DNA Microarray Analysis of Estrogen Responsive Genes in Ishikawa Cells by Glabridin

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ABSTRACT: Based on a previous study, glabridin displayed a dose-dependent increase in estrogenic activity and cell proliferative activity in Ishikawa cells. However, when treated in combination with 17β-E_2, synergistic estrogenic effect was observed but without the same synergistic increase in cell proliferative effect. This study aimed to identify the estrogen and nonestrogen-regulated activities induced by glabridin and in combination with 17β-E_2 in comparison with 17β-E_2. The results showed that 10 µM glabridin and the combination treatment of 100 nM glabridin with 1 nM 17β-E_2 regulated both the genomic and nongenomic estrogen pathways to possibly provide benefits of estrogens in cardiovascular, circulatory, and vasculature systems. Meanwhile, the combination of 100 nM glabridin with 1 nM 17β-E_2 seems to be more suitable to be used as an estrogen replacement. Finally, the results of this study have added on to the present knowledge of glabridin's function as a phytoestrogen and suggested new ideas for the usage of glabridin.

KEYWORDS: Glabridin, microarray, Ishikawa cells

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Background

Licorice root of the *Glycyrrhiza* plant has been used for medicinal purposes for more than 4000 years.[1] The plant is widely used in Asia as herbal medicine, flavoring agent, and sweetener.[2] As a medicinal plant, licorice root has been used for menopausal symptoms in women.[3,4] The estrogenicity of licorice has been attributed to the presence of phytoestrogens such as glabridin, a prenylated isoflavonoid.[5,6]

According to Botta et al,[7] prenylation of an isoflavonoid modulates its response toward estrogen receptors (ER). In 2000, Tamir et al showed that glabridin expressed ER-dependent growth-promoting effect at 10 to 10 mM and ER binding activity in T47D human breast ductal carcinoma cells with an IC_{50} of 5 mM. The ER binding activity of glabridin was found to be similar to values of other known phytoestrogens, such as genistein, which is 10^4 lower than that of 17β-E_2. At the same time, glabridin also had ER-independent antiproliferative activity at concentrations of >15 mM. Tamir et al[6] also showed that glabridin activated creatine kinase (CK) activity in skeletal and cardiovascular tissues while increasing uterine weight of rats displaying the potential of glabridin to bring out the positive effect of 17β-E_2 in the bone, cardiovascular tissues, and the uterus. This selective ER modulator activity of glabridin showed that it acts as a phytoestrogen and suggested that glabridin may serve as a natural estrogen agonist in preventing the symptoms and diseases associated with estrogen deficiency.

Later studies have shown more similarities between glabridin and 17β-E_2. In 2004, Somjen et al discovered that both glabridin and 17β-E_2 stimulated DNA synthesis in human endothelial cells and exhibited biphasic effect on proliferation of human vascular smooth muscle cells. Meanwhile, in animal studies, both glabridin and 17β-E_2 also stimulated the activity of CK in the aorta and left ventricle of the heart of both intact and ovariectomized female rats.[8] Later on, Choi[9] discovered that glabridin (1-10 µM) significantly increased alkaline phosphatase (ALP) activity, cell proliferation, collagen content, and osteocalcin secretion in MC3T3-E_1 osteoblast cells, supporting its use for the prevention of osteoporosis and inflammation in the bone. The increase in ALP activity and collagen was thought to be related to estrogenic effects as they were inhibited by 1 µM tamoxifen.[9]

In the previous study,[10] it was found that glabridin displayed a dose-dependent increase in ALP activity from 1 pM to a maximum induction at 10 µM. At 10 µM, glabridin induced cell proliferation of 25.04 ± 3.33% increase from the untreated control. When treated in combination with 17β-E_2, synergistic effect was observed and the greatest synergy occurred at the combination treatment of 100 nM glabridin with 1 nM 17β-E_2 (synergy quotient [SQ] of 1.217). At this combination treatment, there was a slight decrease in cell proliferation (16.98 ± 3.76% increase from control) compared with 1 nM 17β-E_2 (19.67 ± 3.42% increase), although it was not significant. However, when compared with 10 nM 17β-E_2 (28.79 ± 1.26% increase), the combination treatment of 100 nM glabridin with 1 nM 17β-E2 had a significantly lowered induction of cell proliferation.[10] Based on the results from the previous study, 5 different treatments were chosen for further

downstream study. The treatments selected were the untreated control, 10 µM glabridin, 100 nM glabridin, 1 nM 17β-E_2, and the combination treatment of 100 nM glabridin with 1 nM 17β-E_2. The cells were treated for 72 hours and the RNA was extracted for microarray and quantitative reverse transcription polymerase chain reaction (RT-qPCR) analysis to evaluate on the estrogen responsive genes and possible activities.

Materials and Methods

Chemicals

Minimum Essential Medium (MEM), 17β-E_2, and dextran-coated charcoal (DCC) were purchased from Sigma, USA. Fetal bovine serum (FBS), fetal calf serum (FCS), antibiotic-antimycotic, and L-glutamine were obtained from Gibco, USA. Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F-12) was sourced from Nacalai Tesque, Japan. Glabridin was purchased from Wako, Japan. Phosphate-buffered saline was from OXOID, Lenexa, KS, USA. Thiazolyl blue tetrazolium bromide (MTT) was obtained from Amresco, USA. Premarin was obtained from Wyeth Pharmaceuticals, USA. Receptor cofactor assay was purchased from Cosmo Bio Co., Ltd, Japan. All other chemicals were purchased from Merck, Germany.

Cell culture and maintenance of Ishikawa cells

Ishikawa cells (99040201; European Collection of Authenticated Cell Cultures [ECACC]), which are human endometrial adenocarcinoma cells, were maintained in MEM supplemented with 10% FBS, 1% antibiotic-antimycotic, and 1% L-glutamine. Cells were passaged twice weekly. Before 2 days of the start of the experiment, near-confluent cells were changed to an estrogen-free basal medium (EFBM). The EFBM consists of DMEM/F-12 media supplemented with 5% DCC-stripped FCS, 1% antibiotic-antimycotic, and 1% L-glutamine. After 24 hours, the cells were harvested with 0.25% EDTA-trypsin and seeded in 96-well flat-bottomed microtiter plates, in 100 µL of EFBM per well.

Preparation of test compounds

All the test compounds were dissolved in ethanol (EtOH) and aliquoted accordingly. Prior to use, the test compounds were diluted appropriately with EFBM until the final concentration of EtOH was at 0.1% (v/v). All test compounds were stored at −20°C.

Treatment

Approximately 1.0×10^6cells was seeded into T-75 flasks in 10 mL of EFBM. After 24 hours, when the cells had attached, the EFBM was replaced with 10 mL of EFBM containing test compounds at the appropriate concentrations. After addition of test compounds, the cells were incubated at 37°C in a 5% CO_2 incubator for 72 hours. All experimental conditions were assayed in triplicate. The test compounds treated to the cells were as follows: untreated control (MEM with 0.1% EtOH), 10 µM glabridin, 100 nM glabridin, 1 nM 17β-E_2, and 100 nM glabridin and 1 nM 17β-E_2. All treatments were performed in triplicate.

RNA extraction

RNA extraction was performed using the QIAshredder spin column (Qiagen, USA) and RNeasy Mini Kit (Qiagen, USA) as per manufacturer's protocol. The eluted RNA was quantified using DU 730 Life Science UV/Vis Spectrophotometer (Beckman Coulter, USA). Only RNA with an A_{260}/A_{280} ratio of 1.9 to 2.1 and A_{260}/A_{230} ratio of 1.7 to 2.1 was used for subsequent experiments.[11]

Deoxyribonuclease treatment

The extracted RNA was subjected to deoxyribonuclease (DNase) treatment using the RapidOut DNA Removal Kit (Thermo Scientific, USA) as per manufacturer's protocol. Briefly, 10 µg of RNA in nuclease-free water was added to 5 µL of 10× DNase buffer with $MgCl_2$, 2.5 µL of RNase-free DNase I and adjusted with nuclease-free water up to 50 µL.

Microarray analysis

The extracted messenger RNA (mRNA) (250 ng) was converted to complementary DNA (cDNA) using GeneChip 3′ IVT Express Kit (Affymetrix, USA) as per manufacturer's protocol (Affymetrix, USA). Next, antisense RNA (aRNA) amplification was performed using the GeneChip 3′ IVT Express Kit (Affymetrix, USA). After aRNA synthesis, the aRNA was placed on ice and quantified immediately for fragmentation. Hybridization was performed using the GeneChip PrimeView array (Affymetrix, USA) in the Affymetrix GeneChip Hybridization Oven 645 (Affymetrix, USA). After washing and staining, the probe arrays were scanned using the Affymetrix GeneChip Scanner 3000. A total of 3 biologically independent assays were performed.[12]

Microarray data analysis

For the analysis, each probe set was analyzed as an individual entity, based on its Affymetrix ID number (Affy ID), regardless of the multiplicity of probe sets representing any given gene product and were considered as representing an individual gene until the completion of the analysis.[12] For analysis of gene expression, GeneSpring GX 12.6.1 Software (Agilent, USA) was used. The microarrays were analyzed via robust

multiarray analysis. The normalized probe sets were subjected to analysis of variance, unequal variance (Welch) statistical test, Tukey post hoc with asymptomatic *P* value computation, and Benjamini-Hochberg false discovery rate (FDR) for multiple testing correction. The entity list obtained from the different treatments was then filtered using volcano plots. The statistical test chosen for independent comparison of the treatments with the untreated control was *t* test unpaired unequal variance (Welch) with asymptomatic *P* value computation as well as Benjamini-Hochberg FDR for multiple testing correction. The fold change cutoff was set as 1.5, and the *P* value cutoff was set as .05.

Primer design

Primers specific for the gene of interest was designed using Primer3 software (http://biotools.umassmed.edu/bioapps/primer3_www.cgi). The details of primers designed are listed in Table S1 for the genes of interest and Table S2 for the candidate reference genes.

Quantitative reverse transcription polymerase chain reaction

The mRNA extracted was converted to cDNA using Omniscript Reverse Transcriptase (Qiagen, USA), according to manufacturer's protocol. The RT-qPCR was performed using the qPCRBIO SyGreen Mix Lo-ROX 2X master mix (PCR Biosystems, UK) following manufacturer's protocol and conditions, as shown in Table S3. All experiments were performed using a Mastercycler RealPlex (Eppendorf, USA). No RT and no DNA template control were included to ensure that the samples were not contaminated with either genomic DNA or primer dimers. All RT-qPCRs were run in triplicate.

RT-qPCR data analysis

Quantification cycle (Cq) values for all the genes from all the samples were compiled and imported to qbase^{PLUS} data analysis software (Biogazelle, Belgium). Each gene was adjusted for amplification efficiency according to their own standard curve and was scaled to the untreated control. The lower and upper boundaries of Cq range for positive controls were 5 and 35, respectively. The acceptable replicate variability (difference in Cq) was <0.5. For normalization, the genes of interest were normalized with the 2 reference genes that were chosen from 8 different housekeeping genes. For statistical analysis, unpaired *t* test was performed with multiple correction testing on the RT-qPCR data after normalization with the reference genes.

Selection of a suitable reference gene

The Cq values collected across all samples were used to evaluate the stability of the candidate reference genes. Several programs were used for the identification of suitable reference genes used in this study[13]: (1) BestKeeper[14] (http://www.gene-quantification.com/bestkeeper.html), (2) NormFinder[15] (http://moma.dk/normfinder-software), and (3) geNorm[16] in qbase^{PLUS} (Biogazelle, Belgium). The suitable reference genes were selected based on the results generated from all 3 programs.

Results and Discussions

Microarray analysis showed that the treatment of 10 μM glabridin was relatively similar to 1 nM 17β-E2

Based on the results from ALP and MTT assay from a previous study,[10] glabridin exhibited weak estrogenic activity by increasing ALP activity and it also increased cell proliferation. However, in the presence of 17β-E_2, the activity of glabridin differed from other isoflavones in that it decreased cell proliferation while increasing ALP activity. The presence of synergism suggests that mechanisms of action of glabridin and 17β-E_2 are likely to occur at different sites and/or through different pathways. As this is the first study to investigate the use of glabridin and 17β-E_2 as an estrogen replacement therapy (ERT) agent, additional work is required to determine the activities of each of the drugs alone before activities of their synergistic effects can be determined.[17] Hence, microarray was used to study the mechanisms and therapeutic effects exhibited by glabridin and the combination treatment in comparison with 17β-E_2. Five different treatments were chosen for further downstream study via microarray. The treatments selected were the untreated control, concentration of glabridin which induced the highest estrogenic activity (10 μM glabridin), and the combination treatment of 100 nM glabridin with 1 nM 17β-E_2 which induces a synergistic increase in estrogenic activity (SQ = 1.217) but with a lower induction of cell proliferation as compared with 17β-E_2 alone, 100 nM glabridin, and 1 nM 17β-E_2. The cells were treated for 72 hours and the RNAs were extracted for microarray and RT-qPCR analyses. All RNA samples had an RNA integrity number greater than 9.4 (Figure S1).

The principal component analysis plot (Figure 1) shows that most of the replicates were strongly correlated with each other. Replicates in each of the treatment groups clustered together and away from other treatments, except for 100 nM glabridin which seemed closely correlated with the control. The heat map (Figure 2) suggests that the overall gene expression profile of 10 μM glabridin was similar to 1 nM 17β-E_2, whereas 100 nM glabridin was similar to the control. Displaying similar activities as 17β-E_2 suggests that glabridin could exert similar activities as 17β-E_2. This supports many of the past studies which have suggested that glabridin displayed similar effects to 17β-E_2.[6,8,9,18]

Volcano plot was used to identify the set of genes that were differently regulated from the control cells with the

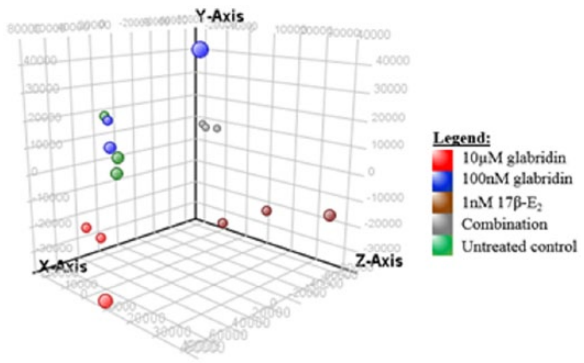

Figure 1. Principal component analysis plot of the 5 different treatment groups: 1 nM 17β-E_2, 10 μM glabridin, 100 μM glabridin, control, and the combination treatment of 1 nM 17β-E_2 and 100 nM glabridin.

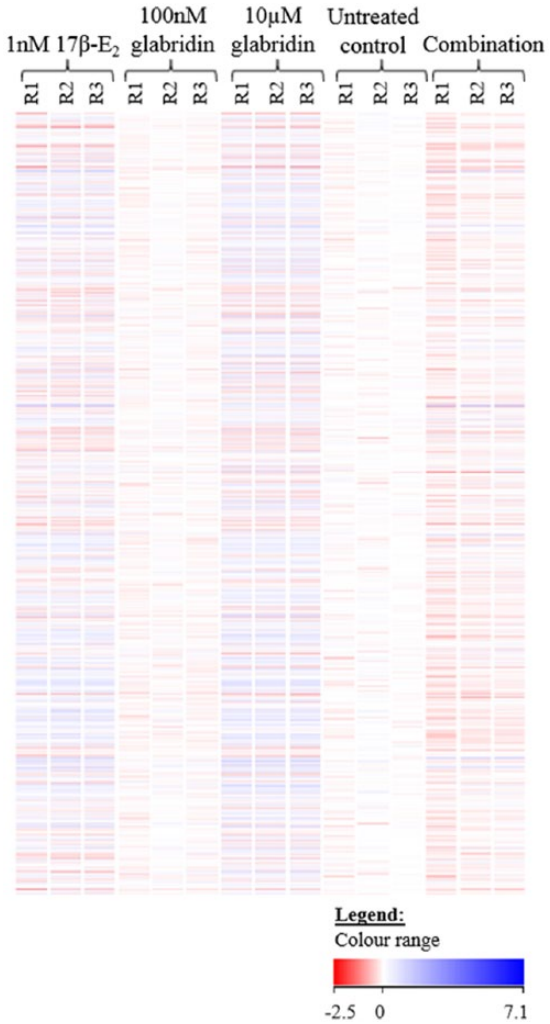

Figure 2. Heat map of biological replicates of Affymetrix gene expression profiles from 5 different treatment groups: 1 nM 17β-E_2, 100 nM glabridin, 10 μM glabridin, control, and the combination treatment of 100 nM glabridin with 1 nM 17β-E_2.

inclusion criteria set as ≥1.5 fold change with a *P* value of ≤.05. The differentially regulated probe sets were deposited in National Center for Biotechnology Information's Gene Expression Omnibus (GEO)[19] and are accessible through GEO Series accession number GSE68333 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE68333). The volcano plot showed that there was no significant difference in any probe sets between 100 nM glabridin and the control. Among the 49 395 probe sets on the GeneChip PrimeView array, a total of 8204 probe sets were regulated by treatment of glabridin (10 μM and 100 nM), 1 nM 17β-E_2, and the combination treatment by 100 nM glabridin with 1 nM 17β-E_2.

HPRT1 and PPIA represent suitable reference gene

For the determination of the most suitable reference genes, geNorm (based in qbase^{PLUS}), NormFinder, and BestKeeper were used. Based on geNorm, all the candidate reference genes were stably expressed (average geNorm $M \leq 0.2$) (Figure S2). The most stable reference gene was *PPIA*, followed by *HPRT1, B2M, GAPDH*, and *RPL13A*. Meanwhile, *HSP90AB1, ACTB*, and *TBP* represented the 3 least stable reference genes. In general, geNorm *V* should be <0.15. In this study, geNorm *V* was <0.15 in general, but when comparing a normalization factor based on the 2 or 3 most stable targets with that of 3 or 4 targets, there was not much added value of including an additional reference gene. Therefore, the use of only 2 reference genes was sufficient.

Based on BestKeeper, *PPIA* ($R = 0.859$, $P = .001$) and *HPRT1* ($R = 0.743$, $P = .002$) represent the top 2 most stably expressed reference genes based on the coefficient of correlation (*R*),[14] as shown in Tables S1 and S2. The most stable reference genes were chosen based on the following 3 criteria: (1) high *R* value, (2) power (x-fold) of lesser than 2, and (3) standard deviation (±Cq) of lesser than 1. According to NormFinder, *PPIA* represents the most stable gene ($M = 0.003$), followed by *HPRT1* ($M = 0.005$), as shown in Table S3. Also, the best combination of 2 genes which had the most stable value was *PPIA* and *HPRT1*, with a stability value of 0.003. Based on the results from geNorm (qbase^{PLUS}), NormFinder, and BestKeeper, it was concluded that the 2 most suitable reference targets to be used for this study were *HPRT1* and *PPIA*.

Validation of microarray by RT-qPCR

To validate the results obtained from the microarray, 12 genes—9 upregulated genes and 3 downregulated genes—were selected to be tested by RT-qPCR. The comparison of the relative expressions of the target genes from microarray and RT-qPCR is tabulated in Table 1. Consistent with the result of the microarray, *PGR* represented the most highly expressed gene and it was expressed more in 1 nM 17β-E_2 than the combination treatment of 100 nM glabridin with 1 nM 17β-E_2. As observed in Table 1, the trend of the genes of interest correlated with the microarray data. Hence, it was concluded that the microarray result shown in the study was suitably validated by RT-qPCR.

Table 1. Comparison of the relative expressions of the target genes from microarray and quantitative reverse transcription polymerase chain reaction.

GENES	MICROARRAY			QBASE^{PLUS}		
	1 NM 17β-E_2	10 MM GLABRIDIN	COMBINATION OF 1 NM 17β-E2 AND 100 NM GLABRIDIN	1 NM 17β-E_2	10 MM GLABRIDIN	COMBINATION OF 1 NM 17β-E2 AND 100 NM GLABRIDIN
*ALDH3*A1	—	4.08	—	<1.19>	3.33	<1.34>
ALPPL2	6.79	—	4.88	6.39	<1.17>	<4.87>
ANPEP	12.05	1.61	11.2	16.15	<0.98>	11.22
C-FOS	—	–1.58	–2.06	<1.18>	<1.24>	0.48
C-JUN	—	—	–2.17	<0.88>	<0.89>	0.58
COL1A2	5.26	1.94	5.59	2.58	<2.06>	<1.94>
DHRS3	7.83	1.88	6.16	6.92	2.06	8.9
ESR1	1.59	—	1.56	2.05	<0.893>	<1.81>
HES2	6.66	1.53	6.85	<1.89>	<0.51>	6.6
NRIP1	2.96	—	3.21	3.22	2.23	<2.48>
PGR	120.41	8.16	117.95	47.3	5.37	44.86
PPARGC1A	–1.78	–1.61	–1.94	0.47	0.66	0.38

— represents values that were not available as they either had fold change <1.5 or the *P* value was >.05, whereas values in < > represent values that were not statistically significant at $P < .05$.

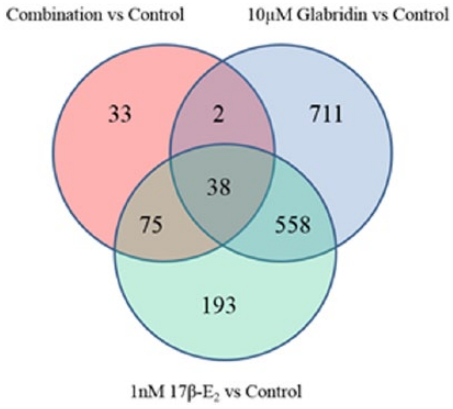

Figure 3. Venn diagram showing the overlap between probe sets regulated by the different treatments (1 nM 17β-E_2, 10 μM glabridin, and the combination treatment of 100 nM glabridin with 1 nM 17β-E_2) on Ishikawa cells.

Upregulation of PGR confirms activation of classical estrogen signaling of glabridin

To further classify the effects of the different treatments that were not regulated by 17β-E_2, the probe sets were compared via a Venn diagram. The Venn diagram in Figure 3 shows the probe sets that are overlapping between the different treatments on Ishikawa cells. Among the 38 commonly regulated probe sets (Figure 3), *PGR* represents the most upregulated gene. They were significantly involved in gene ontologies such as blood vessel development, multicellular organismal development, multicellular organismal process, developmental process, single-multicellular organism process, positive regulation of cell cycle, and regulation of epithelial cell proliferation. These probe sets were also involved in the pathways of the nuclear receptors and metabolism of steroid hormone pathways. Among the pathways that were regulated, ovarian infertility genes' pathway was the most significantly regulated.

PGR which codes for progesterone receptor was the most upregulated gene in all the different treatment groups (Table 2). The increase in *PGR* expression by 17β-E_2 had been well established in many steroid-sensitive tissues. In Ishikawa cells, the upregulation of *PGR* indicates estrogenic response as past literature showed that 17β-E_2 upregulates *PGR*, whereas progesterone downregulates it.[20,21] The upregulation of *PGR* also represents a marker for estrogen action that can occur via the classical estrogen signaling through ER-α, ER-β or as a result of mechanical stimulation by progesterone.[22,23] Similarly, in this study, *PGR* was upregulated a lot higher compared with *ESR1*. The upregulation of *PGR* by the different treatments confirmed that they displayed estrogenic effects in Ishikawa cells via the classical estrogen pathway. This also showed that *PGR* is more suitable as a gene marker for detecting estrogenic activity in Ishikawa cells, compared with *ESR1*.

For global functional classifications, functional annotations derived from GeneSpring's Gene Ontology Analysis

Table 2. A list of the top 10 highly upregulated and downregulated probe sets in Ishikawa cells based on microarray from the different treatments that were significant compared with the control.

PROBE SET ID	ENTREZ GENE	GENE SYMBOL	FOLD CHANGE		
			100 NM GLABRIDIN WITH 1 NM 17β-E_2	1 NM 17β-E_2	10 MM GLABRIDIN
Top 10 upregulated probe sets					
11726168_at	5241	*PGR*	117.95	120.41	8.16
11755866_a_at	10439	*OLFM1*	20.31	20.12	—
11717127_a_at	290	*ANPEP*	11.20	12.05	—
11728894_at	54626	*HES2*	6.85	6.66	—
11724762_a_at	64798	*DEPTOR*	6.47	—	—
11716835_a_at	9249	*DHRS3*	6.16	7.83	—
11721092_a_at	7057	*THBS1*	—	7.53	—
11727334_at	251	*ALPPL2*	—	6.79	—
11726360_a_at	218	*ALDH3A1*	—	—	4.08
11754344_s_at	81848	*SPRY4*	—	—	3.56
Top 10 downregulated probe sets					
11746122_s_at	9877	*ZC3H11A*	—	—	3.82
11716915_a_at	57509	*MTUS1*	—	—	3.34
11755635_s_at	728642///984	*CDK11A///CDK11B*	—	—	3.14
11729051_a_at	8496	*PPFIBP1*	—	—	3.01
11731416_at	4719	*NDUFS1*	—	—	2.76
11723403_at	10891	*PPARGC1A*	1.94	2.97	2.69
11718964_at	57532	*NUFIP2*	—	—	2.34
11718603_a_at	222166	*C7orf41*	—	—	2.29
11764058_at	5621	*PRNP*	—	2.88	—
11744091_s_at	552889	*ATXN7L3B*	—	—	2.28

— represents values that were not available as they either had fold change <1.5 or the *P* value was >.05.

database were used. Figure 4 shows the effect of the distribution of the different treatments on biological processes, cellular components, and molecular functions in Ishikawa cells. Focusing on the probe sets regulated, Tables 2 and 4 presented the top 10 most highly upregulated and downregulated probe sets, respectively, by the treatment. *PGR* represents the most highly regulated gene in all treatments. Most of the genes, such as *PGR, ANPEP, OLFM1, HES2*, and *DHRS3*, were similarly regulated in both the combination treatment and 1 nM 17β-E_2. This could be because their effect was highly influenced by the presence of 1 nM 17β-E_2. Except for *PGR*, there were no other similarities between the top 10 highly upregulated genes between the 3 different treatments. Meanwhile, most of the most highly downregulated genes were by 10 μM glabridin. *PPARGC1A* was the only most highly downregulated gene that was common to all treatments.

Table 3 tabulates the effect of the different treatments on gene ontologies related to activities in the circulatory, cardiovascular, and vascular systems. These activities had been, in the past, associated with its estrogenic action. As shown in Table 1, the combination treatment significantly regulated the most number of gene ontologies compared with 10 μM glabridin and 1 nM 17β-E_2. Meanwhile, Table 4 shows the regulation of *NRIP1* by the combination treatment and 1 nM 17β-E_2 alone. This gene was not regulated by 10 μM glabridin. This gene is involved in the estrogen pathway and was one of the genes chosen for validation of microarray.

Glabridin at 10 μM displayed similar effect on the estrogen pathway as 1 nM 17β-E_2

For the curation of biological pathways of the differentially regulated probe sets, WikiPathways (www.wikipathways.org) was used.[24] Similar gene expression profiles between 10 μM glabridin and 1 nM 17β-E_2 as compared with the control suggested that their activities might be analogous. Estrogen pathway is one of the commonly regulated pathways by 10 μM glabridin and 1 nM 17β-E_2. From Figure 5, it could be seen that 10 μM glabridin and 1 nM 17β-E_2 were clustered close together in comparison with the control, whereas the combination treatment of 100 nM glabridin with 1 nM 17β-E_2 regulated the genes in a different manner.

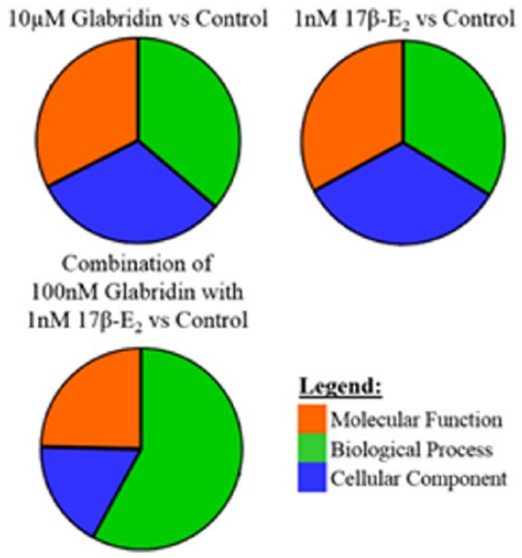

Figure 4. The gene ontology of the different treatments (1 nM 17β-E_2, 10 μM glabridin, and the combination treatment of 100 nM glabridin with 1 nM 17β-E_2) against the untreated control.

Many of the genes are regulated in a similar manner between 10 μM glabridin and 1 nM 17β-E_2. Based on the gene list—GSE68333, the effect of glabridin and the combination treatment on the estrogen pathway which could be classical, estrogen response element (ERE)–independent genomic, or nongenomic were drawn out in the form of pathways with the regulation of the different genes involved marked accordingly (Figure 6A and B). Although 10 μM glabridin did not upregulate *ESR1*, in a previous study, we found that glabridin induced a dose-dependent increase in ER-α-SRC-1 and coactivator complex activity.[10] Hence, the proposed pathway for estrogenic action of glabridin in Ishikawa cells is shown in Figure 6A. Moreover, 10 μM glabridin regulated several downstream effectors that are involved in the classical and ERE-independent pathways,[25] which were also regulated by 1 nM 17β-E_2, such as *SP-1* of the classical pathway and *PGR* which is involved with the ER pathway in general.

C-FOS, C-JUN, and NRIP1 might be involved in the decrease in cell proliferation in the combination treatment of glabridin with 17β-E_2

Based on a past study,[10] the synergistic effect on ALP induction could have occurred via the ER-α-SRC-1 and coactivator. Meanwhile, glabridin and 17β-E_2 induced dose-dependent

Table 3. Gene ontologies (GO) of 10 μM glabridin, 1 nM 17β-E_2, and the combination treatment of 100 nM glabridin with 1 nM 17β-E_2 that have been associated with its estrogenic action.

GO ID	GO ACCESSION	GO TERM	10 MM GLABRIDIN	COMBINATION OF 1 NM 17β-E_2 AND 100 NM GLABRIDIN	1 NM 17β-E_2
			P VALUE	*P* VALUE	*P* VALUE
957	GO:0001568	blood vessel development	1.40E−02	1.14E−06	2.97E−03
1269	GO:0001944	vasculature development	2.35E−02	1.95E−06	—
24971	GO:0048514	blood vessel morphogenesis	—	4.22E−05	—
32123	GO:0072358	cardiovascular system development	3.35E−04	1.96E−05	1.25E−03
32124	GO:0072359	circulatory system development	3.35E−04	1.96E−05	1.25E−03

Table 4. Regulation of *NRIP1* gene by the combination of 100 nM glabridin with 1 nM 17β-E_2 and 1 nM 17β-E_2.

PROBE SET ID	COMBINATION OF 100 NM GLABRIDIN WITH 1 NM 17β-E_2			1 NM 17β-E_2			GENE SYMBOL	ENTREZ GENE
	P VALUE	FOLD CHANGE	REGULATION	*P* VALUE	FOLD CHANGE	REGULATION		
11720153_s_at	.01	3.212	Up	.01	2.960	Up	*NRIP1*	8204

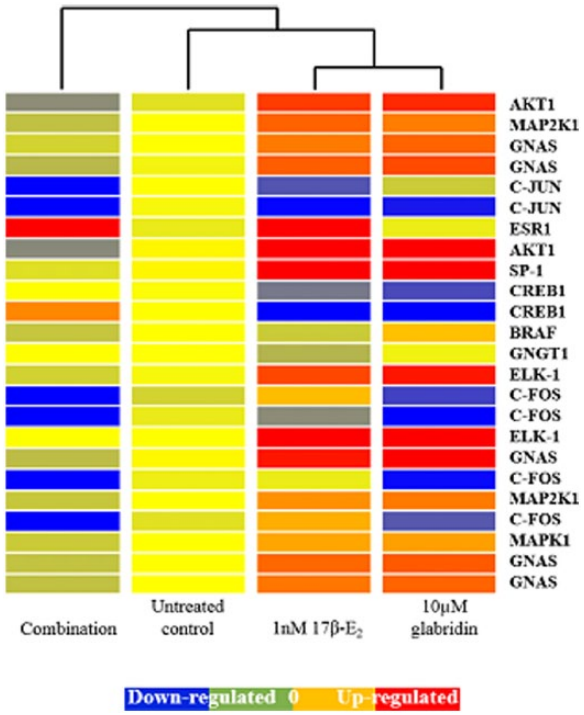

Figure 5. Hierarchical clustering of the estrogen pathway of the different treatments (untreated control, 1 nM 17β-E_2, 10 μM glabridin, and the combination treatment of 100 nM glabridin with 1 nM 17β-E_2).

increase in both ALP activity and cell proliferation in Ishikawa cells, and the combination study results showed an increase in ALP activity but with a lower induction of cell proliferation. This suggested that glabridin could act in a different manner from 17β-E_2. Therefore, it was postulated that this difference could be via ER-α–independent mechanisms in Ishikawa cells.[10]

Compared with the difference in gene expressions, the combination treatment of 100 nM glabridin with 1 nM 17β-E_2 downregulated genes, such as *C-FOS* and *C-JUN*, which are transcription factors that heterodimerize to form a part of the nuclear activator protein 1 (AP-1) complex that regulates the expression of genes involved in many rapid cellular events such as proliferation, differentiation, and apoptosis apoptosis.[26] Although 10 μM glabridin also decreased *C-FOS* expression, the induction of cell proliferation was comparable with that of 17β-E_2 treatment. This suggests both that *C-FOS* and *C-JUN* have to decrease in expression to reduce the induction of cell proliferation. Table S4 shows that *C-JUN* represented the most highly regulated probe set among the probe sets that were differentially regulated by the combination of 1 nM 17β-E_2 and 100 nM glabridin alone that was not regulated by others. The probe sets that were only regulated by the combination treatment were observed to regulate gene ontologies such as the regulation of cell death and the regulation of programmed cell death (Table S5). Similarly, Table S6 also shows that pathways such as regulation of cell death and regulation of programmed cell death were involved.

The microarray result also showed that the combination upregulated *NRIP1* (Table 4), which is a ligand-dependent corepressor for most of the nuclear receptors. *NRIP1* had also been shown to act as a corepressor that specifically antagonizes ER transcription via interaction with AP-1 protein in vivo.[27,28] Previously, it had been reported that retinoic acid could inhibit cell proliferation in Ishikawa cells and *NRIP1* represents 1 of the 4 most highly regulated genes that was upregulated.[29] Similarly, in a separate study, upregulation of *NRIP1* was found to inhibit the proliferation of microglioma cells.[30] Therefore, the downregulation of *C-JUN* and upregulation of *NRIP1* by the combination of 100 nM glabridin with 1 nM 17β-E_2 might be the cause of the slight decrease in induction of cell proliferation as compared with 1 nM 17β-E_2. Combining with the previous postulation, it was suggested that the downregulation of *C-FOS, C-JUN* and upregulation of *NRIP1* could have occurred via ER-α–independent mechanisms.

Possible effects of the combination treatment on ovulation

Table 4 shows that similar to 1 nM 17β-E_2, the combination treatment of 100 nM glabridin with 1 nM 17β-E_2 upregulated *NRIP1*. *NRIP1* plays an important role in the ovary. Expression of *NRIP1* in the ovary is essential for oocyte release from mature follicles during ovulation, but not for luteinization of mature ovarian follicles.[31,32] This could highlight the importance of *NRIP1* in female fertility. *NRIP1* was also found to have a role in the maintenance of pregnancy.[33] Another gene which was also involved in ovulation from the ovarian follicle is *PGR*.[23]

The gene ontology analysis also showed that the combination treatment displayed effects on ovulation. Among the different treatments, only the combination treatment had an effect on reproduction and ovulation. Meanwhile, 10 μM glabridin had no effect on both reproduction and ovulation, and 1 nM 17β-E_2 only regulated ovulation. The gene ontologies related to steroid and developmental activity based on probe sets that were differently regulated in the combination of 100 nM glabridin with 1 nM 17β-E_2 are shown in Tables 5 and 6, respectively. Together with the gene analysis, this suggested that there is a possibility of the combination treatment to be explored for using in aiding ovulation.

Possible regulation of glabridin and the combination treatment on cardiovascular, circulatory, and vasculatory development

Past studies had shown that premenopausal women had lower chances of cardiovascular diseases as compared with menopausal women, and this effect could be reversed with the intake of ERT.[34,35] This is as estrogen has a significant impact on heart and vasculature.[34] Gene ontology analysis in this study showed

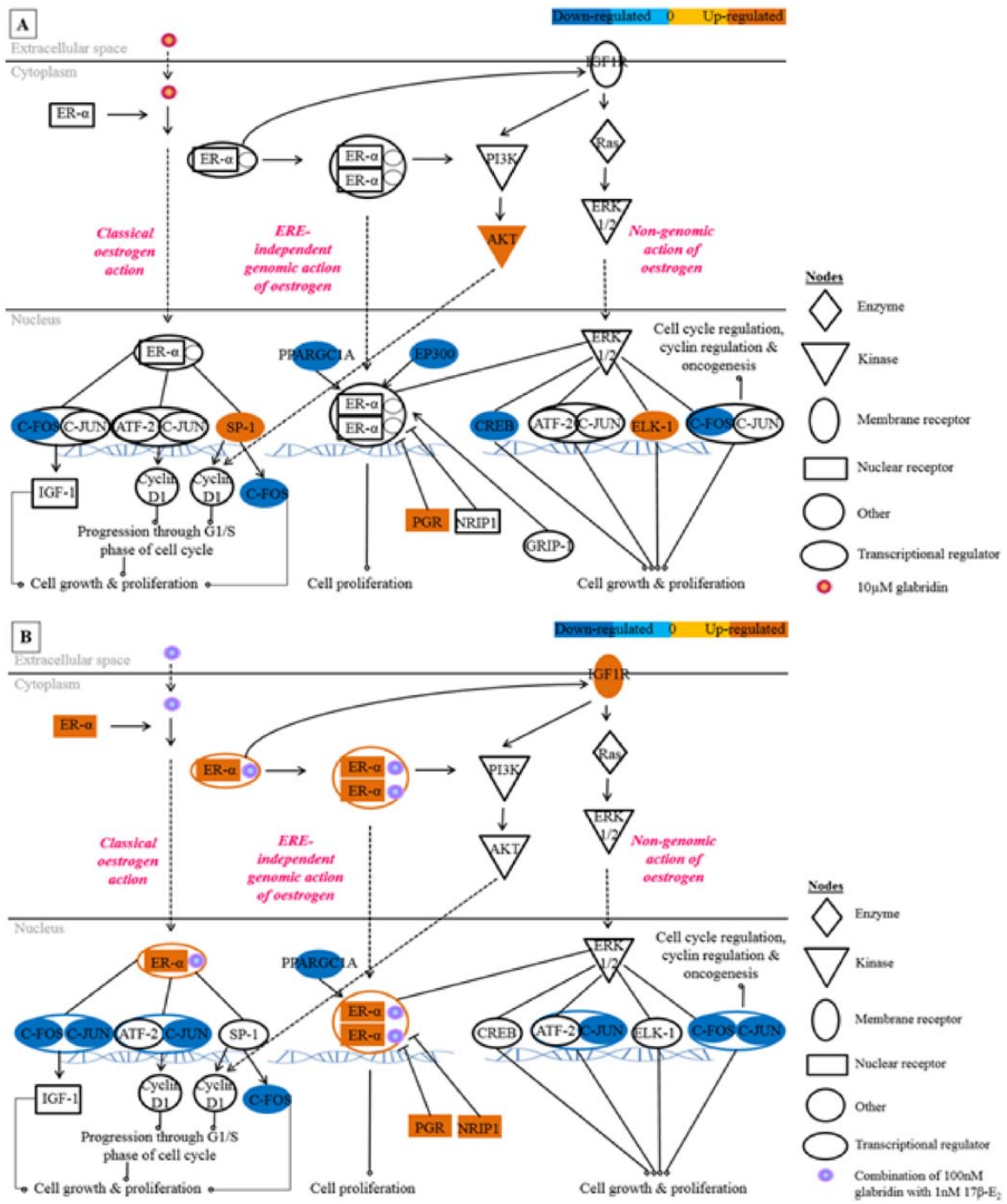

Figure 6. (A) Possible effects of 10 μM glabridin and (B) 100 nM glabridin with 1 nM 17β-E_2 on the estrogen pathway after 72 hours treatment on Ishikawa cells. The pathways drawn were adapted from ER signaling pathways from WikiPathways[47] and Ingenuity Target Explorer.[48]

that similar to 17β-E_2, both 10 μM glabridin and the combination treatment regulated genes involved in the vasculature development, cardiovascular, and circulatory systems that had been associated with estrogenic action (Table 3). Meanwhile, the combination treatment of 100 nM glabridin with 1 nM 17β-E_2 could regulate blood vessel morphogenesis, vasculature development, cardiovascular, and circulatory systems.

This is consistent with a past study by Tamir et al[6] which showed that glabridin activated CK activity in skeletal and cardiovascular tissues. This showed that glabridin was capable of exhibiting the positive effects of 17β-E_2 in cardiovascular tissues. Even in animal studies, glabridin had been shown to stimulate the activity of CK in the aorta and left ventricle of the heart of both intact and ovariectomized female rats.[8] Meanwhile, Somjen et al. (2004) also showed that glabridin stimulated DNA synthesis in human endothelial cells and displayed biphasic effect on proliferation of human vascular smooth muscle cells similar to that of 17β-E_2. The result of this study together with past studies provides evidence that 10 μM glabridin and the combination treatment could bring

Table 5. Gene ontologies (GO) related to steroid activity based on probe sets that were differently regulated in the combination of 100 nM glabridin with 1 nM 17β-E_2.

GO ID	GO ACCESSION	GO TERM	*P* VALUE
3	GO:0000003	reproduction	.03
933	GO:0001542	ovulation from ovarian follicle	.01
1221	GO:0001890	placenta development	.05
2266	GO:0003006	developmental process involved in reproduction	.02
2704	GO:0003707	steroid hormone receptor activity	.03
5328	GO:0007292	female gamete generation	.02
5548	GO:0007548	sex differentiation	.02
6054	GO:0008585	female gonad development	.03
7002	GO:0009725	response to hormone stimulus	1.44E–04
12453	GO:0022414	reproductive process	.02
12458	GO:0022602	ovulation cycle process	.03
13158	GO:0030728	ovulation	1.44E–04
17513	GO:0035239	tube morphogenesis	.02
17568	GO:0035295	tube development	.01
19740	GO:0042698	ovulation cycle	.04
20380	GO:0043401	steroid hormone mediated signaling pathway	.04
20596	GO:0043627	response to estrogen stimulus	2.13E–04
21871	GO:0045137	development of primary sexual characteristics	7.61E–03
23151	GO:0046545	development of primary female sexual characteristics	.04
23255	GO:0046660	female sex differentiation	.04
25000	GO:0048545	response to steroid hormone stimulus	1.95E–06
25047	GO:0048598	embryonic morphogenesis	.03
25051	GO:0048608	reproductive structure development	8.39E–04
25172	GO:0048732	gland development	.02
28359	GO:0060135	maternal process involved in female pregnancy	.01
28784	GO:0060562	epithelial tube morphogenesis	.02
28966	GO:0060745	mammary gland branching involved in pregnancy	.05
29392	GO:0061180	mammary gland epithelium development	.04
29669	GO:0061458	reproductive system development	8.39E–04
37089	GO:2000241	regulation of reproductive process	.03

about the positive effects of 17β-E_2 in the cardiovascular, circulatory, and vasculature systems.

Possible regulation of glabridin and the combination treatment in improving polycystic ovary syndrome conditions

Polycystic ovary syndrome (PCOS) is a condition correlated with molecular defects of insulin signaling pathway at the postreceptor level in tissues such as adipocytes, muscles, and ovary.[36] In this study, both 10 μM glabridin and the combination treatment were reported to play a role in regulating the insulin signaling pathway, MAPK signaling pathways, and adipogenesis (Table 7), which are all related to metabolic syndromes and PCOS.[37]

Besides, this study found that 10 μM glabridin increased *PPARG* expression by 1.562-fold ($P = .01$), thus suggesting that it could help with metabolic disorders by improving insulin

Table 6. Gene ontologies (GO) related to developmental activity based on probe sets that were differently regulated the combination of 100 nM glabridin with 1 nM 17β-E_2.

GO ID	GO ACCESSION	GO TERM	*P* VALUE
957	GO:0001568	blood vessel development	1.14E−06
1009	GO:0001655	urogenital system development	1.05E−03
1011	GO:0001657	ureteric bud development	.03
1153	GO:0001822	kidney development	.05
1220	GO:0001889	liver development	.03
1221	GO:0001890	placenta development	.05
1269	GO:0001944	vasculature development	1.95E−06
2266	GO:0003006	developmental process involved in reproduction	.02
2454	GO:0003205	cardiac chamber development	.03
2480	GO:0003231	cardiac ventricle development	.01
2528	GO:0003279	cardiac septum development	1.54E−03
2529	GO:0003281	ventricular septum development	5.38E−04
5311	GO:0007275	multicellular organismal development	3.63E−09
5416	GO:0007399	nervous system development	.02
5927	GO:0008406	gonad development	4.03E−03
6054	GO:0008585	female gonad development	.03
7151	GO:0009888	tissue development	1.05E−03
14851	GO:0032502	developmental process	4.77E−11
17568	GO:0035295	tube development	.01
21705	GO:0044767	single-organism developmental process	2.17E−06
21871	GO:0045137	development of primary sexual characteristics	7.61E−03
23151	GO:0046545	development of primary female sexual characteristics	.04
24927	GO:0048468	cell development	4.35E−03
28651	GO:0060429	epithelium development	1.49E−03
29228	GO:0061008	hepaticobiliary system development	.03
29392	GO:0061180	mammary gland epithelium development	.04
29519	GO:0061307	cardiac neural crest cell differentiation involved in heart development	.05
29520	GO:0061308	cardiac neural crest cell development involved in heart development	.04
29669	GO:0061458	reproductive system development	8.39E−04
31770	GO:0072001	renal system development	.01
31775	GO:0072006	nephron development	.01
31778	GO:0072009	nephron epithelium development	.01
31840	GO:0072073	kidney epithelium development	.04
31907	GO:0072141	renal interstitial cell development	.04
32123	GO:0072358	cardiovascular system development	1.96E−05
32124	GO:0072359	circulatory system development	1.96E−05
36879	GO:2000026	regulation of multicellular organismal development	1.99E−04

Table 7. Various pathways related to the metabolic syndrome and MAPK pathways that were regulated by the different treatments.

PATHWAYS	10 MM GLABRIDIN	COMBINATION OF 1 NM 17β-E_2 AND 100 NM GLABRIDIN	1 NM 17β-E_2
	P VALUE	*P* VALUE	*P* VALUE
Metabolic syndrome			
Insulin signaling	3.85E–10	.01	1.05E–06
Adipogenesis	4.09E–05	7.24E–03	.03
MAPK pathways			
MAPK cascade	9.19E–05	—	9.76E–06
MAPK targets—nuclear events mediated by MAPKs	1.74E–02	2.49E–04	—
MAPK signaling pathway	5.53E–06	1.31E–04	5.72E–04
p38 MAPK signaling pathway	1.17E–02	—	—

sensitivity and increase uptake of glucose by fat cells via the PPARG pathway.[38] This was contradictory to a past study where Lee et al[39] found that glabridin did not affect the expression of *PPARG* or adipogenesis in adipose tissue of glabridin-treated obese mice. However, in a later study, Ahn et al[40] showed that glabridin and glabridin-rich licorice extract effectively inhibited adipogenesis in 3T3-L1 cells and hence were effective anti-obesity agents.

Another past study had suggested that the upregulation of *PPARG* was indicative of its effect in maintaining glucose homeostasis and improving insulin sensitivity.[37] In addition, Nelson-Degrave et al[41] found that the inactivation of MAPK pathway was implicated in the pathogenesis of excessive ovarian androgen production in PCOS women. Overall, the upregulation of *PPARG* by glabridin together with the regulation of pathways associated with adipogenesis, insulin resistance, and gluconeogenesis, which are metabolic disorders related to PCOS, suggests that further studies on 10 μM glabridin and the combination in these areas could be explored for the improvement of the PCOS-related metabolic syndromes. This is the first study to show that glabridin could regulate pathways related to PCOS and hence suggests the use of glabridin for further study as ERT for patients with PCOS. From this study, it could be observed that although glabridin exhibited a wide array of activities, they seem to be interconnected and results mostly from its ability to act on ER.[42]

Possible nongenomic signaling of glabridin in Ishikawa cells

Figure 6A shows that components of the nongenomic estrogen pathway such as *AKT1, MAPK1, SP-1*, ETS domain-containing protein (*ELK-1*), and dual specificity mitogen-activated protein kinase (*MAP2K1*) were upregulated by 1 nM 17β-E_2 and 10 μM glabridin. Table 7 shows the various MAPK pathways regulated by the different treatments. Based on the results, it could be observed that 10 μM glabridin regulated the most number of MAPK pathways. Hence, it is postulated that glabridin could mediate nongenomic signaling via ER-α[43] or insulinlike growth factor 1 receptor (*IGF1R*)[44] to phophorylate PI3K/AKT and ERK/MAPK pathways. Besides, the nongenomic pathway could also possibly modulate the functions of ER to enhance the classical mechanism of ER action[45] suggesting a possibility for the convergence of genomic and nongenomic actions.[46]

The biphasic effect demonstrated by glabridin in Ishikawa cells suggested that they regulate estrogenic activity and cell proliferation not only through ER in a dose-dependent manner but also through a different molecular mechanism which differs from the ER pathway that modulates estrogenic activities in endometrial cells.[6] This proposes that the nongenomic pathway could be looked into to better understand the effect of glabridin in endometrial cells. Moreover, the differences observed between glabridin and 17β-E_2 suggested that glabridin acts via a different mechanism from that of 17β-E_2.

Conclusions

The microarray results showed that 10 μM glabridin and the combination treatment of 100 nM glabridin with 1 nM 17β-E_2 regulated both the genomic and nongenomic estrogen pathways in Ishikawa cells. Based on the results, both 10 μM glabridin and the combination could possibly provide benefits of estrogens in ERT. The combination might be more suitable as an ERT, whereas glabridin could be explored for the improvement of PCOS-related metabolic syndromes.

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Author Contributions

PSWM, CYY, and VN conceived and designed the experiments. PSWM analyzed the data and wrote the first draft of the manuscript. PSWM, CYY, and YVCP contributed to the writing of the manuscript. PSWM, CYY, YVCP, and VN agree with manuscript results and conclusions. PSWM, CYY, YVCP, and VN jointly developed the structure and arguments for the paper. PSWM and CYY made critical revisions and approved the final version. All authors reviewed and approved the final manuscript.

Disclosures and Ethics

As a requirement of publication, author(s) have provided to the publisher signed confirmation of compliance with legal and ethical obligations including but not limited to the following: authorship and contributorship, conflicts of interest, privacy and confidentiality, and (where applicable) protection of human and animal research subjects. The authors have read and confirmed their agreement with the ICMJE authorship and conflict of interest criteria. The authors have also confirmed that this article is unique and not under consideration or published in any other publication, and that they have permission from rights holders to reproduce any copyrighted material. Any disclosures are made in this section. The external blind peer reviewers report no conflicts of interest.

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