A polyacetylene compound from herbal medicine regulates genes associated with thrombosis in endothelial cells

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Supplementary data

Materials: All solvents for purification were in the HPLC grade and were purchased from Fisher Scientific. The Toki-shakuyaku-san (TSS) formulation was purchased from Honso Pharmaceutical Co. (Nagoya, Japan). The TSS formulation (lot number: 05I120) was standardized by ferulic acid (test result 0.024%; specification 0.019-0.033%) and paeoniflorin (test result 1.53%; specification 1.24-1.94%). Unless specified otherwise, all other chemicals and reagents were obtained through Fisher Scientific and used without further purification.

Cell Culture: Primary Human Umbilical Vein Endothelial Cells (HUVEC) were purchased from BioWhittaker. Cells were propagated in Endothelium Growth Medium-2 (EGM-2, BioWhittaker) in a 37 °C humidified 5% CO_2 incubator, and subcultured at 70 - 80% confluency. Cells at the passages 2 - 4 were used for the experiments.

Gene expression profiling*:* Cells were treated with TSS (100 μg/ml) or DMSO vehicle controls for 4h in a 37 °C humidified 5% CO_2 incubator. 100 μg/ml is a sub-toxic concentration of TSS as determined by MTS cell proliferation assay (Promega);[1] IC_{50} of TSS was above 500 μg/ml (data not shown). Total RNA was purified with Qiagen

RNeasy mini kit. Expression profiling was carried out by following Affymetrix GeneChip Expression Profiling Manual. Briefly, synthesis of biotinylated cRNA via double strand cDNA with T7 promoter was carried out with Affymetrix One-Cycle Target Labeling and Control Reagent kit. Biotinylated cRNA samples were fragmented. Fragmented samples were submitted to Rockefeller University Genomics Center for hybridization to Human Genome U133 Plus 2.0 array, staining, washing, and scanning. The resulting data were analyzed with Affymetrix GeneChip® Microarray Suite Software Version 5.0 (MAS5). Two independent experiments were carried out for each treatment condition: TSS treated and DMSO (control) treated HUVEC (total 4 chips). This enabled four comparisons between TSS-treated and control groups.

In the gene expression profiling of TDEYA, i.e., the purified active compound, cells were treated with TDEYA (5 μg/ml) or DMSO vehicle controls for 4h in a 37 °C humidified 5% CO_2 incubator. The rest of the procedure is same as one described for the profiling of TSS.

Table S1. HUVEC genes differentially regulated by TSS (in Blue), and their profiles in the TDEYA-treated HUVEC (in Green).

UniGene	Descriptions	TSS	Log Ratio	Log p-value	TDEYA	Log Ratio	Log p-value
Hs.12813	**TIPARP: TCDD-inducible poly(ADP-ribose) polymerase**	**I**	**0.70**	**0.00002**	**I**	**1.15**	**0.00002**
Hs.196384	**PTGS2: Prostaglandin-endoperoxide synthase 2 (COX2)**	**I**	**1.03**	**0.00002**	**I**	**0.98**	**0.00002**
Hs.594481	**SERPINB2: Serpin peptidase inhibitor, clade B (ovalbumin), member 2**	**I**	**0.98**	**0.00002**	**I**	**1.00**	**0.00002**
Hs.433791	**TMEM46: Transmembrane protein 46**	**I**	**0.85**	**0.00002**	**I**	**0.60**	**0.00004**
Hs.326035	**EGR1: Early growth response 1**	**I**	**0.75**	**0.00002**	**I**	**1.20**	**0.00056**
Hs.72912	**CYP1A1: Cytochrome P450, family 1, subfamily A, polypeptide 1**	**I**	**1.85**	**0.00003**	**I**	**1.93**	**0.00003**
Hs.73853	BMP2: Bone morphogenetic protein 2	I	0.63	0.00003	NC	-0.18	0.83320
Hs.25590	**stanniocalcin 1**	**I**	**0.70**	**0.00006**	**I**	**0.53**	**0.00006**
Hs.1048	KITLG: KIT ligand	I	0.70	0.00058	NC	0.10	0.36975
Hs.534333	NDUFA2: NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 2,	I	0.83	0.00076	NC	0.25	0.19141
Hs.9914	FST: Follistatin	I	0.65	0.00078	NC	0.63	0.26196
Hs.154654	**CYP1B1: Cytochrome P450, family 1, subfamily B, polypeptide 1**	**I**	**2.13**	**0.00133**	**I**	**2.70**	**0.00002**
Hs.651231	**HSPG2: Heparan sulfate proteoglycan 2 (perlecan)**	**D**	**-0.68**	**0.99904**	**D**	**-0.78**	**0.99996**
Hs.594486	unknown	D	-1.90	0.99944	NC	-0.58	0.92567
Hs.29055	GNL3L: Guanine nucleotide binding protein-like 3 (nucleolar)-like	D	-0.75	0.99955	NC	-0.08	0.55649
Hs.557980	**unknown**	**D**	**-0.68**	**0.99961**	**D**	**-0.33**	**0.99941**
Hs.355753	GOLGA7: Golgi autoantigen, golgin subfamily a, 7	D	-1.18	0.99998	NC	-0.10	0.56784

Genes on this Table showed Change p-values of >0.999 (down regulated genes) or <0.001 (up-regulated genes) in the TSS profile; these are higher stringency criteria than those used in the MAS5. The profiles of these genes in TDEYA-treated HUVEC are also shown.

Out of 17 genes on this table, 10 genes were also differentially regulated by TDEYA (shown in **bold**).

Real-time PCR assay. Real-time PCR assay was conducted as follows. HUVEC were plated onto each well of 6-well plate at ~20% confluency in 2 ml EGM/well and incubated for 24 hours to allow them to recover from passage. Cells were treated with 20 μl of TSS fractions for 4 hours. RNeasy mini kit (Qiagen) was used to purify the total RNA. RNA samples were quantified using UV absorbance at 260 nm. Samples with the 260nm/280nm ratio ~1.8 or higher were used for the subsequent study. The first strand cDNA samples were synthesized with Gibco BRL Superscript Choice system and oligo $(dT)_{12\text{-}18}$ primer. TaqMan® Gene Expression Assays (Applied Biosystems) were carried out on Applied Biosystems 7500 Real-Time PCR system using pre-optimized assays for SerpinB2, COX2, Perlecan, TIPARP, and GAPDH (endogenous control). The $\Delta\Delta C_T$ method was employed to quantify differential gene regulation. The raw data were first normalized by the endogenous control (GAPDH) for individual samples. Then the relative quantification (RQ) values, i.e., the fold-change ratios, were obtained by comparing the normalized data against the DMSO vehicle control. At least duplicate experiments were carried out for each sample during the fractionation. Minimum triplicate experiments were carried out for the final data presented in Figure 1c and Figure S1. Student's paired t-Test, with a two-tailed distribution, was used to assess the statistical significance of the difference between control and drug treated samples.

Real-time PCR results of all tested genes (SerpinB2, COX2, perlecan, and TI-PARP) were consistent with the DNA microarray profile (Figure 1c and Figure S1), although there were minor differences in terms of the magnitude of changes in expression.

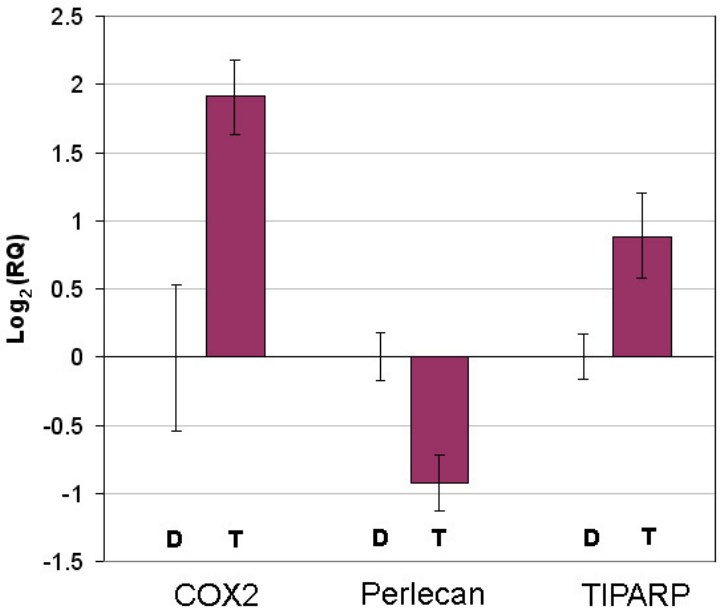

Figure S1. Real-time PCR validation of genes differentially regulated by TDEYA: COX2, Perlecan, and TIPARP. D (DMSO); T (TDEYA). GAPDH was used as the endogenous control. Log2(RQ) corresponds to the Log Ratio value in the expression profile (Table 1). RQ stands for relative quantitation (i.e., the fold-change ratio) in real-time PCR analysis.

Real-time PCR guided fractionation of TSS: Fractionation was carried out with 500 g of TSS. Five different solvent systems (hexane, ethyl acetate, chloroform, methanol, and 2:1 mixture of chloroform-methanol) were tested for the initial extraction. Both extracts and insoluble materials were tested by the real-time RT-PCR of SerpinB2 as described above. It was found that the chloroform-methanol (2:1) mixture extracts the SerpinB2 regulatory activity most efficiently. This chloroform-methanol extract was partitioned between 1-butanol/EtOAc (2:1) and water. The activity was then found in the organic layer. Further fractionation of this organic layer was carried out with silica gel chromatography. Three fractions, namely, 1%, 10% and 20% methanol/CH_2Cl_2 fractions, were obtained. The activity was found in the 1% methanol/CH_2Cl_2 fraction (2 g of crude mixture). Peaks in this 1% MeOH/CH_2Cl_2 fraction were isolated with semi-preparative C18 column with a

linear gradient (10-100% MeOH in water, 0.1% trifluoroacetic acid, 3 ml/min). The presence of trifluoroacetic acid in the solvent system did not affect the gene regulatory activity; the activity was acid stable. The activity was found in a peak eluted at 18 min in our HPLC condition.

High resolution LC/MS: LC/MS spectra were acquired on an Agilent Technologies 6210 Time-of-Flight mass spectrometer equipped with an Agilent Technologies 1200 capillary HPLC system. Chromatography was performed on a Zorbax 0.5mm x 150mm SB-C18 column (#5064-8256) using water containing 0.1% formic acid and 50µM ammonium formate (Solvent A) and methanol containing 0.1% formic acid and 50µM ammonium formate (Solvent B) at a flow rate of 12ul/minute. The gradient program was as follows: 10% B (0-2min), 10-100% B (2-20 min), 100% B (20-50 min). Total run time was 50 min. The temperature of the column was held at 40°C for the entire run. Sample ionization was accomplished using an Agilent Technologies electrospray source with data collection in the positive ion mode. Ionization source parameters were the following: nebulizer pressure of 20psi, drying gas temperature of 300°C, drying gas flow rate of 8.0 L/min, and capillary voltage was set to 3500V. The mass spectrometer was set to acquire data with a fragmentor voltage of 165V. A mass range of 100 to 3200 m/z was scanned using 10,000 transients per scan. The length of transients was 104992. The reference masses used were triethylamine with M+H at 102.12827 and HP 922 with M+H at 922.009798 m/z. The instrument was controlled with Agilent Mass Hunter Workstation A.02.02 and the data was processed using Applied Biosystems Analyst QS 1.1 software. The molecular formula of the purified compound was determined to be $C_{18}H_{22}O_4$, using

the following ions: ammonium adduct ion [M+NH_4^+], m/z 320.1863 (calcd 320.1856), sodium adduct ion [M+Na^+], m/z 325.1413 (calcd 325.1410) and the protonated adduct ion [M+H^+], m/z 303.1594 (calcd 303.1590).

NMR: For the initial structural characterization, the purified material was dissolved in methanol-d_4. NMR spectra were measured by Brüker Avance 500MHz NMR Spectrometer equipped with a dual [^{13}C, ^{1}H] CryoProbe. The dual [^{13}C, ^{1}H] CryoProbe, in which the receiver and transmitter coils are cooled to approximately 20K to reduce contributions to noise in the signal obtained,[2] allowed rapid measurement of ^{1}H, HSQC, and COSY (total less than two hours) with ~0.1 mg of the purified material. ^{13}C-NMR was measured by overnight experiment (Figure S2). Data were acquired and processed with the Brüker XWIN-NMR software package.

In order to make a direct comparison with the published ^{13}C-NMR data of TDEYA, which had been measured in $CDCl_3$,[3] we re-purified 1 mg of the active compound and measured ^{13}C-NMR in $CDCl_3$. The resulting data, together with other spectroscopic data, unequivocally confirmed the identity of the active compound as TDEYA (Table S2).

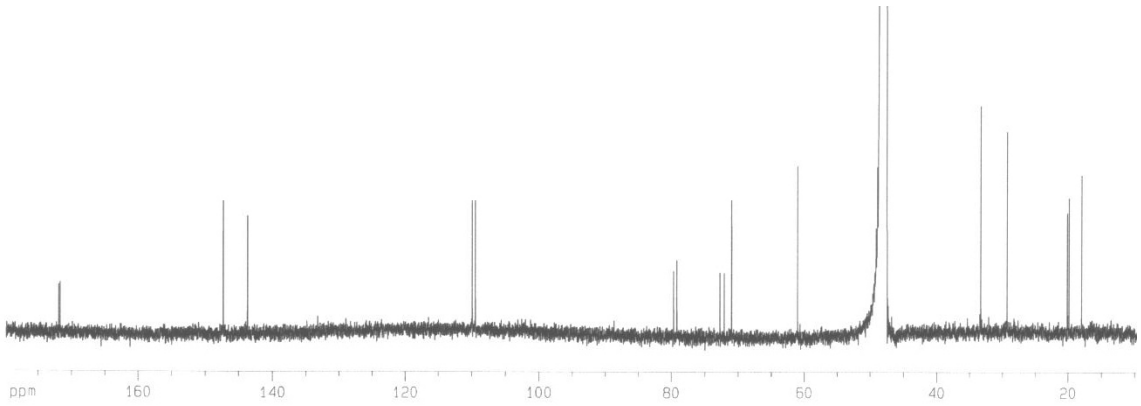

Figure S2. The ^{13}C-NMR spectrum of the purified active material (~0.1 mg) in methanol-*d_4*.

Table S2. ^{13}C NMR comparison of TDEYA and the purified material in $CDCl_3$.

Carbon	TDEYA (Published)	Purified Active Compound
1	60.6	60.636
2	33.1	33.092
3	70.4	70.370
4	33.1	33.089
5	29.1	29.148
6	146.6	146.524
7	109.5	109.560
8	79.3	79.298
9	72.4	72.305
10	73.2	73.156
11	80.0	80.049
12	109.9	109.919
13	143.5	143.508
14	18.9	18.916
CH3	21.1	21.115
CH3	20.9	20.941
O=C-O	170.9	171.010
O=C-O	170.6	170.617

References:

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3. Kano, Y.; Komatsu, K.; Saito, K.; Bando, H.; Sakurai, T., *Chem. Pharm. Bull.* **1989,** *37,* 193.