotoxic activities of amentoflavone are mediated by increasing of PTEN expression levels due to hPPARγ activation.

Key Words : PPARγ, Flavonoid, Amentoflavone, PTEN, Cancer

Introduction

Peroxisome proliferator-activated receptors (PPARs) are a subfamily of nuclear receptors (NRs). The NR family is one of the largest families of transcription factors. Members of this family are activated by small lipophilic molecules, including hormones and vitamins.[1,2] PPARs play critical roles in the regulation of cellular differentiation and development and are, therefore, therapeutic targets in metabolic disorders such as obesity, type 2 diabetes, atherosclerosis, and cancer.[3-6]

PPARs are activated by natural ligands, including fatty acids and cholesterol metabolites.[7] Activated PPARs form a heterodimeric complex with retinoid-X receptors (RXRs) that recruits co-activators to promoter regions of genes involved in the control of lipid metabolism, and thereby regulates their transcription.[8,9] Three PPAR subtypes, *i.e.*, PPARα, PPARγ, and PPARδ, have been identified in humans, and their structures and functions are well known. The structures of PPARs and their therapeutic and biological actions have been studied in detail since their initial discovery. PPARα is highly expressed in the liver, heart, kidney and intestine, and plays an important role in fatty acid metabolism. PPARδ is expressed in a broad range of tissues, and in its activated form, affects glucose and lipid metabolism. Recently, it has been reported that an antagonist of PPARδ is effective against cancer; therefore, PPARδ could be a target in cancer therapy.[10] PPARγ, the most well-known PPAR, is present in adipocytes in high concentrations. Because it is highly expressed in adipocytes, PPARγ has long been considered a typical therapeutic target for type-2 diabetes.

Human PPARγ (hPPARγ) regulates the proliferation, apoptosis, and differentiation of various human cancer cells, including lung, breast, colon, and prostate cancer cells.[11-13] Activated hPPARγ may act as both a tumor suppressor and a tumor promoter.[14] It has been reported that activation of hPPARγ causes an increase in PTEN (phosphatase and tensin homologue) protein levels or a decrease in transforming growth factor β1 (TGFβ1) levels, resulting in tumor suppression through induction of apoptosis, inhibition of cellular growth, and/or promotion of cellular differentiation of cancer cells (Figure 1).[15-17]

Recent efforts by a number of research groups have focused on the design of novel agonists that exert dual agonistic activity on hPPARα and hPPARγ. The ligands of PPARγ are diverse and include thiazolidinediones (TZDs), polyunsaturated fatty acids, and non-steroidal anti-inflammatory drugs (NSAIDs). These ligands have high binding affinities for the

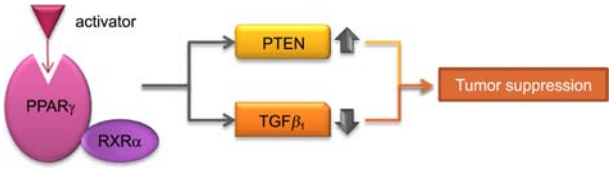

Figure 1. Schematic depiction of the mechanism of tumor suppression by PPARγ.

LBD of PPARγ. Indomethacin, an NSAID, is a representative hPPARγ ligand that inhibits the growth of human colorectal cancer cells by directly activating hPPARγ.

Flavonoids exhibit a wide range of activities, including antioxidant, antiviral, antibacterial, and anticancer activities.[18,19] Flavonoids are agonists of NR family members, and the crystallographic complex structure of the human estrogen receptor (ER), an NR, with the isoflavonoid, genistein, has been solved.[20-22] Thus, it is likely that flavonoids may also function as hPPARγ activators, promoting anticancer effects. Amentoflavone has agonist activity toward hPPARγ, but the mechanism underlying this activity is unclear.[23,24]

In our previous study, we verified that amentoflavone is an activator of hPPARγ and probed the molecular basis of its action.[25] We proposed a model of amentoflavone and hPPARγ binding in which amentoflavone forms three hydrogen bonds with the side chains of His323, Tyr327, and Arg280 in hPPARγ and participates in two hydrophobic interactions. It was also demonstrated that amentoflavone bound hPPARγ with high (picomolar) affinity and increased the binding between hPPARγ and steroid receptor coactivator-1 (SRC-1) by approximately 4-fold. Here, we investigated the mechanism of anticancer activity of amentoflavone. To verify the mechanism of action of amentoflavone in cancer cells, we investigated whether amentoflavone treatment affects the expression of hPPARγ using reverse transcription-polymerase chain reaction (RT-PCR) and real-time quantitative PCR (qPCR).

Methods

Extraction and Isolation of Amentoflavone. The whole plant of Selaginella tamariscina (600 g) was extracted with methanol at room temperature yielding 50.54 g of residue. The methanol extract was re-suspended in water and partitioned sequentially with dichloromethane, ethyl acetate, and *n*-butanol. The ethyl acetate fraction (3.0g) was placed a silica gel (300 g, 4.8 × 45 cm) column and eluted using a chloroform/methanol/H_2O (120:10:1, 80:10:1, 50:10:1, 20:10:1, 10:10:1, and 0:10:0, v/v) gradient system. Based on their TLC pattern, the fractions were combined to yield subfractions, which were designated E1-10. Subfraction E7 (296.33 mg) was finally purified by repeated column chromatography over a silica gel, RP-18, and Sephadex LH 20, yielding amentoflavone (82.23 mg). The UV, ^{1}H NMR, and ^{13}C NMR data for amentoflavone were identical to those reported in the literature.[26]

Determination of Cytotoxic Activity Against Human Cancer Cells. 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 cytotoxic activity of the amentoflavone 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 flavonoids 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.

MTT Assay for Cytotoxicity. Human keratinocyte HaCa T 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 amentoflavone 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 amentoflavone 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.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) and Real-Time Quantitative PCR (RT-qPCR). In order to conduct RT-PCR, total RNA was isolated using the Easy-BLUE™ total RNA extraction kit (iNtRon Biotechnology, Korea), according to the manufacturer's protocol. Reverse transcription was performed using ProSTAR™ (Stratagene, La Jolla, CA), and the cDNA was amplified with PCR. The GAPDH primer has been previously described[27] and primers specific for Fas were synthesized according to sequences published earlier.[28] The specific primers for SYBR green-based RT-qPCR were as follows: 5'-CCT ATT GAC CCA GAA AGG GA-3'/5'-CCA TTA CGG AGA GAT CCA CG-3' for hPPARγ. RT-qPCR was performed as previously described.[27]

Western Blot Analysis. Cultured MCF-7 and HeLa cells were treated with amentoflavone (20 μM and 40 μM) for

overnight. Cells were washed with phosphate buffer saline (PBS) and detached by EDTA-trypsin (Gibco, invitrogen, N.Y., USA). The collected cells were centrifuged at 5000 rpm for 5 min at 4 °C. Cell pellets were re-suspended in 100 μL of lysis buffer (1% Triton X-100, 1% deoxycholate, and 0.1% NaN_3) and incubated for 30 min on ice. After centrifugation of cell lysates at 12000 rpm for 10 min, the concentration of protein was determined by Bradford assay (Bio-Rad, Hercules, CA, USA). Each protein extract (20 μg) was separated on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA, USA). The transblotted membranes were incubated with 5% skim milk in Tris-buffered saline Tween-20 (TBST) for blocking non-specific binding at RT for 1 hour. After blocking with TBST, each membrane incubated with respective primary antibody specific for PTEN (1:1000, Millipore, Billerica, MA, USA) and β-actin (1:5000, Sigma-Aldrich, St.Louis, MO, USA). After incubated with primary antibodies, membranes were washed with TBST and incubated with secondary antibodies conjugated with anti-mouse IgG peroxidase (1:30000, Sigma-Aldrich, St. Louis, MO, USA). The immunoblots were detected by chemiluminescence detection system (ECL, GE Healthcare, Buckinghamshire, UK).[28,29]

Results and Discussion

Cytotoxic Activity of Amentoflavone. The cytotoxic activity of amentoflavone was tested against five human cancer cell lines (MCF-7, A549, HeLa, MDA-MB231, and PC3) using the tetrazolium-based colorimetric MTT assay. The cytotoxicities of amentoflavone against the different cell lines, expressed as 50% inhibitory concentrations (IC_{50}s), are presented in Table 1, and the titration curves are shown in Figure 2. Indomethacin is a representative hPPARγ ligand that inhibits the growth of human colorectal cancer cells by directly activating hPPARγ. As we reported previously, indomethacin inhibited HeLa cell proliferation with an IC_{50} of 25 μM, but had no effect against the four other cancer cell lines. Amentoflavone exerted potent cytotoxic effects against both MCF-7 and HeLa cells, with IC_{50}s of 25 and 20 μM, respectively. It has been reported that amentoflavone has anti-inflammatory activity and pregulates hPPARγ in TNFα activated A549 cells.[28,29] However, in our results, amentoflavone did not show noticeable cytotoxic activity against A549 cell line. Collectively, these observations suggest that amentoflavone is a potent agonist of hPPARγ with cytotoxic activities against human breast and cervical cancers.

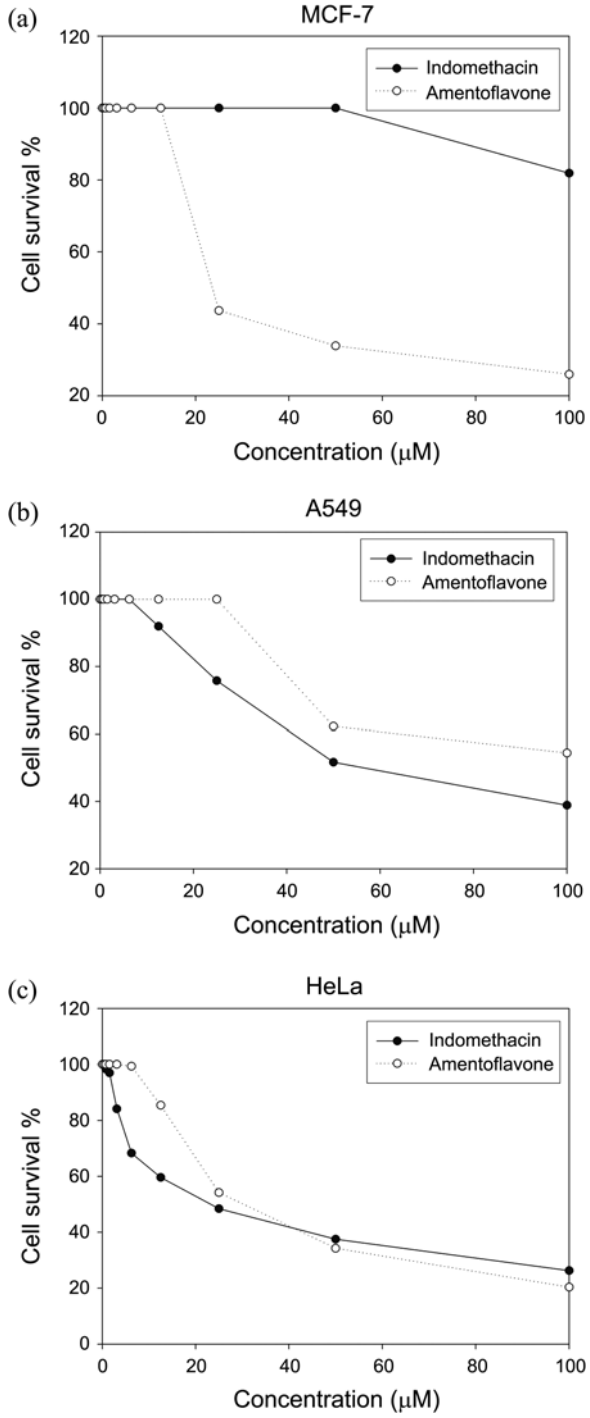

Figure 2. Cytotoxic activities of amentoflavone and indomethacin against three cancer cell lines. (a) MCF-7 human breast cancer cells. (b) A549 human lung cancer cells. (c) HeLa human cervical cancer cells.

Table 1. Cytotoxic activities of the hPPARã agonist indomethacin and amentoflavone

Compound	Cytotoxic activities (IC_{50}, μM)				
	MCF-7	A549	HeLa	MDA-MB-231	PC3
Indomethacin	> 100	50	25	> 100	> 100
Amentoflavone	25	100	20	> 100	> 100

Cytotoxicity against Normal Cells. The cytotoxicity of amentoflavone was investigated using the HaCa T human keratinocyte cell line. As shown in Figure 3, which depicts the survival (%) of HaCa T cells as a function of the concentration of the compound, amentoflavone was not cyto-

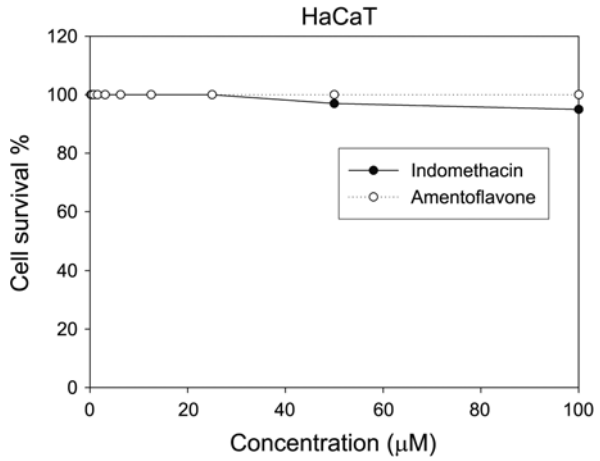

Figure 3. Cytotoxicity of three compounds against normal human keratinocytes (HaCa T).

toxic against at concentrations as high as 100 μM, indicating a toxicity profile suitable for an anticancer agent. Compared to Indomethacin which was used as a positive control, amentoflavone is less toxic at high concentration.

Amentoflavone Increases PTEN Expression Through Activation of hPPARγ. Although our data indicated that the hPPAR agonist amentoflavone exerted cytotoxic activity, this effect might be achieved via either hPPAR-dependent or -independent mechanisms. To verify the mechanism of action of amentoflavone in cancer cells, we investigated whether amentoflavone treatment affects the expression of hPPARγ using reverse transcription-polymerase chain reaction (RT-PCR) and real-time quantitative PCR (qPCR).[33]

First, using conventional RT-PCR to observed hPPARγ mRNA expression, we found that amentoflavone induced a substantial, concentration-dependent increase in hPPARγ mRNA levels in MCF-7 and HeLa cells following a 24-h exposure. qPCR analysis confirmed a marked increase in

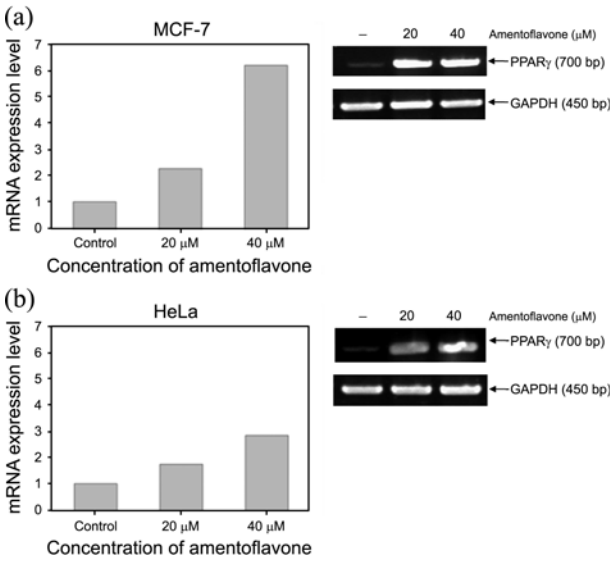

Figure 4. Analysis of amentoflavone-induced hPPARγ mRNA expression in cancer cells by RT-PCR and qPCR. (a) MCF-7 cells with amentoflavone. (b) HeLa cells with amentoflavone.

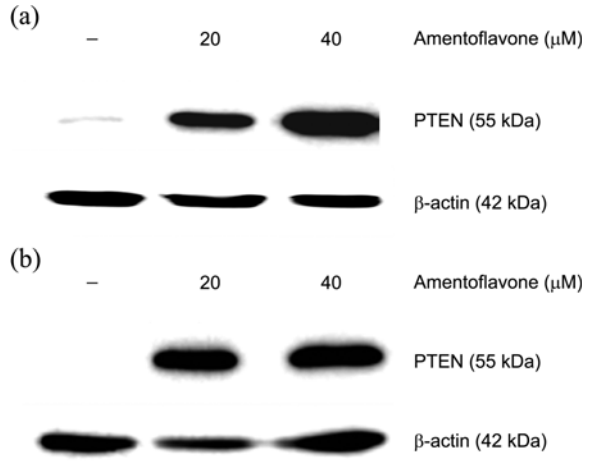

Figure 5. Western blots showing the concentration-dependent effects of amentoflavone on the expression levels of PTEN in two cancer cells. (a) MCF-7 cells treated with amentoflavone. (b) HeLa cells treated with amentoflavone.

hPPARγ mRNA levels in the two cell lines after a 24-h treatment with amentoflavone. The hPPARγ mRNA levels in each cancer cell line are depicted in Figure 4. These results demonstrate that hPPARγ expression in MCF-7 and HeLa cells was specifically stimulated by amentoflavone, and suggest that amentoflavone-induced cytotoxic activities are mediated by activation of hPPARγ in these two cancer cell lines.

As mentioned before, hPPARγ activation is due to the increasing of PTEN levels in cancer cells. To investigate whether PTEN levels are increased by amentoflavone, western blotting was performed for two cancer cells treated with amentoflavone. As shown in Figure 5, we confirmed that amentoflavone increases the expression levels of PTEN by hPPARγ activation.

Conclusion

It is well known that amentoflavone has many pharmacological activities. In this study, we investigated the biological activities of amentoflavone and showed that amentoflavone has strong cytotoxicities against MCF-7 and HeLa cancer cell lines. It has been reported that hPPARγ's effects on apoptosis and cell differentiation are beneficial in chemotherapy against a number of human cancers, including lung, breast, colon, cervical, and prostate cancers. One known mechanism of hPPARγ in cancer is related to its regulation of the tumor suppressor, PTEN. The results shown in this study verified that amentoflavone acts through activation of hPPARγ and hPPARγ activation is due to upregulation of PTEN to exert potent cytotoxic effects in human breast cancer and cervical cancers.

Acknowledgments. This work was supported by Priority Research Centers Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (2009-0093824) and by

National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (2011-0022873).

References

1. McKenna, N. J.; O'Malley, B. W. *Endocrinology* **2002**, *143*, 2461-2465.
2. Hansen, M. K.; Connolly, T. M. *Curr. Opin. Investig. Drugs* **2008**, *9*, 247-255.
3. Berger, J. P.; Akiyama, T. E.; Meinke, P. T. *Trends Pharmacol. Sci.* **2005**, *26*, 244-251.
4. Kersten, S.; Desvergne, B.; Wahli, W. *Nature* **2000**, *405*, 421-424.
5. Berger, J.; Moller, D. E. *Annu. Rev. Med.* **2002**, *53*, 409-435.
6. Chinetti-Gbaguidi, G.; Fruchart, J. C.; Staels, B. *Curr. Opin. Pharmacol.* **2005**, *5*, 177-183.
7. Chinetti, G.; Lestavel, S.; Bocher, V.; Remaley, A. T.; Neve, B.; Torra, I. P.; Teissier, E.; Minnich, A.; Jaye, M.; Duverger, N.; Brewer, H. B.; Fruchart, J. C.; Clavey, V.; Staels, B. *Nat. Med.* **2001**, *7*, 53-58.
8. Lee, C. H.; Olson, P.; Evans, R. M. *Endocrinology* **2003**, *144*, 2201-2207.
9. Bocher, V.; Pineda-Torra, I.; Fruchart, J. C.; Staels, B. *Ann. N. Y. Acad. Sci.* **2002**, *967*, 7-18.
10. Markt, P.; Petersen, R. K.; Flindt, E. N.; Kristiansen, K.; Kirchmair, J.; Spitzer, G.; Distinto, S.; Schuster, D.; Wolber, G.; Laggner, C.; Langer, T. *J. Med. Chem.* **2008**, *51*, 6303-63017.
11. Rosen, E. D.; Spiegelman, B. M. *J. Biol. Chem.* **2001**, *276*, 37731-37734.
12. Lehrke, M.; Lazar, M. A. *Cell* **2005**, *123*, 993-999.
13. Tontonoz, P.; Spiegelman, B. M. *Annu. Rev. Biochem.* **2008**, *77*, 289-312.
14. Krishnan, A.; Nair, S. A.; Pillai, M. R. *Curr. Mol. Med.* **2007**, *7*, 532-540.
15. Steck, P. A.; Pershouse, M. A.; Jasser, S. A.; Yung, W. K.; Lin, H.; Ligon, A. H.; Langford, L. A.; Baumgard, M. L.; Hattier, T.; Davis, T.; Frye, C.; Hu, R.; Swedlund, B.; Teng, D. H.; Tavtigian, S. V. *Nat. Genet.* **1997**, *15*, 356-362.
16. Herpin, A.; Lelong, C.; Favrel, P. *Dev. Comp. Immunol.* **2004**, *28*, 461-485.
17. Ye, J. *Biochem. Biophys. Res. Commun.* **2008**, *374*, 405-408.
18. Cazarolli, L. H.; Zanatta, L.; Alberton, E. H.; Figueiredo, M. S.; Folador, P.; Damazio, R. G.; Pizzolatti, M. G.; Silva, F. R. *Mini Rev. Med. Chem.* **2008**, *8*, 1429-1440.
19. Rajnaryana, K.; Sripalreddy, M.; Chalavadi, M. R.; Krishna, D. R. *Indian J. Pharmacol.* **2001**, *33*, 2-16.
20. Crozier, A.; Jaganath, I. B.; Clifford, M. N. *Nat. Prod. Rep.* **2009**, *26*, 1001-1043.
21. Butt, M. S.; Sultan, M. T. *Crit. Rev. Food Sci. Nutr.* **2009**, *49*, 463-473.
22. Manas, E. S.; Xu, Z. B.; Unwalla, R. J.; Somers, W. S. *Structure* **2004**, *12*, 2197-2207.
23. Lee, J. Y.; Kim, J. K.; Cho, M. C.; Shin, S.; Yoon, D. Y.; Heo, Y. S.; Kim, Y. *J. Nat. Prod.* **2010**, *73*, 1261-1265.
24. Lee, J. Y.; Jung, K. W.; Woo, E. R.; Kim, Y. *Bull. Korean Chem. Soc.* **2008**, *29*, 1479-1484.
25. Lee, J. Y.; Kim, J.-K.; Lee, S.; Lee, E.; Shin, S.; Jin, Q.; Yoon, D.-Y.; Woo, E. R.; Kim, Y. *Bull. Korean Chem. Soc.* **2012**, in press.
26. Silva, G. L.; Chai, H.; Gupta, M. P.; Farnsworth, N. R.; Cordell, G. A.; Pezzuto, J. M.; Beecher, C. W.; Kinghorn, A. D. *Phytochemistry* **1995**, *40*, 129-134.
27. Lee, H. S.; Cho, M. C.; Paek, T. W.; Choe, Y. K.; Kim, J. W.; Hong, J. T.; Myung, P. K.; Paik, S. G.; Yoon, D. Y. *J. Immunol. Methods* **2005**, *296*, 125-134.
28. Banerjee, T.; Valacchi, G.; Ziboh, V. A.; van der Vliet, A. *Mol. Cell. Biochem.* **2002**, *238*, 105-110.
29. Selvam, C.; Jachak, S. M. *J. Ethnopharmacol.* **2004**, *95*, 209-212.