The Anti-Inflammatory Effect of IH-901 in HT-29 Cells

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Abstract

20-O-(β-D-Glucopyranosyl)-20(S)-protopanaxadiol (IH-901) is one of the major metabolites of ginsenosides from Panax ginseng, and is suggested that IH-901 has been associated with various pharmacological and physiological activities. In this study, we demonstrate that IH-901 induced anti-inflammation in HT-29 human colon adenocarcinoma cells. Our results showed that IH-901 inhibited cell proliferation of HT-29 in a time- and dose-dependent manner. We also found that IH-901 was significantly decreased expression of iNOS compared with non-treated. We observed effect of IH-901 related with inflammatory genes using by cDNA microarray. We were known that the 34 inflammatory genes such as E2F, CDK6, TNF-α, and PKC were down-regulated. Thus, these results suggest that IH-901 may have a potential preventive factor to improving cancer induced by chronic inflammation.

Keywords: IH-901, Inducible Nitric Oxide Synthase (iNOS), Nitric Oxide (NO), Inflammation, HT-29 human colon adenocarcinoma cells

Panax ginseng C.A. Meyer (ginseng) has been used as a traditional medicine in Asian Countries for more than 2,000 years, and is now used numerous countries for the treatment of various diseases. The major components of ginseng are ginsenosides which have steroid-like structure, and are divided into three types according to their aglycones; protopanaxadiol, protopanaxatriol and oleanoic acid ginsenosides. Recently, it was reported that ginsenosides of protopanaxadiol (IH-901) by intestinal bacteria in human and rat. IH-901 is discovered as one of the major metabolites in urine and blood after administration of ginseng extract. It is non-toxic, and suppresses uptake of glucose by tumor cells, exhibits anti-metastatic effects and reverses multidrug resistance in tumor cells. Lee, S. J. et al., IH-901 induces apoptosis and suppresses growth of leukemia cells by activation of caspase-3 protease, which occurs via mitochondrial cytochrome C release independently of Bcl-2 modulation.

Nitric Oxide (NO) is synthesized from the oxidation of guanino group of L-arginine by diverse NADPH-dependent enzymes called nitric oxide synthase (NOS). The three difference isoforms of NOS are neuronal NOS (nNOS), endothelial NOS (eNOS) and inducible NOS (iNOS). nNOS and eNOS are generally expressed neurons and endothelial cells depended on calcium and calmodulin, whereas iNOS is induced by inflammatory cytokines such as tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ), interleukin (IL)-1 and lipopolysaccharide (LPS) instead of calcium. Recent some studies suggested that increased expression and activation of iNOS are associated with development of colon cancer. For example, Ambs, S. et al., suggested that iNOS expressed low level of activity in the colon normal tissue contiguous to the neoplastic lesions, but they found very high level in the colon adenocarcinoma. According to study of Rao, C. V. et al., 20(S)-protopanaxadiol inhibited iNOS induced by azoxymethane (AOM) in colonic aberrant crypt foci. Also, it is known that over-production NO by iNOS promotes inflammation, cancer progress, tumor angiogenesis and activation of proto-oncogene, and inhibits apoptosis.

It has been studied that ginsenosides down-regulated expression of iNOS and COX-2 and suppressed NO level. The main focus of this study was investigated anti-inflammatory effect of IH-901 using by HT-29 human colon adenocarcinoma cells.

Inhibition of Proliferation on IH-901 in HT-29 Cells

We investigated proliferation inhibitory effect of IH-901 in HT-29 human colon adenocarcinoma cells using by MTT assay. HT-29 was treated with IH-901 at various concentrations from 20 to 320 µM and incubated 12 hrs. As shown in Figure 1, the results showed that IH-901 inhibits significantly cell growth in a dose- and time-dependent manner.
Inhibition of iNOS mRNA on IH-901 in HT-29 Cells

To measure expression of iNOS mRNA, HT-29 cells were treated with IH-901 at 20, 80 and 320 µM for 12 hrs. After treatment, we investigated the expression of iNOS by RT-PCR. As shown in Figure 2, expression of iNOS in non-treated cells was increased, whereas expression of iNOS mRNA in response to IH-901 was significantly decreased in a dose-dependent manner. Compared to control, level of iNOS mRNA treated with IH-901 at 20, 80 and 320 µM was decreased by 53%, 57% and 81%, respectively (Figure 2).

Gene Expression Profiling in HT-29 Cells

To find gene associated with effect of IH-901 in HT-29 cells, we investigated using cDNA microarray. Gene expression profiles of interest were up-regulated and down-regulated in experimental group compared with non-treated group. The up- and down-regulated genes are listed in Table 1 and 2. Gene expression Profiles were showed up-regulated genes of 26 including major histocompatibility complex (MHC) class I, MHC class II and interleukin 2 receptor (Table 1). Also, Table 2 was demonstrated that 34 genes such as tumor necrosis factor (TNF), cAMP responsive element binding protein (CREB), and protein kinase C were down-regulated. Z scores analysis of ginseng- and control groups was performed and individual gene was plotted. To examine the relationships between ginseng- and control group, we used a hierarchical clustering and revealed up- and down-regulated genes on the basis of similar expression patterns, and the data is presented in a matrix format (Figure 3 and 4). The expression level of genes was visualized in color related with its median expression level across all samples. Red represented up-regulated expression, green represents down-regulated expression and block represented the median expression level.

Discussion

Ginsenosides are major metabolite of Panax Ginseng C.A. Meyer, and these have been used in traditional medicine for treatment of many disorders. Also, it is known that ginsenosides have biological and pharmacological effects such as anti-inflammation, anti-cancer, anti-oxidation, anti-stress and immunomodulation. IH-901 is one of novel ginsenosides metabolite formed by intestinal bacteria after oral administration.
administration of ginseng extract, and it has been
supposed that IH-901 is important form of proto-
panaxadiol saponin absorbed from the intestine 3,15.
Several researchers reported that IH-901 has different
pharmacological activities such as inhibition of pro-
liferation, metastasis and invasion and induction of
apoptosis in cancer16-20. For example, IH-901 induced
apoptotic cell death through down-regularion of
apoptosis related protein such as c-Myc and Cyclin
D1, and inhibited proliferation in tumor cell20.

The purpose of this study is to determine effect of
IH-901 related with inflammatory response in HT-29
human colon adenocarcinoma cells.

In our experimental present study, we also observed
inhibitory effect of IH-901 on the growth of HT-29
human colon adenocarcinoma cells using MTT assay.
We had shown that IH-901 was decreased prolif-
eration of cancer cell in a time- and dose-dependent
manner (Figure 1).

iNOS, pro-inflammation enzyme, is involved in
regulation of physiological and pathological pro-
cesses responsible for inflammation and cancer10.
Many studies reported that increased levels of iNOS
have been discovered in various cancers and inhibi-
tion of iNOS induces apoptotic cell death in human
cancer cells16. Also, over-production of NO by iNOS
induces tumorigenic process including DNA damage,
lipid peroxidation and promotes neovascularization in
some cancer16,17.

Previous studies had reported that ginsenosides
have effect of anti-inflammation. Oh, G. S. et al.10,
studied that protopanaxatriol blocked activation of
LPS induced iNOS and COX-2 in RAW 264.7 mu-
rine macrophage cells via glucocorticoid receptor-
independent pathway. Also, Lee, S. J. et al.23, demon-
strated that IH-901 inhibited activation of iNOS and
production of NO by promotion of oxygenase-1 (HO-
1) expression. Therefore, to investigate anti-inflam-

Table 1. Up-regulated gene expression in HT-29 cells.

<table>
<thead>
<tr>
<th>Up-regulated gene</th>
<th>Abb</th>
<th>Z ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apoptosis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>deleted in colorectal cancer</td>
<td>DCC</td>
<td>1.51</td>
</tr>
<tr>
<td>deoxyribonuclease</td>
<td></td>
<td>, lysosomal adenine A2a receptor</td>
</tr>
<tr>
<td>Cell cycle</td>
<td></td>
<td></td>
</tr>
<tr>
<td>budding uninhibited by benzimidazoles 1 (yeast homolog), beta; MAD3-like protein kinase mRNA</td>
<td>BUB1B</td>
<td>2.18</td>
</tr>
<tr>
<td>Immune</td>
<td></td>
<td></td>
</tr>
<tr>
<td>interleukin 11</td>
<td>IL-11</td>
<td>1.99</td>
</tr>
<tr>
<td>major histocompatibility complex, class I, A</td>
<td>HLA-A</td>
<td>2.18</td>
</tr>
<tr>
<td>major histocompatibility complex, class II, DQ beta 1</td>
<td>HLA-DOB1</td>
<td>2.52</td>
</tr>
<tr>
<td>interleukin 2 receptor, alpha</td>
<td>IL2RA</td>
<td>2.75</td>
</tr>
<tr>
<td>ferritin, heavy polypeptide 1</td>
<td>FTH1</td>
<td>3.33</td>
</tr>
<tr>
<td>Signal Transduction</td>
<td></td>
<td></td>
</tr>
<tr>
<td>chemokine (C-C motif) receptor 5</td>
<td>CCR5</td>
<td>2.22</td>
</tr>
<tr>
<td>thyroid stimulating hormone receptor</td>
<td>TSHR</td>
<td>3.65</td>
</tr>
<tr>
<td>Transcription</td>
<td></td>
<td></td>
</tr>
<tr>
<td>protein phosphatase 1, regulatory subunit 10</td>
<td>PPP1R10</td>
<td>1.55</td>
</tr>
<tr>
<td>similar to latent transforming growth factor beta binding protein 1</td>
<td>TGFBI</td>
<td>1.65</td>
</tr>
<tr>
<td>CCAAT/enhancer binding protein (C/EBP), delta</td>
<td>CEBPD</td>
<td>1.86</td>
</tr>
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<td>excision repair cross-complementing rodent repair deficiency, complementation group 2</td>
<td>ERC2</td>
<td>1.89</td>
</tr>
<tr>
<td>TEA domain family member 4; RTEF1; TEF-3</td>
<td>TEAD4</td>
<td>1.89</td>
</tr>
<tr>
<td>nuclear transcription factor Y, alpha; Hap2</td>
<td>NFYA</td>
<td>3.11</td>
</tr>
<tr>
<td>POU domain, class 6, transcription factor 1</td>
<td>POU6F1</td>
<td>4.09</td>
</tr>
<tr>
<td>Others</td>
<td></td>
<td></td>
</tr>
<tr>
<td>prodynorphin</td>
<td>PDYN</td>
<td>1.52</td>
</tr>
<tr>
<td>ubiquitin specific protease 6 (Tre-2 oncogene)</td>
<td>USP6</td>
<td>1.78</td>
</tr>
<tr>
<td>SH3-domain binding protein 2</td>
<td>SH3BP2</td>
<td>1.84</td>
</tr>
<tr>
<td>phosphogluconate dehydrogenase</td>
<td>PGD</td>
<td>1.85</td>
</tr>
<tr>
<td>T-cell receptor, alpha (V, D, J, C)</td>
<td>TRA@</td>
<td>1.87</td>
</tr>
<tr>
<td>similar to protein tyrosine phosphatase, non-receptor type 13</td>
<td>PTPN13</td>
<td>1.88</td>
</tr>
<tr>
<td>damage-specific DNA binding protein 1 (127 kD)</td>
<td>DDB1</td>
<td>2.04</td>
</tr>
<tr>
<td>tyrosine kinase 2</td>
<td>TK2</td>
<td>2.14</td>
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matory effect of IH-901, we monitored expression of iNOS in HT-29 cells. RT-PCR analysis exposed that IH-901 inhibited expression of iNOS (Figure 2).

Also, we observed gene expression profiles related with inhibitory effect of IH-901 on colon cancer using by cDNA microarray. In this study, several genes were up- or down-regulated, and these genes were arranged according to their functions (Table 1 and 2). These genes were categorized according to their functions; apoptosis, cell cycle, immune, signal transduction, transcription and others. In DNA microarray results, we are known that genes connected with inflammation such as Tumor Necrosis Factor, Alpha-Induced Protein 1 (TNFAIP1), interleukin 10, E2F transcription factor 3 (E2F3), MAX and cAMP Responsive Element Binding Protein-like (CREPB1) were down-regulated in HT-29 cells, and genes related with anti-inflammation such as deleted colorectal cancer (DCC), major histocompatibility complex, class I-A (HLA-A), T-cell receptor alpha (V, D, J, C) (TCA@) and interleukin 2 receptor alpha (IL2RA) were up-regulation.

Our results demonstrated that IH-901 significantly inhibited proliferation in HT-29 human colon adenocarcinoma cells. Also, IH-901 possesses anti-inflammatory capacity, resulting in down-regulation of iNOS expression and NO production in HT-29 cells. And, we were known that IH-901 inhibited pro-inflammatory factors based on results of cDNA microarray. In conclusion, we suggested that effect of IH-901 may be important in the understanding of a novel mechanism for the anti-inflammatory action.

Table 2. Down-regulated gene expression in HT-29 cells.

<table>
<thead>
<tr>
<th>Down-regulated gene</th>
<th>Abb</th>
<th>Z ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Anti-apoptosis</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Presenilin 1</td>
<td>PSEN1</td>
<td>−1.67</td>
</tr>
<tr>
<td>myeloid cell leukemia sequence 1 (BCL2-related)</td>
<td>MCL1</td>
<td>−1.78</td>
</tr>
<tr>
<td>interleukin 10</td>
<td>IL-10</td>
<td>−2.55</td>
</tr>
<tr>
<td>synuclein, alpha (non A4 component of amyloid precursor)</td>
<td>SNCA</td>
<td>−3.24</td>
</tr>
<tr>
<td><strong>Cell cycle</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cyclin B1</td>
<td>CCNB1</td>
<td>−1.66</td>
</tr>
<tr>
<td>cyclin D2</td>
<td>CCND2</td>
<td>−1.82</td>
</tr>
<tr>
<td>cyclin-dependent kinase 6</td>
<td>CDK6</td>
<td>−2.57</td>
</tr>
<tr>
<td>E2F transcription factor 3</td>
<td>E2F3</td>
<td>−4.26</td>
</tr>
<tr>
<td><strong>Immune</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>tumor necrosis factor, alpha-induced protein 1</td>
<td>TNFAIP1</td>
<td>−2.88</td>
</tr>
<tr>
<td>similar to mitogen-activated protein kinase kinase kinase 2</td>
<td>MAP4K2</td>
<td>−3.72</td>
</tr>
<tr>
<td><strong>Transcription</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>interferon regulatory factor 1</td>
<td>IRF1</td>
<td>−1.69</td>
</tr>
<tr>
<td>sterol regulatory element binding transcription factor 1</td>
<td>SREBTF-1</td>
<td>−3.82</td>
</tr>
<tr>
<td>MAX protein; helix-loop-helix zipper protein</td>
<td>MAX</td>
<td>−5.13</td>
</tr>
<tr>
<td><strong>Signal Transduction</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mitogen-activated protein kinase 10</td>
<td>MAPK10</td>
<td>−1.60</td>
</tr>
<tr>
<td>mitogen-activated protein kinase 11</td>
<td>MAPK11</td>
<td>−1.72</td>
</tr>
<tr>
<td>anti-Mullerian hormone receptor, type II</td>
<td>AMHR2</td>
<td>−1.80</td>
</tr>
<tr>
<td>cAMP responsive element binding protein-like 1</td>
<td>CREBP1</td>
<td>−2.37</td>
</tr>
<tr>
<td>small inducible cytokine A5; RANTES</td>
<td>CCL5</td>
<td>−2.73</td>
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<tr>
<td>regulator of G-protein signaling 1</td>
<td>RGS1</td>
<td>−3.28</td>
</tr>
<tr>
<td><strong>Others</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ribosomal protein S25</td>
<td>RPS254</td>
<td>−1.50</td>
</tr>
<tr>
<td>milk fat globule-EGF factor 8 protein; breast epithelial antigen BA46 mRNA</td>
<td>MFGE8</td>
<td>−1.54</td>
</tr>
<tr>
<td>glutathione synthetase</td>
<td>GSS</td>
<td>−1.71</td>
</tr>
<tr>
<td>insulin-like growth factor binding protein 3</td>
<td>IGFBP3</td>
<td>−1.80</td>
</tr>
<tr>
<td>phosphodiesterase I/nucleotide pyrophosphatase 2 (autotaxin)</td>
<td>ENPP2</td>
<td>−1.95</td>
</tr>
<tr>
<td>surfactant, pulmonary-associated protein C</td>
<td>SFTPC</td>
<td>−1.96</td>
</tr>
<tr>
<td>serine protease inhibitor, Kunitz type, 2</td>
<td>SPINT2</td>
<td>−1.99</td>
</tr>
<tr>
<td>integrin, beta 3 (platelet glycoprotein IIIa, antigen CD61)</td>
<td>ITGB3</td>
<td>−2.05</td>
</tr>
<tr>
<td>protein kinase C</td>
<td>PRKC</td>
<td>−2.11</td>
</tr>
<tr>
<td>defensin, alpha 6, Paneth cell-specific</td>
<td>DEFA</td>
<td>−2.81</td>
</tr>
<tr>
<td>integrin, beta 8</td>
<td>ITGB8</td>
<td>−3.31</td>
</tr>
<tr>
<td>P glycoprotein 1/multiple drug resistance 1; MDR1</td>
<td>ABCB1</td>
<td>−3.72</td>
</tr>
</tbody>
</table>
Methods

Reagents

IH-901 was a product of Ambo Institute (Seoul, South Korea). IH-901 was dissolved in Dimethyl sulfoxide (DMSO) to a concentration of 0.1%. 3-(4, 5-dimethylthiazol 1-yl)-2, 5-diphenyltetrazolium bromide (MTT), Sulfanilamide, N-(1-Naphthyl) ethylenediamine dihydrochloride and Phosphoric acid were purchased from Sigma Aldrich (Steinheim, Germany). iNOS and GAPDH were obtained from...
Bioneer Corporation (Daejeon, South Korea). RPMI-1640, Fetal Bovine Serum (FBS) was from Wel-GENE (Daegu, South Korea).

Cell Culture

HT-29 human colon adenocarcinoma cells were cultured in the RPMI-1640 containing 10% heat-inactivated fetal bovine serum, 2% 200 mM L-glutamine and 2% penicillin and streptomycin solution (10,000 units/mL penicillin and 10 mg/mL streptomycin). The cultures were maintained at 37°C in humidified 5% CO2.

MTT Assay

Cells were plated in 96-well plates at 1.0 x 10^4 cells/well in a 200 µL medium for cell viability measurement. 24 hrs later, cells were washed and changed the fresh medium, and they were treated with IH-901 at 40, 80, 160 and 320 µM for 12 hrs. After treatment, 10 µL of MTT stock solution (0.5 mg/mL) was added to each well and incubated at 37°C for 3 hrs. The formazan product was dissolved in 200 µM DMSO, and shook for 10 min. After 10 min, it measured optical density using the Microplate Leader (Molecular Devices, Spectra Max, Union City, CA, USA) at 540 nm.

RNA Preparation

Cells were seeded in 6-well plate at 2.5 x 10^4 cells/well in a 2 mL medium for RT-PCR and cDNA microarray analysis. 24 hrs later, cells were washed and changed the fresh medium, and they were treated with various concentration of IH-901 for 12 hrs. RNA isolated using 1 mL of TRIzol reagent (Invitrogen, CA, USA). The RNA pellets were dried and dissolved in Diethylpyrocarbonate (DEPC) treated water (Invitrogen, CA, USA) and incubated at 55 to 60°C for 30 minutes. The total RNA was quantified using the NanoDrop ND-1000 Spectrophotometer (NanoDrop, DE, USA).

RT-PCR

After extraction and quantification of RNA, RT-PCR reaction was performed as described in the methodology of arraying was based on the procedures of DeRisi, J. et al. 20.

Human cDNA Microarray

A human cDNA Microarray was primarily derived from a commercially available master set of approximately 15,000 human verifies-sequences (Research Genetics, Inc, AL, USA). The human cDNA clone set was sorted for a list of genes (1,152 elements) representing families correlated with differentiation, development, proliferation, transformation, cell cycle progression, immune response, transcription and transcription factors, oncogenes, and molecules related to cell growth and maintenance. PCR-amplified cDNAs were spotted on nylon membranes. The general methodology of arraying was based on the procedures of DeRisi, J. et al. 20.

cDNA Radiolabeling

After quantification, 2-3 µg of total RNAs prepared from blood was used for each sample for adjustment of different diagnosis. To synthesize 33P-labeled cDNAs, quantified RNA were labeled in a reverse transcription reaction containing 8 µL of 5X first standard PCR buffer (Invitrogen, Milano, Italy), 4 µL of 24-mer poly dT primer (Invitrogen, CA, USA), 4 µL of 0.1 M Dithiothreitol (DTT) (Invitrogen, Milano, Italy), 1 µL of RNaseOUT (LIT), 1 µL of DEPC treated water and 5 µL of 3,000 Ci/mmol a-33P dCTP to a final volume of 20 µL. Two µL of Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase (Invitrogen, CA, USA) was then added and the samples were incubated for 30 minutes at 42°C, followed by the addition of 2 µL of M-MLV reverse transcriptase and another 30 minutes at 42°C. 2.5 µL of 0.5 M Ethylene-Diamine-Tetra-Acetic Acid (EDTA) was added to chelate divalent cations. After the addition of 5 µL 0.1 M NaOH, the samples were incubated at 65°C for 30 minutes to hydrolyze remaining RNA. Following the addition of 12.5 µL of 1 M Tris HCl (pH 8.0), the samples were purified using purification columns (Bio-rad, CA, USA). After purification, each sample was put in 4 mL of hybridization buffer and reacted with nylon membrane during 24 hrs.

Hybridization and Scanning

cDNA microarrays were pre-hybridized in hybridization buffer containing 4 mL Microhyb and 10 µL of 8 mg/mL poly dA (Invitrogen, CA, USA). Both Human Cot 1 and poly dA were denatured at 95°C for 5 min prior to use. After 4 hrs of pre-hybridization
at 42°C, approximately 10^7 cpm/mL of heat-denatur-
ed (95°C, 5 minutes) probe was added, and incubation
continued at 42°C for 17 hrs. Hybridized arrays were
washed 3 times in 2X SCC and 0.1% SDS for 15
minutes at room temperature. The microarrays were
exposed to phosphorimager screens for 1-5 days, and
the screens were then scanned using a FLA-8000 (Fuji
Photo Film Co., Japan) at 50 µm resolution.

Data Analysis

Microarray images were trimmed and rotated for
further analysis using L-Processor system (Fuji Photo
Film Co., Japan). Gene expression of each micro-
array was captured by the intensity of each spot
produced by radioactive isotopes. Pixels per spot
were counted by Arraygauge (Fuji Photo Co., Japan)
and exported to Microsoft Excel (Microsoft, WA,
USA).

Statistical Methods

Data analysis of microarray was used by Microsoft
Excel (Microsoft, WA, USA). To normalize each
membrane or each gene across membranes, we used
global normalization basic methods, which calculate
the mean or median of the signal intensities of each
individual experimental data set and then calculate
the mean of the means (or grand mean) for all of
the included experiments. Each individual data set is then
mathematically adjusted such that the mean of that
data set equals the calculated grand mean. Raw
intensity values obtained from the previous step were exported to EXCEL and normalized with Z trans-
formation by subtracting with each average of gene
expression raw data, log values and Z scores were averaged by using the mean ± S.D.

Hierarchical clustering was determined using soft-
ware programs developed at Stanford University22.
And cluster analysis was performed on Z-transformed microarray data by using two programs avail-
able as shareware from Michael Eisens’ laboratory
(http://rana.lbl.gov). Clustering of changed in gene
expression was determined by using public domain cluster
based on pairwise complete-linkage cluster
analysis. Gene expression raw data, log values and Z
scores were averaged by using the mean ± S.D.

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0002). This work was partially supported by the
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grant funded by the Korea Government (MOST) (No.
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ginseng saponin, activates estrogen receptor in human
breast carcinoma MCF-7 cells. J Steroid Biochem Mol

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lipopolysaccharide/interferon-gamma stimulated BV-

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presses phorbol ester-induced matrix metallopro-
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protein-1 and mitogen-activated protein kinase signa-
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