JOURNAL OF NATURAL PRODUCTS

Article

pubs.acs.org/jnp

Asperjinone, a Nor-Neolignan, and Terrein, a Suppressor of ABCG2-Expressing Breast Cancer Cells, from Thermophilic *Aspergillus terreus*

Wen-Ying Liao,[†,‡] Chia-Ning Shen,[‡] Li-Hua Lin,[§] Yu-Liang Yang,[⊥] Hsin-Ying Han,[‡] Jing-Wei Chen,[†] Sheng-Chu Kuo,*[,†] Shih-Hsiung Wu,*[,§,∥] and Chih-Chuang Liaw*[,∇]

[†]Graduate Institute of Pharmaceutical Chemistry, China Medical University, Taichung 402, Taiwan, Republic of China

[‡]Genomics Research Center, Academia Sinica, Taipei 115, Taiwan, Republic of China

[§]Graduate Institute of Biochemical Science, National Taiwan University, Taipei 106, Taiwan, Republic of China

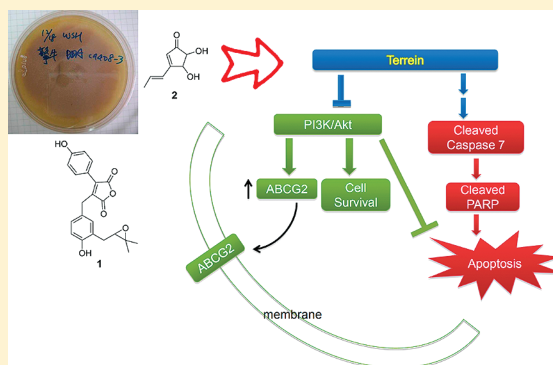
[⊥]Agricultural Biotechnology Research Center, Academia Sinica, Taipei 115, Taiwan, Republic of China

[∥]Institute of Biological Chemistry, Academia Sinica, Taipei 115, Taiwan, Republic of China

[∇]Department of Marine Biotechnology and Resources, National Sun Yat-sen University, Kaohsiung 804, Taiwan, Republic of China

Supporting Information

ABSTRACT: Breast cancer cells express ABCG2 transporters, which mediate multidrug resistance. Discovering a novel compound that can suppress ABCG2 expression and restore drug sensitivity could be the key to improving breast cancer therapeutics. In the current work, one new nor-neolignan, asperjinone (**1**), as well as 12 other known compounds, was isolated from *Aspergillus terreus*. The structure of the new isolate was determined by spectroscopic methods. Among these isolates, terrein (**2**) displayed strong cytotoxicity against breast cancer MCF-7 cells. Treatment with terrein (**2**) significantly suppressed growth of ABCG2-expressing breast cancer cells. This suppressive effect was achieved by inducing apoptosis via activating the caspase-7 pathway and inhibiting the Akt signaling pathway, which led to a decrease in ABCG2-expressing cells and a reduction in the side-population phenotype.



Breast cancer is the leading cause of cancer death in women worldwide,[1,2] and multidrug resistance remains one of the key reasons for chemotherapy failure.[3,4] The ATP binding cassette transporter ABCG2, also known as breast cancer resistance protein (BCRP)/mitoxantrone resistance protein (MXR)/placental ABC protein (ABCP), was first discovered in doxorubicin-resistant breast cancer cells and has been shown to effectively reduce the intracellular concentrations of several prominent anticancer chemotherapeutic agents such as topotecan, mitoxantrone, and doxorubicin.[5] Furthermore, the expression of ABCG2 has been found to correlate with the side-population (SP) phenotype,[6] which was first defined by Goodell et al.[7] as the ability of hematopoietic stem cells to exclude Hoechst 33342 or rhodamine 123. Recently, the subset of ABCG2-expressing breast cancer cells displaying SP phenotype was found to express characteristics of cancer stem cells (CSCs), which underlie the causes of breast cancer recurrence and chemoresistance.[8−11] Therefore, discovering a novel compound that can suppress ABCG2 and restore drug sensitivity could be a key to improving breast cancer therapeutics.

Fungi inhabit a wide range of environments, including extreme environments such as hypersaline waters, tropical forests, and deserts. It has been estimated that there are around 1.5 million different fungal species on earth, although only about 5% have been taxonomically described.[12] A wide variety of fungally derived bioactive compounds have been described, including, for example, a number of antibacterial agents (e.g., cephalosporins), immunosuppressive drugs (e.g., cyclosporins), and anticancer candidates (e.g., plinabulin and fumagillin).[13] Among these fungi, *Aspergillus* has been recognized as a valuable source of novel bioactive compounds. For example, the cholesterol-lowering drug lovastatin was first isolated from *Aspergillus terreus*. *A. terreus* has been found to produce a number of other potent bioactive metabolites,[12] such as terreineol, terreulactone A, terrein, terreic acid, aspulvinones, and butyrolactone I. Some of these compounds display strong cytotoxic activity against colon, pancreatic, lung, and prostatic cancer cell lines.[14,15]

On the basis of our cytotoxic screening of the ethyl acetate extracts from a series of indigenous Formosan thermophilic fungi, we found the extract of *A. terreus* (C9408-3) showed significantly cytotoxicity toward human A549 lung cancer cells at an IC_{50} of 0.8 ± 0.3 μg/mL. In the current study, we identified a novel nor-neolignan, asperjinone (**1**), and 12

Received: October 27, 2011
Published: February 23, 2012

ACS Publications © 2012 American Chemical Society and American Society of Pharmacognosy

known compounds from thermophilic *A. terreus* (Figure 1). Among them, terrein (**2**) displayed cytotoxicity against MCF-7

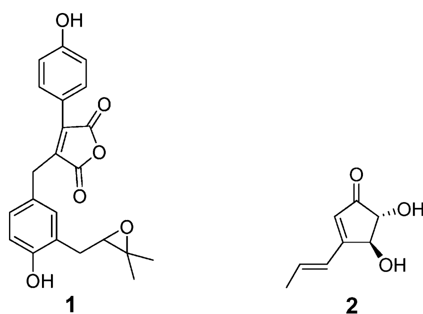


Figure 1. Structure of the natural compounds isolated from *A. terreus*.

breast cancer cells. Although other groups have already shown that terrein (**2**) can be isolated from *A. terreus*,[16−18] this is the first time that terrein (**2**) has been shown to display cytotoxic potency comparable to that of paclitaxel. Treatment with terrain (**2**) significantly suppressed the growth of ABCG2-expressing breast cancer cells, which was mediated by apoptosis through activating the caspase-7 pathway and inhibiting Akt signaling. These findings suggest that terrein (**2**) may serve as a potential ABCG2 inhibitor for the treatment of drug-resistant breast cancer cells.

■ RESULTS AND DISCUSSION

Following bioactivity-guided fractionation using an in vitro measure of cytotoxicity, 13 compounds were isolated from an ethyl acetate extract of *A. terreus*, including one novel nor-neolignan as well as three butyrolactones, five asterric acids, three quinones, and one polyketide (Figure 1 and Supporting Information Figure S1).

Compound **1** was obtained as a yellewish oil, and its molecular formula was determined to be $C_{22}H_{20}O_6$ by FTMS. The IR spectrum showed the presence of phenolic OH at 3433 cm^{-1} and an ester/lactone carbonyl at 1761 and 1683 cm^{-1}. The ^{13}C NMR spectrum of **1** (Table 1) exhibited the presence of 22 carbon resonances, containing two carbonyl carbons (δ_C 166.8 and 165.7), seven quaternary aromatic carbon atoms, seven aromatic methines, one oxygen-bearing quarternay carbon (δ_C 77.0), one oxygen-bearing methine (δ_C 68.8), two methylenes, and two methyls. The ^1H NMR spectrum of **1** (Table 1) revealed the presence of a 1,4-disubstituted phenolic moiety at δ_H 7.63 and 7.01, a 1,2,4-trisubstituted phenol at δ_H 6.99, 6.99, and 6.66, one methylene at δ_H 3.98 and 3.97, one oxymethine at δ_H 3.76, one methylene at δ_H 2.94 and 2.67, and two methyl groups at δ_H 1.33 and 1.22. The HMBC correlations between δ_H 2.94 and 2.67 (H_2-7″)/δ_C 77.0 (C-9″), 68.8 (C-8″), 129.6 (C-2″), and 152.2 (C-4″) and between δ_H 1.33 (H_3-11″) and 1.22 (H_3-10″)/δ_C 77.0 (C-9″) and 68.8 (C-8″) suggested the presence of an epoxide-substituted isoprenyl side chain (Figure 2a), of which the stereochemistry is uncertain due to the amount limitation. The HMBC correlations between δ_H 7.63 (H-2′, 6′)/δ_C 140.7 (C-2) and between δ_H 3.97 and 3.98 (H_2-5)/δ_C 166.8 (C-4), 140.7 (C-2), 137.5 (C-3), 129.6 (C-2″), and 127.5 (C-1″) suggested the presence of a tetrasubstituted double bond linked to the aromatic moieties (Figure 2b). In addition, the molecular formula $C_{22}H_{20}O_6$ of **1** required 13 indices of hydrogen deficiency (IHD). Apart from a total of 12 IHD deduced from the aforementioned moieties (two aromatic rings, two carbonyls, two olefinic carbons, and an epoxide moiety), **1** should possess one more ring bridged between two aromatic rings. Exhaustive analysis of the NMR data of compound **1** exhibited some similarity to butyrolactone III, a synthesized analogue of butyrolactone I with an epoxide-substituted isoprenyl side chain,[19] differing by an oxygen-bearing quaternary carbon (δ_C 86−84) in butyrolactones.[19,20] From these results, including the remaining IHD and the carbonyl group (δ_C 165.7), a furan-2,5-dione moiety was proposed to connect to both benzene moieties in **1**. The structure of **1** was deduced as 3-(3-((3,3-dimethyloxiran-2-yl)methyl)-4-hydroxybenzyl)-4-(4-hydroxyphenyl)furan-2,5-dione and was named asperjinone.

The 12 known compounds were identified as terrein (**2**),[16−18] butyrolactone I,[19] butyrolactone IV,[19] butyrolactone V,[21,22] geodin hydrate,[23] methyl 3,5-dichloroasterric acid,[24] asterric acid,[25] dihydrogeodin,[26] 2-(3,5-dichloro-2,6-dihydroxy-4-methylbenzoyl)-5-hydroxy-3-methoxybenzoic acid,[26] ques-

Table 1. ^1H and ^{13}C NMR Spectroscopic Data (600 MHz, acetone-d_6) for Asperjinone (**1**)

position	δ_C	type	δ_H (J in Hz)	HMBC[a]
1	165.7	C		
2	140.7	C		
3	137.5	C		
4	166.8	C		
5	29.2	CH_2	3.97, d (11.2)	C-2, 3, 4, 1″, 2″
			3.98, d (11.2)	
1′	119.0	C		
2′, 6′	131.5	CH	7.63, d (8.1)	C-2, 1′, 2′, 4′
3′, 5′	115.8	CH	7.01, d (8.1)	C-1′, 4′
4′	160.3	C		
1″	127.5	C		
2″	129.6	CH	6.99, m	C-4″, 6″, 7″
3″	120.9	C		
4″	152.2	C		
5″	117.0	CH	6.66, d (8.6)	C-3″, 4″
6″	127.3	CH	6.99, m	C-5
7″	31.2	CH_2	2.67, dd (16.9, 8.0)	C-2″, 3″, 4″, 8″, 9″
			2.94, dd (16.9, 5.0)	
8″	68.8	CH	3.76, m	
9″	77.0	C		
10″	19.7	CH_3	1.22, s	C-8″, 9″, 11″
11″	25.3	CH_3	1.33, s	C-8″, 9″, 10″

[a]HMBC correlations, optimized for 6 Hz, are from proton(s) stated to the indicated carbon.

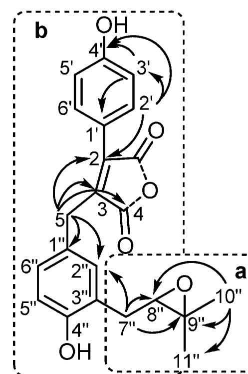


Figure 2. Structural fragments and HMBC correlations (→) of compound **1**.

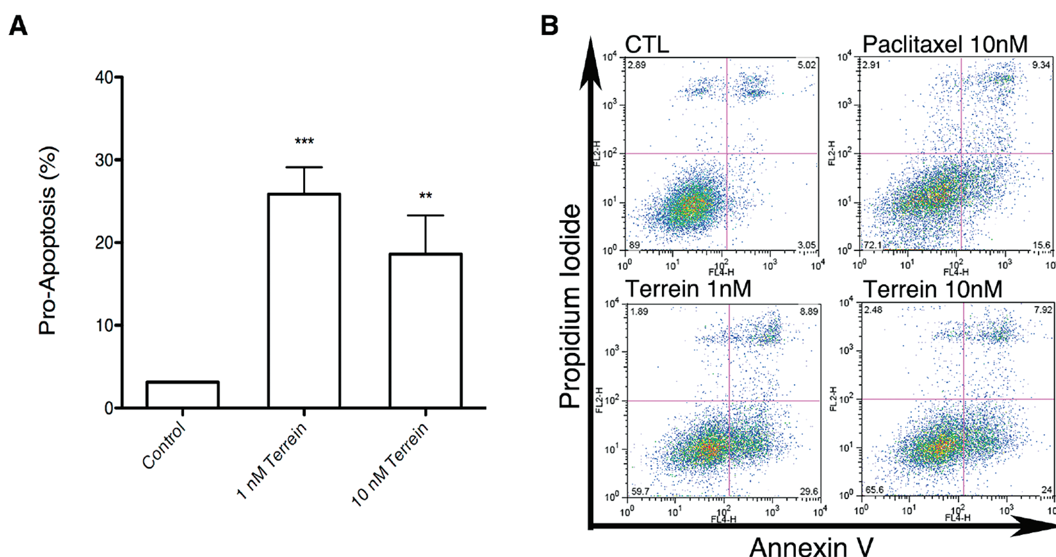


Figure 3. Terrein induced cell apoptosis. (A) Apoptosis was measured by annexin-V and PI staining after 96 h of treatment with 1 or 10 nM terrein. Data shown represent at least three independent experiments. (B) Dot plots displaying the percentages of apoptotic cells in paclitaxel- or terrein-treated MCF-7 cell cultures after 96 h of incubation.

tin,[27] ω-hydroxyemodin-5-methyl ether,[28,29] and ω-acetylcarviolin[28,29] by comparison of their UV, IR, ^{1}H NMR, ^{13}C NMR, and MS data with those reported in the literature (see Supporting Information).

The cytotoxicity of the 13 compounds isolated from *A. terreus* was determined, and it was shown that terrein (**2**) was 100-fold more potent than paclitaxel. The IC_{50} value of terrein against breast cancer MCF-7 cells was 1.1 nM, and that of paclitaxel was 0.1 μM. Incubation of MCF-7 cells with 1 nM terrein reduced the growth rate by 70% (Supporting Information Figure S7). None of the other compounds related were active at 100 μM. Furthermore, terrein also displays cytotoxicity against pancreatic cancer PANC-1 cells (IC_{50} value, 9.8 μM) and liver cancer HepG2 cells (IC_{50} value, 66.8 nM).

To determine whether this reduction in cell growth induced by terrein was mediated by apoptosis, FACS analysis was performed using propidium iodide (PI) and annexin-V staining. We found that treating MCF-7 cells with either 1 or 10 nM terrein induced generation of annexin-V-positive pro-apoptotic cells (10 nM paclitaxel treatment served as a positive control; Figure 3). Western blot analysis further confirmed that treatment with 1 nM terrein generated the cleaved form of caspase-7 and poly(ADP-ribose) polymerase (PARP) (Figure 4A). These results suggested that the reduction in the cell growth rate induced by terrein was mediated by apoptosis via activating the caspase-7 pathway.

It has been shown that expression of ABCG2 transporters mediates multidrug resistance of breast cancer cells.[30,31] We therefore determined whether terrein treatment could reduce ABCG2 expression in MCF-7 cells. Based on Western blot analysis, terrein treatment largely downregulated ABCG2 (10 nM paclitaxel treatment served as a positive control; Figure 4A). We further confirmed, utilizing FACS analysis, that either 1 or 10 nM terrein could significantly reduce the ABCG2-positve subpopulation of MCF-7 cells (10 nM paclitaxel treatment served as a positive control; Figure 4B). We also investigated the effect of terrein treatment on the growth of $ABCG2^+$ subpopulations. Figure 4B indicates that the cytotoxic effects of paclitaxel or terrein on $ABCG2^+$ subpopulations of MCF-7 cells were achieved in a dose-dependent manner during the 96 h incubation. Significant reduction in the $ABCG2^+$ subpopulation of MCF-7 cells was obtained after treatment with 1 or 10 nM terrein.

The SP phenotype of breast cancer cells is mediated primarily via the expression of the ATP binding cassette transporter ABCG2.[6,32−37] A recent study further confirmed that MCF-7 SP cells are composed of tumorigenic $CD24^{-/low}CD44^+$ breast cancer stem cells and express tumor antigen Mucin 1 (Muc1).[38] We therefore investigated whether the SP phenotype of MCF-7 cells was affected by paclitaxel or terrein treatment. MCF-7 cells were incubated with Hoechst 33342 in the presence of 10 μM fumitremorgin C (FTC, an inhibitor of ABCG2 transporter, used as the control treatment) or 150 μM verapamil, paclitaxel, terrein for 90 min. The percentages of SP cells were then analyzed using flow cytometry. SP cells generally maintained a discrete population on the lower-left side of the FACS analysis plot. As shown in Figure 5A, the percentage of SP cells in the control group was about 2.7%. In the terrein-treated group, the percentage of SP cells was reduced to 0.8%. The ability of terrein to suppress the SP phenotype suggested that it could inhibit the growth of breast cancer stem cells. The PI3K/Akt signaling pathway is known to play a key role in regulating expression and membrane localization of ABCG2 in stem cells and in cancer cells.[39] To determine whether terrein treatment could reduce ABCG-expressing cells via inhibiting the Akt pathway, Western blot analysis was performed. We found that a 6 h treatment of MCF-7 cells with terrein significantly downregulated Akt and phosphorylated Akt (10 μM LY294002, a PI3K/Akt pathway inhibitor, served as a positive control; Figure 5B). The results suggest that the ability of terrein to downregulate ABCG2 and to suppress the SP phenotype is possibly due to the downregulation of Akt activity.

Terrein was first identified from *A. terreus* in 1935.[16] Previous reports have shown that terrein is a potent inhibitor of 20S proteasome,[40] can suppress keratinocyte proliferation,[41] and can block melanogenesis.[42] However, the mechanism governing these actions remains to be determined. Previous work has shown that Akt/PKB inactivation may inhibit melanocyte proliferation and decrease melanin synthesis. Our current work

dx.doi.org/10.1021/np200866z | *J. Nat. Prod.* 2012, 75, 630−635

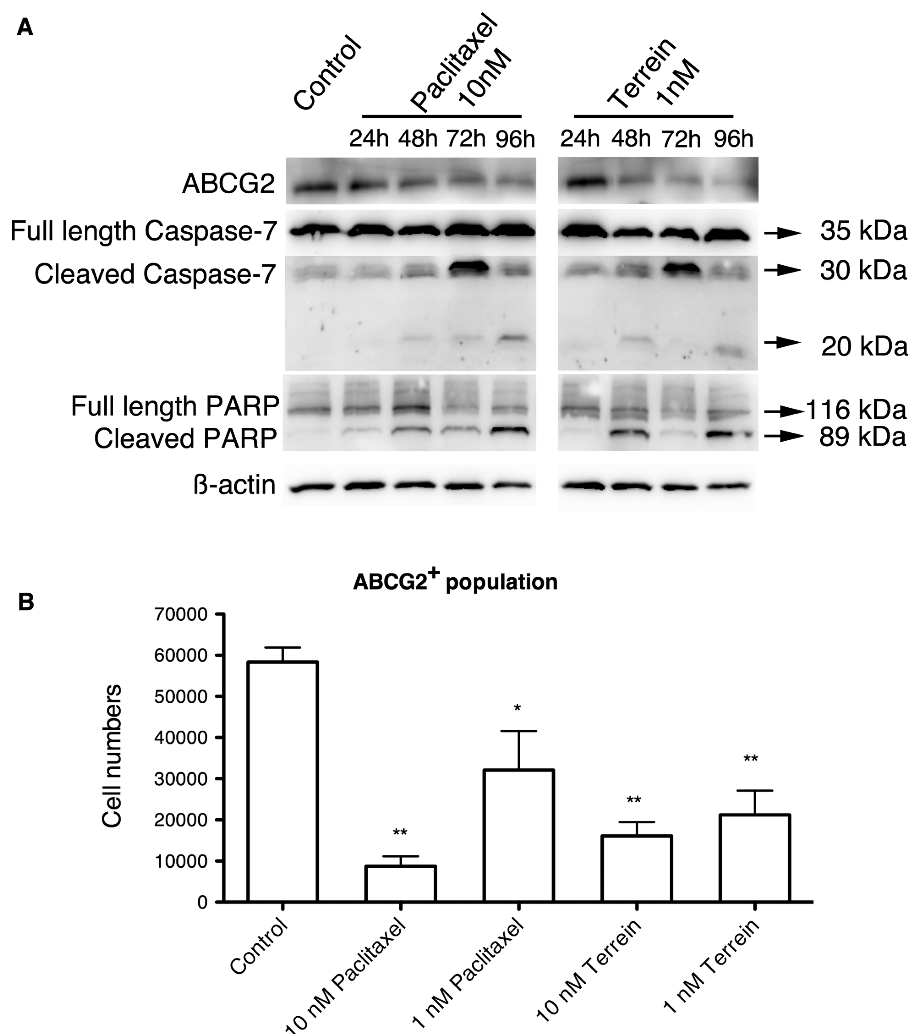


Figure 4. Terrein activated the cleavage of caspase-7 and PARP and reduced ABCG2-expressing cells. (A) MCF-7 cells (2.0×10^5 cells) were treated with 10 nM paclitaxel or 1 nM terrein for 24, 48, 72, and 96 h before protein extraction. Western blots probed with anti-ABCG2, caspase-7, and PARP antibodies. Equal amounts of protein were used (40 μg per lane). (B) Total cell numbers of the ABCG2$^+$ population. MCF-7 cells (2.0×10^4 cells in 12-well plates) were stained with ABCG2 after 96 h of drug treatment. Data shown were derived from at least three independent experiments and expressed as the mean ± SD. $^{**}p < 0.01$ or $^{*}p < 0.05$ versus control cells.

showed that terrein was able to downregulate Akt and phosphorylated Akt, which may explain why it can inhibit melanogenesis.

■ EXPERIMENTAL SECTION

General Experimental Procedures. Melting points were measured using a Yanaco MP-500D melting point apparatus and are uncorrected. Optical rotations were measured using a P-1020 polarimeter (Jasco, Easton, MD, USA). UV spectra were recorded on a U-2800 UV–vis spectrophotometer (Hitachi, Tokyo, Japan). IR spectra were taken using an IR Prestige-21 FT-IR spectrometer (Shimadzu, Kyoto, Japan). CD spectra were obtained on a J-715 spectropolarimeter (Jasco, Easton, MD, USA). 1D and 2D NMR spectra were recorded with a Bruker 400 AV (Academia Sinica, Taipei, Taiwan), Bruker 500 AVII (Academia Sinica), and Bruker Avance III 600 MHz NMR (UCSD, USA) with a 1.7 mm Micro-CryoProbe at 300 K using standard pulse sequences provided by Bruker Daltonics (Billerica, MA, USA). HRFABMS were measured with a MAT 95XL spectrometer (ThermoQuest Finnigan, Bremen, Germany), and ESIMS/MS were obtained on a HCT Ultra PTM Discovery system (Bruker Daltonics). HRFTMS were acquired using a Nanomate electrospray ionization robot (Advion, Ithaca, NY, USA) for consecutive electrospray into the MS inlet of the 6.42 T Finnigan LTQ-FT-ICR MS (Thermo-Electron Corporation, San Jose, CA, USA) running Tune Plus software version 1.0 and Xcalibur software version 1.4 SR1 (UCSD, USA). Sephadex LH-20 (Amersham Pharmacia Biotech, Piscataway, NJ, USA) and silica gel 60 (230–400 or 70–230 mesh; Merck & Co, Whitehouse Station, NJ, USA) were used for column chromatography; precoated Si gel plates (silica gel 60 F_{254}; Merck & Co) were used for analytical TLC. Compounds were detected by spraying with 50% H_2SO_4 aqueous solution and then heating on a hot plate. HPLC was performed on an L-2130 pump equipped with an L-2420 UV–vis detector (Hitachi). Sunfire C_{18} (5 μm, 250 × 4.6 mm i.d.) and semipreparative Sunfire C_{18} (5 μm, 250 × 19 mm i.d.) columns (Waters, Milford, MA, USA) were used for analytical and preparative purposes, respectively. FTC was purchased from CalBiochem (San Diego, CA, USA). LY294002 was purchased from Sigma-Aldrich (St. Louis, MO, USA).

Fungal Material. The thermophilic fungal strain *A. terreus* was isolated from the soil of fumaroles from the hot springs zone of the Yangmingshan mountain area, Taipei, Taiwan. The fungal strain was identified by the late Professor Kuei-Yu Chen according to various morphological, biochemical, and physiological characteristics. To investigate the taxonomic position of the strain, the 28S rDNA-D1/D2 gene was amplified using the PCR method with primers ITS1 and ITS4. Then the PCR products were sequenced by Genomics BioScience and Technology Co., Ltd. The conserved regions of the

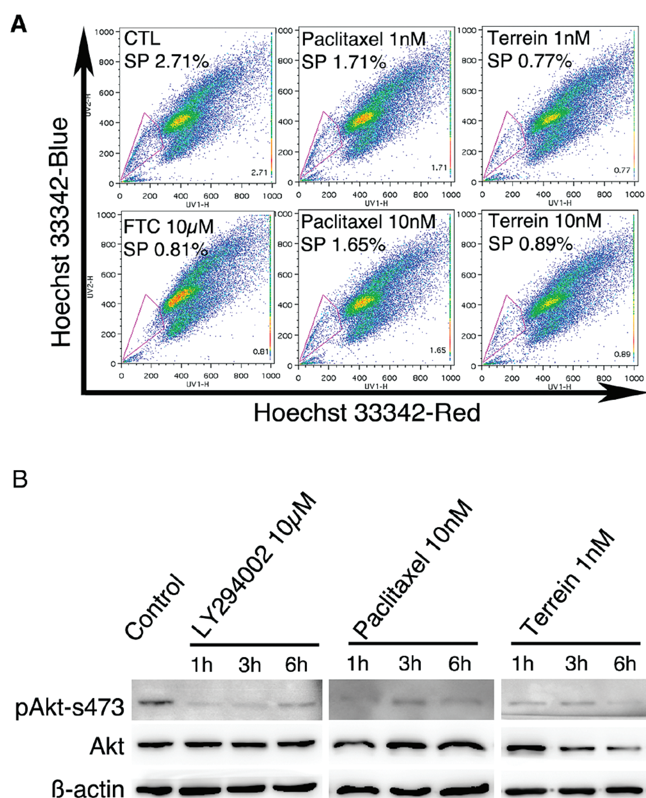


Figure 5. Terrein reduced the SP phenotype and inhibited Akt activity. (A) MCF-7 cells were incubated with Hoechst 33342 in the presence of 10 μM FTC, paclitaxel (1, 10 nM), or terrein (1, 10 nM) for 90 min. The dot plots show that terrein induced the eradication of SP cells. The percentages of SP cells are shown in the upper left. The data shown represent at least three independent experiments. (B) MCF-7 cells (2.0×10^5 cells) were treated with 10 μM LY294002, 10 nM paclitaxel, or 1 nM terrein for 1, 3, and 6 h before protein extraction. Western blots probed with antiphospho-Akt Ser473, total Akt, and β-actin antibodies. Equal amounts of protein were used (40 μg per lane).

18S, 5.8S, and 28S rRNA genes of the strain were compared with other sequences in the public database using the BLAST program to show the highest similarity to strain *A. terreus* NTGMP05 (100.0%). A voucher specimen (C9408-3) was deposited at the Institute of Biological Chemistry, Academia Sinica, Taiwan.

Extraction and Isolation. The fungal strain *A. terreus* (C9408-3) was cultured at 40 °C for 7 days on potato dextrose agar plates. A total of 400 plates of fungal cultures were used for extraction. The mass mycelium and medium were extracted with EtOAc (15 L × 3). The EtOAc extract (3.28 g) was fractionated using a Sephadex LH-20 column eluted with MeOH to yield 20 fractions. Fraction 6 (Asp-f6) was further separated by column chromatography on silica gel with $CHCl_3$−MeOH (15:1) to afford terrein (**2**; 5.2 mg). The residue of Asp-f6 was purified by RP-HPLC (CH_3CN−H_2O, 50:50) to obtain butyrolactone IV (7.1 mg) and butyrolactone I (2.1 mg). Fraction 7 (Asp-f7) was purified by RP-HPLC (Sunfire C18, 250 × 19 mm, 3.0 mL/min, CH_3CN−H_2O [0.05% TFA], 54:46) to obtain asterric acid (8 mg), methyl-3,5-dichloroasterric acid (4.2 mg), butyrolactone V (3.7 mg), and asperjinone (**1**; 1.0 mg). Fraction 8 was purified by RP-HPLC (Sunfire C18, 250 × 19 mm, 3.0 mL/min, CH_3CN−H_2O [0.05% TFA], 54:46) to obtain geodin hydrate (4.5 mg). Fraction 10 was purified by RP-HPLC (Sunfire C18, 250 × 19 mm, 3.0 mL/min, CH_3CN−H_2O [0.05% TFA], 60:40) to obtain 2-(3,5-dichloro-2,6-dihydroxy-4-methylbenzoyl)-5-hydroxy-3-methoxybenzoic acid (8.6 mg) and dihydrogeodin (5.8 mg). Fraction 13 was purified by RP-HPLC (Discovery HS C18, 250 × 10 mm, 3.0 mL/min, CH_3CN−H_2O, 50:50) to obtain ω-acetylcarviolin (9 mg) and questin (2.1 mg). Fraction 16 was purified by column chromatography on silica gel with $CHCl_3$−MeOH (12:1) to obtain ω-hydroxyemodin-5-methyl ether (3.7 mg).

Asperjinone (1): light yellow oil; UV (MeOH) λ_{max} (log ε) 222 (3.58) nm, 280 (3.19) nm; IR (KBr) ν_{max} 3433, 1761, 1683 cm^{-1}; ^1H NMR (acetone-d_6, 600 MHz) and ^{13}C NMR (acetone-d_6, 150 MHz), see Table 1; (+)-FT-MS m/z 381.1366 $[M + H]^+$, 403.1180 $[M + Na]^+$ (calcd 381.1338 and 403.1158 for $C_{22}H_{21}O_6$ and $C_{22}H_{20}O_6Na$, respectively).

Cell Culture. The human breast cancer cell line MCF-7 was obtained from the Bioresource Collection and Research Center (Hsin-Chu, Taiwan) and maintained in α-minimum essential medium (α-MEM) (Invitrogen, Carlsbad, CA, USA) supplemented with 2 mM L-glutamine (Sigma-Aldrich), 1.5 g/L sodium bicarbonate, 0.1 mM nonessential amino acids (Invitrogen, Carlsbad, CA, USA), 1.0 mM sodium pyruvate (Invitrogen, Carlsbad, CA, USA), and 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA).

Cytotoxicity Assays. Isolated compounds were dissolved with dimethyl sulfoxide (DMSO). MCF-7 cells (3.0×10^4 per well) were seeded into 96-well microplates and were treated with a serial dilution of terrein for 48 h. Cytotoxicity was determined using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay (Sigma-Aldrich). Paclitaxel, with an IC_{50} value of 0.1 μM against MCF-7 cells, was used as the positive control.

Analysis of Side-Population Phenotype. MCF-7 cells (1×10^6 cells) were trypsinized and washed three times with PBS before being resuspended in Hanks' balanced salt solution (HBSS; Invitrogen, Carlsbad, CA, USA) containing 2% FBS and 10 mM HEPES, followed by incubation with Hoechst 33342 dye (5 μg/mL) (Sigma-Aldrich) at 37 °C for 90 min with or without FTC (10 μM), paclitaxel (10 nM), or terrein (1 or 10 nM). SP analyses were carried out using a FACSAriaII cell sorter (BD Biosciences, Franklin Lakes, NJ, USA).

Flow Cytometric Analysis. MCF-7 cells were trypsinized and washed three times with PBS before resuspension in HBSS containing 2% FBS and 10 mM HEPES (Invitrogen, Carlsbad, CA, USA). The cell density was adjusted to 10^6/100 μL in staining buffer before being stained with anti-ABCG2 antibodies (5D3; R&D Systems, Minneapolis, MN, USA) for 30 min followed by PE-conjugated horse anti-mouse IgG (BD Biosciences, Franklin Lakes, NJ, USA) for another 15 min. Stained cells were analyzed utilizing a FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) after the addition of PI (2 μg/mL) to exclude dead cells.

Western Blot Analysis. Whole-cell extracts were prepared by lysing the cells with RIPA buffer containing 150 mM NaCl, 50 mM Tris HCl (pH 8), 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, and protease inhibitor and phosphatase inhibitor cocktails (Sigma-Aldrich). The cell extracts were separated by 10% SDS-PAGE and transferred onto a polyvinylidene fluoride membrane (Millipore, Billerica, MA, USA). Samples were incubated in blocking buffer (0.1% Tween 20 and 5% nonfat milk powder in Tris-buffered saline [TBS]) for 1 h at room temperature. Afterward, the membrane was incubated with primary antibody in blocking buffer overnight at 4 °C before being washed twice with TBST (0.1% Tween in TBS) and incubated with the appropriate secondary antibody in blocking buffer for 1 h at room temperature. The blot was developed using ECL Western blotting substrate (Millipore) and analyzed using a luminescent image analyzer (LAS-4000 mini; FujiFilm, Tokyo, Japan). The primary antibodies were used at the following dilutions: rat anti-ABCG2, 1:100 (Abcam, Cambridge, UK); rabbit anti-PARP, 1:1000 (Cell Signaling Technology, Beverly, MA, USA); rabbit anti-caspase 7, 1:1000 (Cell Signaling Technology); rabbit anti-Akt and phosphate Akt-ser 473, 1:1000 (Cell Signaling Technology); and mouse anti-β-actin, 1:10000 (Sigma-Aldrich).

■ ASSOCIATED CONTENT

Supporting Information

1D and 2D selective NMR spectra of asperjinone, as well as the fully assigned NMR data of the known compounds, are available free of charge via the Internet at http://pubs.acs.org.

dx.doi.org/10.1021/np200866z | J. Nat. Prod. 2012, 75, 630−635

AUTHOR INFORMATION

Corresponding Author

*(C.-C.L.) Tel: +886-7-5252000, ext 5058. Fax: +886-7-5255020. E-mail: ccliaw@mail.nsysu.edu.tw. (S.-H.W.) Tel: +886-2-27855696, ext 7101. Fax: +886-2-27889759. E-mail: shwu@gate.sinica.edu.tw. (S.-C.K.) Tel: +886-4-22053366, ext 5608. Fax: +886-4-22078083. E-mail: pharmakuo@gmail.com .

Author Contributions

Co-authors Wen-Ying Liao and Chia-Ning Shen contributed equally to this study.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

The authors would like to thank Prof. Pieter C. Dorrestein (UC San Diego) for collecting NMR and HRMS data. This work was supported in part by National Science Council Grant NSC100-2320-B-110-004 to C.C.L. and by Academia Summit Project Grants 5202402020-0 to C.N.S. and 5202401023-2 to S.H.W.

REFERENCES

(1) Amir, E.; Freedman, O. C.; Seruga, B.; Evans, D. G. *J. Natl. Cancer Inst.* **2010**, *102*, 680−691.
(2) Morrison, B. J.; Schmidt, C. W.; Lakhani, S. R.; Reynolds, B. A.; Lopez, J. A. *Breast Cancer Res.* **2008**, *10*, 210.
(3) Gottesman, M. M.; Fojo, T.; Bates, S. E. *Nat. Rev. Cancer* **2002**, *2*, 48−58.
(4) Moitra, K.; Lou, H.; Dean, M. *Clin. Pharmacol. Ther.* **2011**, *89*, 491−502.
(5) Dean, M. *J. Mammary Gland Biol. Neoplasia* **2009**, *14*, 3−9.
(6) Patrawala, L.; Calhoun, T.; Schneider-Broussard, R.; Zhou, J.; Claypool, K.; Tang, D. G. *Cancer Res.* **2005**, *65*, 6207−6219.
(7) Goodell, M. A.; Brose, K.; Paradis, G.; Conner, A. S.; Mulligan, R. C. *J. Exp. Med.* **1996**, *183*, 1797−1806.
(8) Diehn, M.; Cho, R. W.; Lobo, N. A.; Kalisky, T.; Dorie, M. J.; Kulp, A. N.; Qian, D.; Lam, J. S.; Ailles, L. E.; Wong, M.; Joshua, B.; Kaplan, M. J.; Wapnir, I.; Dirbas, F. M.; Somlo, G.; Garberoglio, C.; Paz, B.; Shen, J.; Lau, S. K.; Quake, S. R.; Brown, J. M.; Weissman, I. L.; Clarke, M. F. *Nature* **2009**, *458*, 780−783.
(9) Li, X.; Lewis, M. T.; Huang, J.; Gutierrez, C.; Osborne, C. K.; Wu, M.-F.; Hilsenbeck, S. G.; Pavlick, A.; Zhang, X.; Chamness, G. C.; Wong, H.; Rosen, J.; Chang, J. C. *J. Natl. Cancer Inst.* **2008**, *100*, 672−679.
(10) Al-Hajj, M.; Wicha, M. S.; Benito-Hernandez, A.; Morrison, S. J.; Clarke, M. F. *Proc. Natl. Acad. Sci. U. S. A.* **2003**, *100*, 3983−3988.
(11) Liu, R.; Wang, X.; Chen, G. Y.; Dalerba, P.; Gurney, A.; Hoey, T.; Sherlock, G.; Lewicki, J.; Shedden, K.; Clarke, M. F. *N. Engl. J. Med.* **2007**, *356*, 217−226.
(12) Bräse, S.; Encinas, A.; Keck, J.; Nising, C. F. *Chem. Rev.* **2009**, *109*, 3903−3990.
(13) Greve, H.; Mohamed, I. E.; Pontius, A.; Kehraus, S.; Gross, H.; König, G. M. *Phytochem. Rev.* **2010**, *9*, 537−545.
(14) Nishio, K.; Ishida, T.; Arioka, H.; Kurokawa, H.; Fukuoka, K.; Nomoto, T.; Fukumoto, H.; Yokote, H.; Saijo, N. *Anticancer Res.* **1996**, *16*, 3387−3395.
(15) Suzuki, M.; Hosaka, Y.; Matsushima, H.; Goto, T.; Kitamura, T.; Kawabe, K. *Cancer Lett.* **1999**, *138*, 121−130.
(16) Raistrick, H.; Smith, G. *Biochem. J.* **1935**, *29*, 606−611.
(17) Wang, Y.; Zheng, J.; Liu, P.; Wang, W.; Zhu, W. *Mar. Drugs* **2011**, *9*, 1368−1378.
(18) Wakana, D.; Hosoe, T.; Itabashi, T.; Nozawa, K.; Okada, K.; de Campos Takaki, G. M.; Yaguchi, T.; Fukushima, K.; Kawai, K. *Mycotoxins* **2006**, *56*, 3−6.
(19) Rao, K. V.; Sadhukhan, A. K.; Veerender, M.; Ravikumar, V.; Mohan, E. V. S.; Dhanvantri, S. D. *Chem. Pharm. Bull.* **2000**, *48*, 559−562.
(20) Parvatkar Rajesh, R.; D'Souza, C.; Tripathi, A.; Naik Chandrakant, G. *Phytochemistry* **2009**, *70*, 128−132.
(21) Lin, T.; Lu, C.; Shen, Y. *Nat. Prod. Res.* **2009**, *23*, 77−85.
(22) Haritakun, R.; Rachtawee, P.; Chanthaket, R.; Boonyuen, N.; Osaka, M. *Chem. Pharm. Bull.* **2010**, *58*, 1545−1548.
(23) Ojima, N.; Takahashi, I.; Ogura, K.; Seto, S. *Tetrahedron Lett.* **1976**, *17*, 1013−1014.
(24) Hargreaves, J.; Park, J. O.; Ghisalberti, E. L.; Sivasithamparam, K.; Skelton, B. W.; White, A. H. *J. Nat. Prod.* **2002**, *65*, 7−10.
(25) Curtis, R. F.; Hassall, C. H.; Jones, D. W.; Williams, T. W. *J. Chem. Soc.* **1960**, 4838−4842.
(26) Katoh, T.; Ohmori, O. *Tetrahedron Lett.* **2000**, *41*, 465−469.
(27) Inamori, Y.; Kato, Y.; Kubo, M.; Kamiki, T.; Takemoto, T.; Nomoto, K. *Chem. Pharm. Bull.* **1983**, *31*, 4543−4548.
(28) Turner, W. B. *Fungal Metabolites*; Academic Press, Inc. Ltd.: London, 1971.
(29) Turner, W. B.; Aldridge, D. C. *Fungal Metabolites II*; Academic Press, Inc. Ltd.: London, 1983.
(30) Doyle, L. A.; Yang, W.; Abruzzo, L. V.; Krogmann, T.; Gao, Y.; Rishi, A. K.; Ross, D. D. *Proc. Natl. Acad. Sci. U. S. A.* **1998**, *95*, 15665−15670.
(31) Krishnamurthy, P.; Schuetz, J. D. *Annu. Rev. Pharmacol. Toxicol.* **2006**, *46*, 381−410.
(32) Hirschmann-Jax, C.; Foster, A. E.; Wulf, G. G.; Nuchtern, J. G.; Jax, T. W.; Gobel, U.; Goodell, M. A.; Brenner, M. K. *Proc. Natl. Acad. Sci. U. S. A.* **2004**, *101*, 14228−14233.
(33) Wulf, G. G.; Wang, R. Y.; Kuehnle, I.; Weidner, D.; Marini, F.; Brenner, M. K.; Andreeff, M.; Goodell, M. A. *Blood* **2001**, *98*, 1166−1173.
(34) Bleau, A.-M.; Hambardzumyan, D.; Ozawa, T.; Fomchenko, E. I.; Huse, J. T.; Brennan, C. W.; Holland, E. C. *Cell Stem Cell* **2009**, *4*, 226−235.
(35) Nakanishi, T.; Chumsri, S.; Khakpour, N.; Brodie, A. H.; Leyland-Jones, B.; Hamburger, A. W.; Ross, D. D.; Burger, A. M. *Br. J. Cancer* **2010**, *102*, 815−826.
(36) Zhou, S.; Schuetz, J. D.; Bunting, K. D.; Colapietro, A. M.; Sampath, J.; Morris, J. J.; Lagutina, I.; Grosveld, G. C.; Osawa, M.; Nakauchi, H.; Sorrentino, B. P. *Nat. Med.* **2001**, *7*, 1028−1034.
(37) Jonker, J. W.; Freeman, J.; Bolscher, E.; Musters, S.; Alvi, A. J.; Titley, I.; Schinkel, A. H.; Dale, T. C. *Stem Cells* **2005**, *23*, 1059−1065.
(38) Engelmann, K.; Shen, H.; Finn, O. J. *Cancer Res.* **2008**, *68*, 2419−2426.
(39) Mogi, M.; Yang, J.; Lambert, J.-F.; Colvin, G. A.; Shiojima, I.; Skurk, C.; Summer, R.; Fine, A.; Quesenberry, P. J.; Walsh, K. *J. Biol. Chem.* **2003**, *278*, 39068−39075.
(40) Demasi, M.; Felicio, A. L.; Pacheco, A. O.; Leite, H. G.; Lima, C.; Andrade, L. H. *J. Braz. Chem. Soc.* **2010**, *21*, 299−305.
(41) Kim, D.-S.; Cho, H.-J.; Lee, H.-K.; Lee, W.-H.; Park, E.-S.; Youn, S.-W.; Park, K.-C. *J. Dermatol. Sci.* **2007**, *46*, 65−68.
(42) Park, S.-H.; Kim, D.-S.; Kim, W.-G.; Ryoo, I.-J.; Lee, D.-H.; Huh, C.-H.; Youn, S.-W.; Yoo, I.-D.; Park, K.-C. *Cell. Mol. Life Sci.* **2004**, *61*, 2878−2885.