Supporting Information

Diaporine, a Novel Endophyte-derived Regulator of Macrophage Differentiation

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1. Experiment procedures

1.1 Reagents

Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were supplied by Gibco (Grand Island, N.Y.). PPARγ (E-8), JNK(D-2) and p-JNK(G-7) were purchased from Santa Cruz. ERK1/2 (137F5), p-ERK1/2 (T202/T204) and mentioned loading controls were provided by Cell Signalling. p-PPARγ (S112) was purchased from Abcam. Ssofast evagreen supermix was provided by BIO-RAD (California, USA). Final cytokine concentrations used are described in detail in results specific for each experiment.

1.2 Fungal Material

The strain IFB-3lp-10 was isolated from the healthy leaves of *Rhizophorastylosa* collected from the mangrove forest of Hainan Province of China. The strain was identified as *Diaporthe*sp. by comparing the morphological character and 18SrDNA sequence with that of a standard. The 18SrDNA sequence of the strain has been deposited to the GenBank as JX536253. White-coloured colonies on potato dextrose agar at 28°C grew fast, reaching 20–25 mm in diameter in three days. Perithecia are globular and gregarious with a long neck at the top while the innermost cells are hyaline, and thin-walled. Asci are globular with a thin membrane and furnished an apical ring at the thickened tip. A voucherspecimen has been deposited in our laboratory. After growing on PDA medium at 28 °C for 5 d, the fungus *Diaporthe*sp. IFB-3lp-10 was inoculated into Erlenmeyer flasks (1 L) containing 400 mL of ME liquid medium. After incubation for 4 d at 28 °C on a rotary shaker at 150 rpm, 20 mL of culture liquid was transferred as the seed into 250 mL flasks, each preloaded with the evenly mingled medium (7.5 g of grain, 7.5 g of bran, 0.5 g of yeast extract, 0.1 g of sodium tartrate, 0.01 g of $FeSO_4 \cdot 7H_2O$, 0.1 g of sodium glutamate and 30 mL of H_2O). The fungus was allowed to grow for 30 d at 28 °C with humidity in the range 60–70 %.

1.3 Extraction and Isolation

The air-dried samples (30 kg) were extracted with 95 % EtOH and the organic solvent was evaporated to dryness under vacuum to afford a crude extract (1.2 kg) which gave six fractions (Fr.1, 21g; Fr.2, 75 g; Fr.3, 56 g; Fr.4, 25 g; Fr.5, 36 g; Fr.6, 158 g) upon column chromatography (10×120 cm) on silica gel (6000 g, 200–300 mesh) eluted with a gradient of CH_2Cl_2/MeOH (v/v 100:0, 100:1, 100:2, 100:4, 100:8, 100:20, 0:100, each 20L) based on TLC monitoring. The third fraction was separated on a reversed phase ODS column (4 cm×40 cm) with a gradient of MeOH/H_2O (v/v 20:80, 30:70, 40:60, 50:50, 60:40, 70:30, 80:20, 90:10, 100:0, each 3 L) to give subfractions 3.1–3.9. Purification of subfraction 3.8 and 3.9 by Sephadex LH-20 (MeOH/$CH_2Cl_2$1:1) followed by crystallization from a MeOH/CH_2Cl_2 (1:1) solution gave monoclinic yellow crystals diaporine A (1.6 g).

1.4 Mice and tumor establishment

BALB/c, female mice (aged 6 to 8 weeks) (Vital River Laboratory Animal

Technology Co. Ltd.) were housed in a pathogen-free isolation facility, light/dark cycle of 12/12 h, and fed chow and water ad libitum. $10^6$4T1 breast cancer cells were subcutaneously injected into mice mammary fat pads. Once the tumor (approximately 50 mm^3) appeared the mice were randomly divided into three groups, animals receiving either 1) vehicle as a conctrol 2) compound **1** compound at 0.2 mg/kg bodyweight dose or 3) paclitaxel at 20 mg/kg bodyweight does. Each group consisted of 8 animals. Vehicle or drugs was administered daily for 12 days. Then the animals were sacrificed. tumor weight, body weight and blood counts were monitored for efficacy and toxicity of the compound. F4/80 antibody was used to sort peritoneal macrophages and tumor-associated macrophages by flow cytometry, then harvested to detect macrophages phenotype respectively.

1.5 Semi-quantitative PCR and real-time quantitative PCR

Total RNA was extracted from macrophages by Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Total RNA (1μg) was reverse-transcribed by M-MLV reverse transcriptase (Trans, China) and submitted to real-time quantitative PCR (RT-qPCR). RNA samples were normalized by the housekeeping gene β-actin. This normalization provided the control for the efficiency of RNA extraction as well as the integrity and the amount of RNA. PCR primers were synthesized by Invitrogen Corporation (Carlsbad, CA) and presented in Table S1.

1.6 Cytokine assays by specific ELISA

Interleukin-10 (IL-10) in supernatants was quantified using standard sandwich enzyme-linked immuno sorbent assays (ELISA) according to instruction manuals. Cytokines concentrations are expressed in nanogram per milliliter, as calculated from calibration curves from serial dilutions of murine recombinant standards (eBioscience) in each assay. The sensitivity of ELISA was 20 pg/mL.

1.7 Western Blot

Cell cultures were harvested and then suspended in whole cell lysis buffer purchased from Beyotime (Haimen, Jiangsu, China). Protein concentration was determined by BCA reagent from Pierce (Rockford, IL, USA).The proteins (50 μg per sample) were electrophoresed on 10% sodium dodecyl sulfate polyacrylamide gels, and then electrotransferred onto polyvinylidene fluoride membrane (Amersham, UK).

1.8 Arginase Activity Assay

Arginase activity was measured in 10^4 cell lysed with 25μl of 0.5% Triton X-100 containing 1mM phenylmethylsulfonyl fluoride (PMSF). We added 20μl 25mM Tris-HCl, pH7.5, and 5μl of 10mM $MnCl_2$, and the enzyme was activated by heating for 3 min at 56 °C. Then 25 μl 0.5 M L-arginine was added and hydrolyzes at 37 °C for 90 min. After the reaction was terminated by 200 μl $H_2SO_4/H_3PO_4/H_2O$ addition, 25 μl 2-Isonitrosopropiophenone was used to react with product urea. Then 200μl 95% ethanol was added, followed by the measurement on a spectrophotometer at 540nm, with 570nm as the reference wavelength.

1.9 Flow Cytometry Analysis

Cells were incubated on ice for 30 minutes with the antibodies that mentioned in the results below. Then cells were analyzed on a fluorescence-activated cell sorting (FACS) calibur cytometer using Cellquest software (Becton Dickinson). The statistics presented are based on 10,000 events gated on the population of interest.

1.10 Luciferase reporter gene assay

PPRE luciferase reporter plasmids, PPARγ plasmid, and pRL-renillin were transfected into HEK-293 cells by Lipofectamine 2000 (Invitrogen). Following an overnight transfection, the cells were treated with compound **1** for 24 h. The cells were harvested and luciferase reporter gene assays were carried out using the Dual-Luciferase kit (Promega). Luciferase activity was normalized to renillia activity.

1.11 Nitric oxide detection

Nitric oxide (NO) levels were detected by the Griess reaction. Briefly, macrophages were seeded in 6-well plates at 10^6 cells/well overnight. Then, cells were treated with 10 ng/ml IL-4 and 20 ng/ml LPS, no treated as control. After incubation for 24h, 10 μM **1** was added into IL-4-treated group, the same concentration of DMSO as control. Cultured for 24h and 100 μl of supernatants were collected and mixed with an equal volume of Griess reagent at room temperature for 10 min. the absorbance was measured at 540 nm by microplate reader. Serial dilutions of 0.1 M $NaNO_2$ were used as a standard curve. NO production of each group was expressed referring standard curve.

1.12 Statistical analysis

Statistical analysis was performed using GraphPad Prism 5 software (GraphPad Software, Inc., San Diego, CA). All values were expressed as the mean±standard error (SEM). A value of $P<0.05$, $P<0.01$ or $P<0.001$ was considered statistically significant and was indicated by *,** or *** respectively.

Table S1. Primer sequences used for RT-qPCR

Gene	Forward primer (5' -> 3')	Reverse primer (5' -> 3')
β–Actin	CTAAGGCCAACCGTGAAAAG	ACCAGAGGCATACAGGGACA
TLR4	GGACTCTGATCATGGCACTG	CTGATCCATGCATTGGTAGGT
TRAF6	ATGCAGAGGAATCACTTGGCA	ACGGACGCAAAGCAAGGTT
PPARγ	GGAAGACCACTCGCATTCCTT	GTAATCAGCAACCATTGGGTCA
MyD88	ATGGGCTGTGATCGGAACTG	GTCTTCCCAATAAGCATGTCTCC
c-Fos	GAATCCGAAGGGAACGGAATAAG	CAATCTCAGTCTGCAACGCA
c-Jun	TTCCTCCAGTCCGAGAGCG	TGAGAAGGTCCGAGTTCTTGG
JNK	TGACGTGGACGAGCTTTCAC	GGGTCTTCTTATCCTGGGTGC

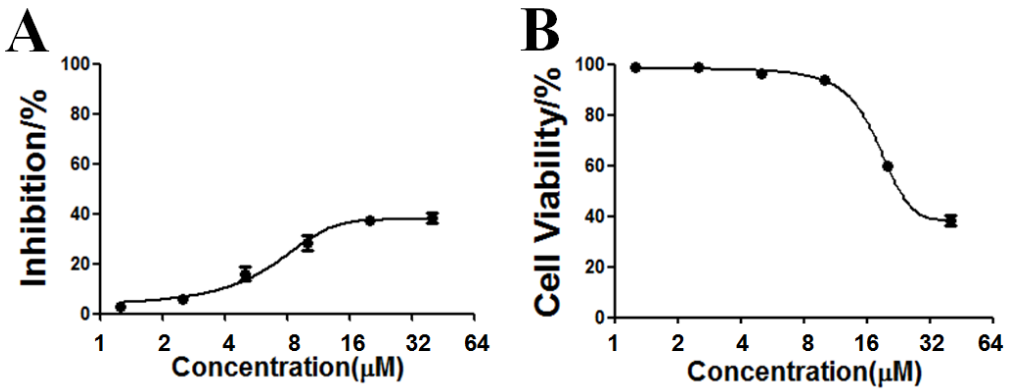

Figure S1 The effect of **1** with different concentrations(1.25, 2.5, 5,10,20,40μM) on arginase activity inhibition (A) and cell viability(B) *in vitro*.

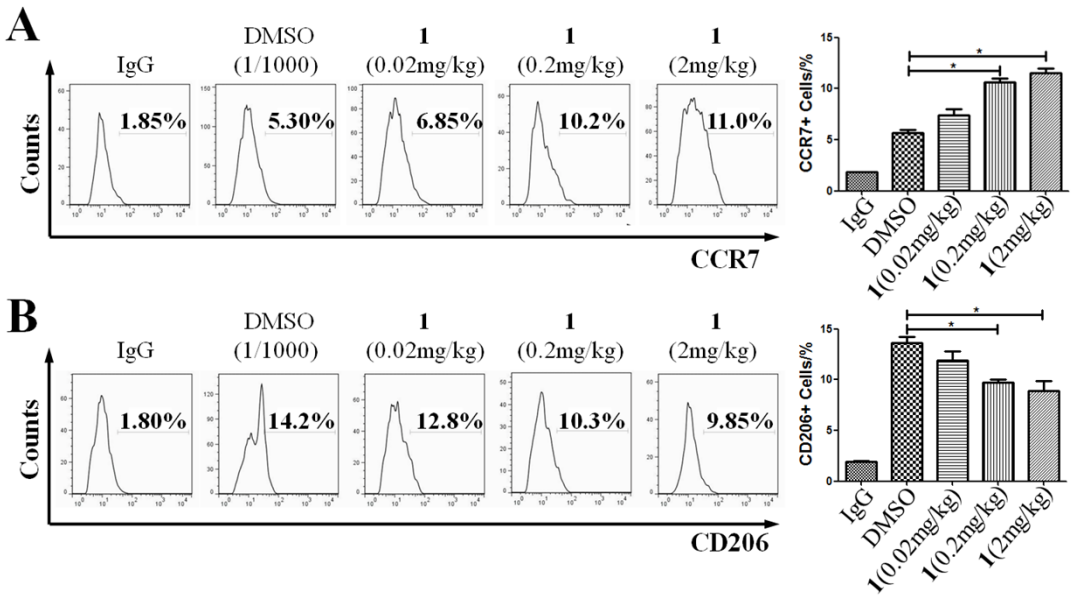

Figure S2 The effect of **1** with different dose (0.02mg/kg, 0.2mg/kg, 2mg/kg) on the expression of CCR7(A) and CD206(B) *in vivo*.

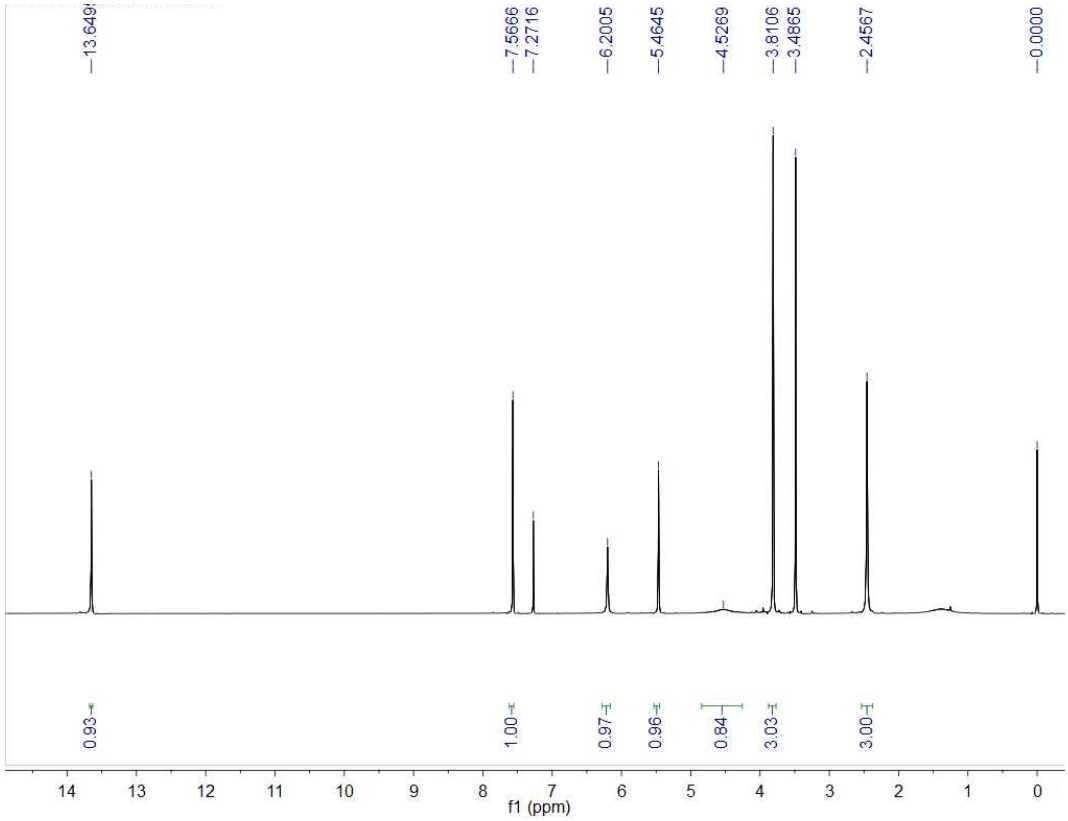

Figure S3. ^{1}H NMR (500 MHz) spectrum of **1**.

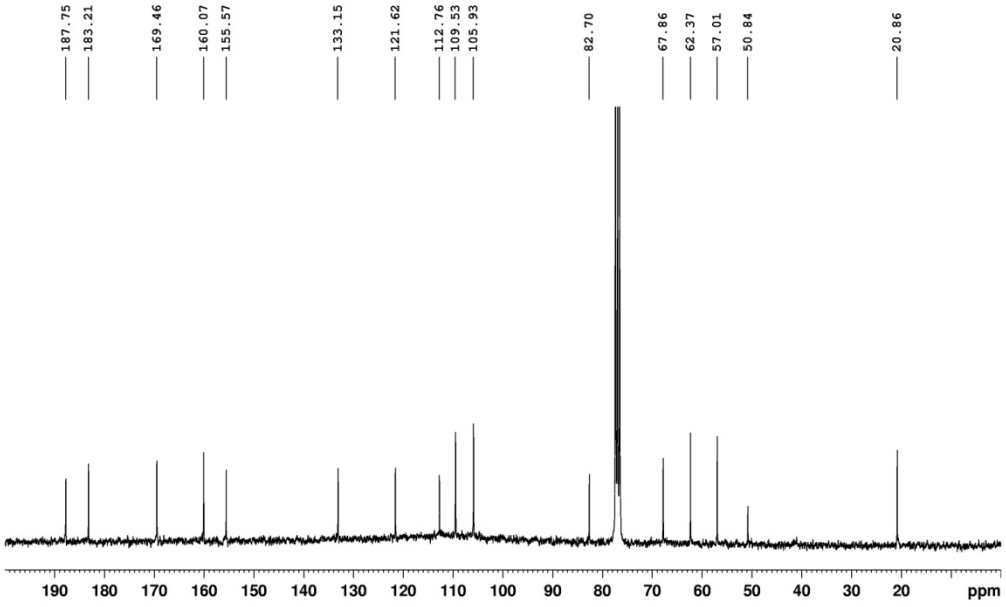

Figure S4. ^{13}C NMR (125 MHz) spectrum of **1**.

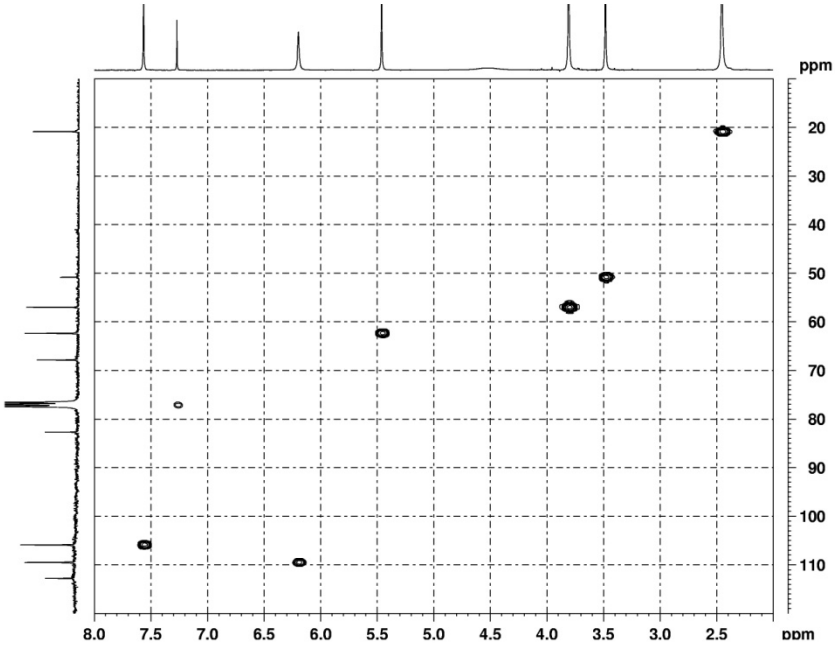

Figure S5. HMQC spectrum of **1**.

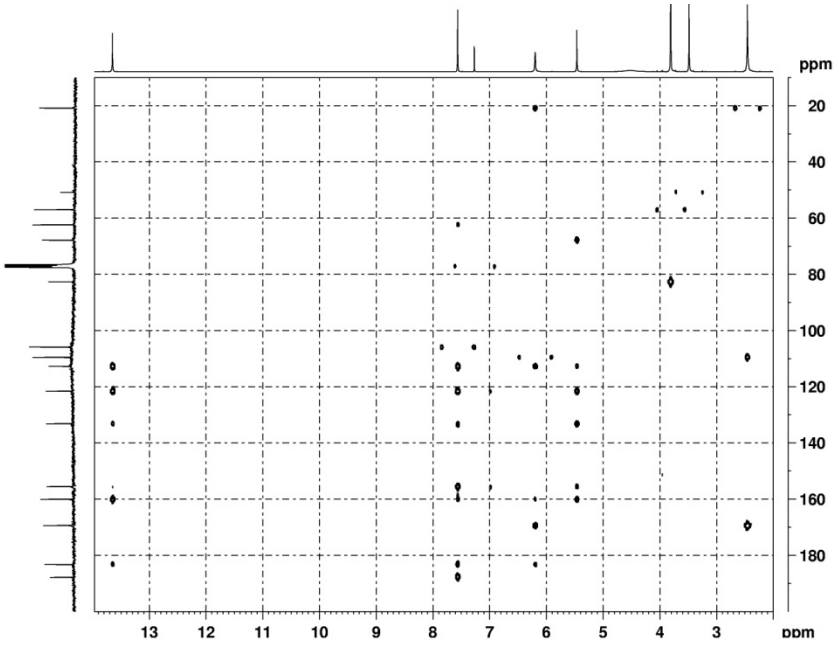

Figure S6. HMBC spectrum of **1**.