(+)-Terrein inhibits human hepatoma Bel-7402 proliferation through cell cycle arrest

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Abstract. Hepatoma is a common malignant tumor. Thus, the development of a high-efficacy therapeutic drug for hepatoma is required. In this study, (+)-terrein isolated from the marine sponge-derived *Aspergillus terreus* PF-26 against cell growth, apoptosis and cell cycle were assessed by MTT and flow cytometry. mRNA array containing 73 cell cycle-related genes and three cell morphology-related genes was generated and its performance evaluated. The cell cycle pathway map was created using the pathview package. The results showed that (+)-terrein inhibited the growth of Bel-7402 cells with alterations in cell morphology and a reduced transcript expression of cell morphology genes (fibronectin, N-cadherin, and vimentin). In addition, flow cytometric analysis revealed that (+)-terrein arrested the Bel-7402 cell cycle without inducing apoptosis. Based on multiple mRNA analysis, the downregulated expression of the *CCND2*, *CCNE2*, *CDKN1C*, *CDKN2B*, *ANAPC*, *PKMYT1*, *CHEK2* and *PCNA* genes was observed in 10 μM (+)-terrein-treated Bel-7402 cells (>2-fold and $P \leq 0.05$), compared with the controls. Thus, the antiproliferative mechanism of (+)-terrein against Bel-7402 cells may be due to the cell cycle arrest by blocking cell cycle gene expression and changing cell morphology.

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Abbreviations: ANAPC5, anaphase-promoting complex subunit 5; Cdk, cyclin/cyclin-dependent kinase; CDKI, Cdk inhibitors; CDKN1C, cyclin-dependent kinase inhibitor 1C; Ct, threshold cycle; DC, dissociation curves; GDC, genomic DNA control; HCC, hepatocellular carcinoma; ink4b, cyclin-dependent kinase 4 ihibitor B; M-CdK, mitotic cyclin-dependent kinase; PCNA, proliferating cell nuclear antigen; SPSS, Statistical Product and Service Solutions; Tm, melting temperature; KEGG, Kyoto Encyclopedia of Genes and Genomes

Key words: (+)-terrein, *Aspergillus terreus*, human hepatoma Bel-7402, cell proliferation, cell cycle arrest

Introduction

Hepatocellular carcinoma (HCC) is the most common malignant tumor and the third leading cause of cancer mortality worldwide (1). In China, HCC causes ~110,000 deaths annually (2). Current chemotherapeutic drugs for treating HCC are restricted in their clinical application because of toxicity and low efficacy (3,4). Arsenic trioxide (As_2O_3) (1-8 μM) arrests HCC (HepG2 and SMMC-7721 cells) in the G_2/M phase (5). However, applications there of are controversial as arsenic compounds exhibit high toxicity. Therefore, developing a novel high-efficacy therapeutic drug for hepatoma is required (4). In 1935, Raistrick and Smith (6) first isolated (+)-terrein from *Aspergillus terreus*. Terreins with distinct configurations have received considerable attention because of their various substantial bioactivities, including anticancer properties against human cells (7-10).

In antitumor therapy, numerous drugs affect tumorigenesis and tumor growth; however, the key is determining which drugs to exploit in the areas of signal transduction, cell-cycle regulation, apoptosis, telomere biology, necrosis, autophagy, cell senescence and angiogenesis (11-13). Angiogenesis is a critical process for tumor growth, invasion, and metastasis (14). Arakawa *et al* (7) determined that (-)-terrein inhibited angiogenin secretion in the tumor angiogenesis of androgen-dependent prostate cancer cells. Apoptosis is a form of cell death (12,15). Furthermore, (+)-terrein suppresses the proliferation of breast cancer cells (9), human cervical carcinoma cells (10), and pulmonary tumor cells (8) by inducing an apoptotic mechanism. These results suggested that terreins inhibit tumor cell growth through multiple strategies.

Findings of studies showed that the isolation and production of (+)-terrein (the molecular formula is $C_8H_{10}O_3$ and the molecular weight is 154; Fig. 1) from the *fungus A. terreus* PF-26 associated with marine sponges were improved to ~9.07 g/l (16-19). However, the activity of (+)-terrein against HCC and its mechanism remain unknown. In this study, the anticancer activity and mechanism of (+)-terrein against HCC were investigated using the Bel-7402 human hepatoma cell line. The results showed that (+)-terrein suppressed Bel-7402 human hepatoma cell growth and proliferation. The

results indicated that 10 μM (+)-terrein induced cell cycle arrest in the G_2/M phase and decreased the cell morphology gene expression of fibronectin, N-cadherin, and vimentin. In addition, the high-throughput platform with parallel detection of multiple mRNAs revealed that treating Bel-7402 cells with (+)-terrein substantially altered the expression of cell cycle-related genes. In addition, (+)-terrein did not induce Bel-7402 cell apoptosis, indicating that (+)-terrein inhibits cell proliferation through distinct mechanisms in different cell strains.

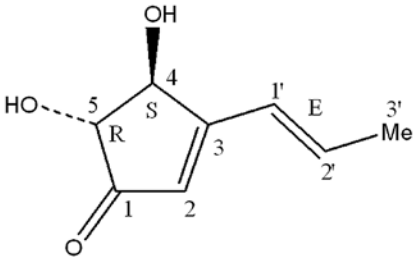

Figure 1. The chemical structure of (+)-terrein.

Materials and methods

Reagents and cell lines. (+)-Terrein (Fig. 1) was isolated from *A. terreus* PF-26, as described previously (18). The isolated (+)-terrein was dissolved in phosphate-buffered saline (PBS, pH 7.2) for subsequent experiments. The human A549 lung adenocarcinoma epithelial cell line was provided by Dr Wei Ma (Shanghai Jiao Tong University, China), and the Bel-7402 human hepatoma cell line was obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). The mentioned cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum and 100 U/ml of penicillin and streptomycin. Unless otherwise mentioned, reagents for cell cultures were purchased from Gibco/Invitrogen (New York, Grand Island, USA) and biochemical reagents were obtained from Sigma (New York, NY, USA) or Ameresco (Solon, OH, USA). The cells were grown in a 5% CO_2 atmosphere at 37˚C.

Cell viability and proliferation. An MTT assay [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] was performed with ~4x10^3 cells/well in 96-well plates. The plates containing the cells were incubated at 37˚C for 24 h, and the cells were treated with (+)-terrein at 37˚C for 48 h. The protocol was performed using an MTT cell proliferation and cytotoxicity detection kit (KeyGen, Nanjing, China) according to the manufacturer's instructions. Briefly, DMEM was supplemented with 50 μl of MTT reagent to each well and incubated at 37˚C for 4 h. Thereafter, the MTT solution was removed. Following the addition of 150 μl of dimethyl sulfoxide (DMSO), the plates were incubated at 37˚C for 15 min to dissolve the formazan crystals. Absorbance of DMSO extracts was detected at 550 nm by using an Enspire 2300 microplate reader (PerkinElmer, Foster City, CA, USA). A total of ~3x10^5 cells/well were inoculated in 6-well plates at 37˚C for 24 h and treated with (+)-terrein at 37˚C for 48 h. The PBS-treated cells served as controls. These cells were used to detect cell morphology, cell cycle, apoptosis, and RNA extraction.

Light microscopy analysis. Cells (3x10^5) were cultured in 6-well plates at 37˚C for 24 h. The Bel-7402 and A549 cells were then treated with 10 μM and 10 mM (+)-terrein at 37˚C for 48 h, respectively, and the PBS-treated cells served as controls. The treated cells were used to observe cell morphology and apoptosis. Light microscopy images of the cells were captured using a Nikon Eclipse Ti-inverted microscope and a Nikon digital sight s-Qi1Mc camera (both from Yokohama, Japan). For each surface, three non-overlapping images were selected.

Cell analysis via flow cytometry. The cells were rinsed once in chilled PBS, digested with 0.25% trypsin (Gibco), and then resuspended in DMEM and 10% serum. The suspended cells were centrifuged at 2,000 x g at 4˚C for 5 min and washed once in cold PBS. The cells were stained with Alexa Fluor® 488 Annexin V and PI by using an Alexa Fluor® 488 Annexin V/Dead cell apoptosis kit (Invitrogen, New York, USA) according to the manufacturer's instructions. The stained cells were analyzed using flow cytometry (FACSAria-II, BD Biosciences, San Jose, CA, USA).

Gene expression analysis of cell cycle and cell morphology. The Bel-7402 cells treated with 10 μM (+)-terrein were trypsinized and washed with a PBS buffer. Fifty microliters of single cell suspension was examined using a cell cycle detection kit (KeyGen). The stained cells were analyzed using flow cytometry (FACSAria II).

Total RNA extraction. The Bel-7402 cells treated with (+)-terrein or PBS were trypsinized and washed with a PBS buffer. The cells were collected using centrifugation at 2,000 x g for 5 min. The cell pellet was then resuspended in RL buffer and centrifuged at 13,000 x g for 5 min. Total RNA was extracted according to the manufacturer's instructions (CWBio, Beijing, China). The purity and concentration of RNA was confirmed by the relative absorbance ratio at 260/280 nm and 260 nm, respectively, by using NanoDrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA).

Reverse transcription. Reverse transcription was performed according to the manufacturer's instructions (Thermo Fisher Scientific). Total RNA (1 μg) was mixed with 4 μl of a 5X reaction buffer, 1 μl of oligo (dT)18 primer, 1 μl of RiboLock RNase inhibitor, 2 μl of a 10 mM dNTP mix, 1 μl of RevertAid reverse transcriptase (100 U/μl), and then, ddH_2O was added to increase the volume to 20 μl. Reverse transcription was performed at 37˚C for 60 min and then at 70˚C for 5 min. The resulting cDNA was stored at -70˚C until use.

Polymerase chain reaction (PCR) array and primer design. Table I shows that 73 potential genes involved in the cell cycle were used as the target mRNAs. *GAPDH*, *b2-MG*, *β-actin*, *RPL27*, *HPRT1*, and *OAZ1* were used as housekeeping genes for the control cells. The primers were designed (CT Bioscience Co., Changzhou, China) to cover all of the transcripts of each gene. Table I shows the RefSeq accession IDs. All of the primers had a similar melting temperature (Tm), and it was not

Table I. Gene information detected by the primers.

Gene ID as in PCR array	Symbol	Gene ID	Gene name
1	ANAPC2	29882	Anaphase promoting complex subunit 2
2	ANAPC4	29945	Anaphase promoting complex subunit 4
3	ANAPC5	51433	Anaphase promoting complex subunit 5
4	BUB1	699	Budding uninhibited by benzimidazoles 1 homolog (yeast)
5	BUB1B	701	Budding uninhibited by benzimidazoles 1 homolog β (yeast)
6	BUB3	9184	Budding uninhibited by benzimidazoles 3 homolog (yeast)
7	CCNE1	898	Cyclin E1
8	CCNE2	9134	Cyclin E2
9	CCND1	595	Cyclin D1
10	CCND2	894	Cyclin D2
11	CCND3	896	Cyclin D3
12	CCNH	902	Cyclin H
13	CDC16	8881	Cell division cycle 16 homolog (*S. cerevisiae*)
14	CDC20	991	Cell division cycle 20 homolog (*S. cerevisiae*)
15	CDC23	8697	Cell division cycle 23 homolog (*S. cerevisiae*)
16	CDC25A	993	Cell division cycle 25 homolog A (*S. pombe*)
17	CDC25B	994	Cell division cycle 25 homolog B (*S. pombe*)
18	CDC25C	995	Cell division cycle 25 homolog C (*S. pombe*)
19	CDC26	246184	Cell division cycle 26 homolog (*S. cerevisiae*)
20	CDC27	996	Cell division cycle 27 homolog (*S. cerevisiae*)
21	CDC6	990	Cell division cycle 6 homolog (*S. cerevisiae*)
22	CDC7	8317	Cell division cycle 7 homolog (*S. cerevisiae*)
23	CDK4	1019	Cyclin-dependent kinase 4
24	CDK6	1021	Cyclin-dependent kinase 6
25	CDK7	1022	Cyclin-dependent kinase 7
26	CDKN1B	1027	Cyclin-dependent kinase inhibitor 1B (p27, Kip1)
27	CHEK1	1111	Checkpoint kinase 1
28	CHEK2	11200	Checkpoint kinase 2
29	E2F1	1869	E2F transcription factor 1
30	E2F2	1870	E2F transcription factor 2
31	E2F3	1871	E2F transcription factor 3
32	HDAC1	3065	Histone deacetylase 1
33	MAD2L1	4085	MAD2 mitotic arrest deficient-like 1 (yeast)
34	MAX	4149	MYC-associated factor X
35	MCM2	4171	Minichromosome maintenance complex component 2
36	MCM3	4172	Minichromosome maintenance complex component 3
37	MCM4	4173	Minichromosome maintenance complex component 4
38	MCM5	4174	Minichromosome maintenance complex component 5
39	MCM6	4175	Minichromosome maintenance complex component 6
40	MCM7	4176	Minichromosome maintenance complex component 7
41	ORC1L	4998	Origin recognition complex, subunit 1
42	ORC2L	4999	Origin recognition complex, subunit 2
43	ORC6L	23594	Origin recognition complex, subunit 6
44	PCNA	5111	Proliferating cell nuclear antigen
45	PKMYT1	9088	Protein kinase, membrane-associated tyrosine/threonine 1
46	RB1	5925	Retinoblastoma 1
47	RBL1	5933	Retinoblastoma-like 1 (p107)
48	SKP2	6502	S-phase kinase-associated protein 2, E3 ubiquitin protein ligase
49	SMC1A	8243	Structural maintenance of chromosomes 1A
50	TOP2A	7153	Topoisomerase (DNA) II α 170 kDa
51	TP53	7157	Tumor protein p53
52	TFDP1	7027	Transcription factor Dp-1

Table I. Continued.

Gene ID as in PCR array	Symbol	Gene ID	Gene name
53	WEE1	7465	WEE1 homolog (*S. pombe*)
54	YWHAE	7531	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation Protein, ε polypeptide
55	CDKN2A	1029	Cyclin-dependent kinase inhibitor 2A
56	CDKN2B	1030	Cyclin-dependent kinase inhibitor 2B (p15, inhibits CDK4)
57	PSMD9	5715	Proteasome (prosome, macropain) 26S subunit, non-ATPase, 9
58	CDKN1C	1028	Cyclin-dependent kinase inhibitor 1C (p57, Kip2)
59	CDC2	983	Cyclin-dependent kinase 1
60	CCNB2	9133	Cyclin B2
61	CCNB1	891	Cyclin B1
62	CDKN1A	1026	Cyclin-dependent kinase inhibitor 1A (p21, Cip1)
63	CDK2	1017	Cyclin-dependent kinase 2
64	CCNA1	8900	Cyclin A1
65	CCNA2	890	Cyclin A2
66	MYC	4609	V-myc myelocytomatosis viral oncogene homolog (avian)
67	CDKN2C	1031	Cyclin-dependent kinase inhibitor 2C (p18, inhibits CDK4)
68	GAPDH	2597	Glyceraldehyde-3-phosphate dehydrogenase
69	ACTB	60	Actin, β
70	B2M	567	β-2-microglobulin
71	HPRT1	3251	Hypoxanthine phosphoribosyltransferase 1
72	OAZ1	4946	Ornithine decarboxylase antizyme 1
73	RPL27	6155	Ribosomal protein L27

located in the genomic repetitive regions. The primers were selected based on criteria such as a typical amplification curve and single peak from the post-PCR melting curve. FN, gene ID 2335; N-cadherin, gene ID 1000; and vimentin, gene ID 7431 which are involved in cell morphology were also investigated.

Real-time PCR analysis. Each cDNA was diluted to 1 ml with ddH_2O and mixed with 1 ml of 2X SYBR Premix Ex Taq™ (Takara, Dalian, China). Twenty microliters of this mixture was added to each well of a 96-well PCR array, except for genomic DNA control (GDC). The 96-well PCR plates containing gene primers were prepared by the CT Bioscience Company (Changzhou, China). The sealed PCR plate was loaded in an Eppendorf Realplex 4S (Hamburg, German). The PCR was performed under the following conditions: 95˚C for 5 min, 40 cycles at 95˚C for 15 sec, 60˚C for 15 sec, and 72˚C for 20 sec. The melting curve procedure (95˚C for 15 sec, 60˚C for 15 sec, and 95˚C for 15 sec) was implemented to analyze the PCR specificity. Dissociation curves (DC) and melting temperatures (Tm) were recorded. Relative changes in gene expression were calculated using the threshold cycle (Ct) method (20). The formula used is presented as follows: n-fold change = $2^{-\Delta\Delta Ct}$ = (Ct target gene - Ct internal control gene) treated sample - (Ct target gene - Ct internal control gene) control sample.

Statistical and pathway analysis. Statistical product and service solutions (SPSS ver. 13.0) was used for the data analysis. All of the experiments were conducted in duplicate. The results are presented as mean ± SD (standard deviation) unless otherwise specified. The P-values were two-tailed. $P \leq 0.05$ was considered to indicate a statistically significant difference. The cell-cycle pathway is a highly regulated process that incorporates three major checkpoints including the participation of several genes (21). The functional pathways associated with the set of differentially expressed genes were analyzed using the Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis (http://www.kegg.jp/kegg/pathway.html). Differentially expressed gene with >2-fold change were analyzed in the cell-cycle pathway (22). The pathway map was created using the Pathview™ package (23).

Results

(+)-Terrein reduces cell growth. The *in vitro* toxicity of (+)-terrein against Bel-7402 cells and the A549 human lung adenocarcinoma epithelial cell line was evaluated using the MTT method [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide] to determine the potential inhibitory concentration. MTT analysis revealed that (+)-terrein inhibited cell viability and proliferation in a concentration-dependent manner. Fig. 2A shows the inhibition of Bel-7402 cells at various (+)-terrein concentrations. The IC_{50} (half maximal inhibitory concentration) value of Bel-7402 was calculated as 11.63 μM ±0.02. The dose-dependent inhibition of Bel-7402 indicated that 1 μM (+)-terrein was non-toxic and 10 μM (+)-terrein was cytotoxic and associated with a survival rate of 57%. The survival rate of the Bel-7402 cells treated with

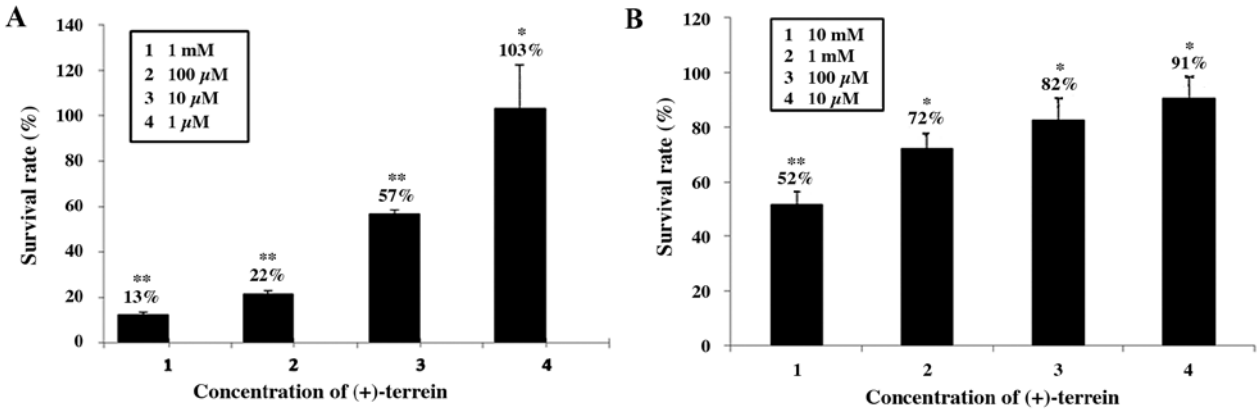

Figure 2. Effect of (+)-terrein on cell viability and proliferation. (A) Effect of (+)-terrein on cell viability and proliferation of Bel-7402 cells. (B) Effect of (+)-terrein on cell viability and proliferation of A549 cells. Bel-7402 and A549 cells were treated with (+)-terrein at various concentrations for 48 h. Viability and proliferation of the cells were examined using the MTT method, and the cell survival rate (%) was calculated. Average values derived from three independent experiments shown as mean ± standard deviations. *Significant difference from control ($P<0.05$). **Significant difference from control ($P<0.01$).

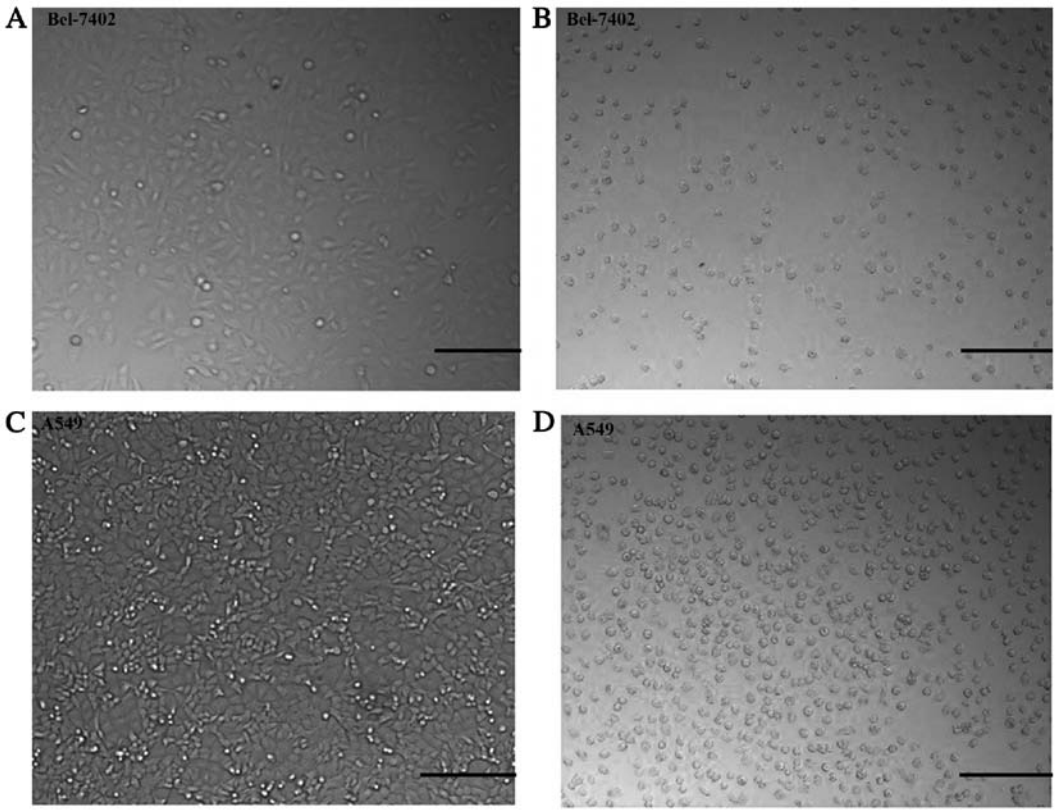

Figure 3. Effect of (+)-terrein on cell morphology of Bel-7402 and A549 cells. Bel-7402 and A549 cells were treated with 10 μM and 10 mM (+)-terrein for 48 h, respectively. Bel-7402 and A549 cells not treated with (+)-terrein served as controls. Bars under each panel represent 50 μm.

100 μM and 1 mM (+)-terrein exhibited inhibitory activities of ~22 and 13%, respectively. A low dose of (+)-terrein did not substantially affect the A549 cells. As indicated in Fig. 2B, (+)-terrein at various concentrations induced inhibitory activity against the A549 cells. (+)-Terrein at concentrations of 10 μM, 100 μM, and 1 mM exhibited weak inhibitory activity, and the survival rate of the treated cells was 91, 82, and 72%, respectively. Moreover, (+)-terrein at 10 mM was cytotoxic to A549 cells, and the survival rate of the treated cells was 52%.

(+)-Terrein induces cell morphology change. We observed a marked phenomenon regarding cell morphology alterations when cells were treated with (+)-terrein. Morphological changes in the Bel-7402 cells exposed to 10 μM (+)-terrein and in the A549 cells exposed to 10 mM (+)-terrein for 48 h were examined using light microscopy (Fig. 3). The Bel-7402 cell morphology alterations were from epithelial-like to spherical, when the cells were treated with 10 μM (+)-terrein (Fig. 3A and B). The morphological changes from epithelial-like to spherical of the A549 cells exposed to 10 mM (+)-terrein for

Table II. Downregulated expression of cell morphology genes in the Bel-7402 cells treated with (+)-terrein.

Gene name	Gene ID	Primers	Expression fold
FN (fibronectin)	2335	F, FN1: 5'-AACCTCGGCTTCCTCCATAA-3' R, FN1: 5'-AACAGTGGGAGCGGACCTA-3'	-3.61
VIM (vimentin)	7431	F, VIM: 5'-GCCAACCGGAACAATGAC-3' R, VIM: 5'-GTGAGGGACTGCACCTGTCT-3'	-2.05
CDH2 (N-cadherin)	1000	F, CDH2: 5'-CTAACCCGTCGTTGCTGTTT-3' R, CDH2: 5'-ACAGAATCAGTGGCGGAGAT-3'	-2.39

The Bel-7402 cells were treated with 10 μM (+)-terrein for 48 h. Each value is the mean of three independent experiments. P≤0.05 and were statistically significant. F, forward; R, reverse.

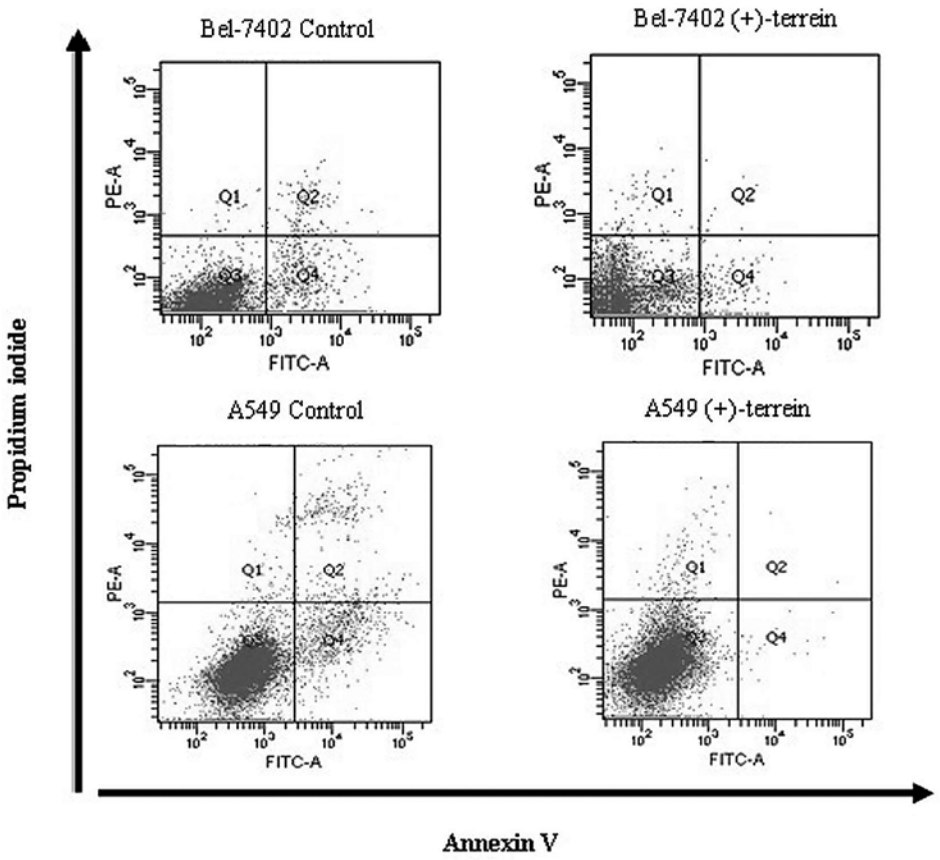

Figure 4. (+)-Terrein induced cell apoptosis and necrosis. Bel-7402 and A549 cells were treated with 10 μM and 10 mM (+)-terrein for 48 h, respectively. Apoptosis and necrosis were monitored using Annexin V/PI analysis. Viable cells were Annexin V/PI-negative (Q_3 quadrant). Early apoptotic cells were Annexin V-positive and PI-negative (Q_4 quadrant). Late apoptotic and necrotic cells were in the Q_1 and Q_2 quadrants, respectively. Each bar is the mean ± SD of five independent experiments.

48 h were examined using light microscopy (Fig. 3C and D). However, 10 μl of (+)-terrein did not significantly affect the morphology of A549 cells (data not shown). The cells were adherent, and all of the experiments were repeated at least five times.

The cell morphology gene (FN, N-cadherin, and vimentin) expression of the Bel-7402 cells treated with 10 μM (+)-terrein was investigated. The results showed that the gene expression of FN, N-cadherin, and vimentin decreased -3.61-, -2.39-, and -2.05-fold, respectively, compared with the control (Table II).

(+)-Terrein inhibits cell apoptosis and necrosis. To determine whether this reduction in cell growth induced by (+)-terrein was mediated by apoptosis, flow cytometric analysis was performed using PI and Annexin-V staining. The results indicated that the apoptotic levels of Bel-7402 and A549 cells treated with (+)-terrein (10 μM and 10 mM) did not increase (Fig. 4), but the number of cells treated with (+)-terrein was less than that of the control cells, according to cell counting (data not shown). The mean percentage ± SD (n=3) of early apoptosis for the Bel-7402 and A549 control cells was

Table III. (+)-Terrein induced cell apoptosis and necrosis.

	Apoptosis (%)		Necrosis (%)	
Cells	Control	Treated cells	Control	Treated cells
Bel-7402	5.33±1.05	2.26±0.50	1.77±0.61	0.83±0.23
A4549	6.17±0.93	0.53±0.31	5.43±2.55	1.93±1.07

Early apoptotic cells (%) were decreased after treatment with (+)-terrein at 10 μM to Bel-7402 or at 10 mM to A549 for 48 h. Cells not treated with (+)-terrein served as controls. Late apoptotic and necrotic cells (%) were decreased after treatment with (+)-terrein at 10 μM to Bel-7402 or at 10 mM to A549 for 48 h. Each value is the mean ± SD of five independent experiments.

Table IV. Effect of (+)-terrein on the cell cycle of the Bel-7402 cells.

Sample	G_0/G_1 (%)	S (%)	G_2/M (%)
Control	59.91±2.8	35.29±2.1	4.8±0.53
Treated cells (10 μM)	56.88±3.5	35.26±2.8	7.36±0.27

The Bel-7402 cells were treated with (+)-terrein at 10 μM for 48 h. Cells not treated with (+)-terrein served as controls. Each value is the mean ± SD of three independent experiments.

5.33±1.05 and 6.17±0.93, respectively, but that for the Bel-7402 and A549 cells after treatment with (+)-terrein for 48 h was 2.26±0.50 and 0.53±0.31, respectively (Table III). Fig. 4 shows that (+)-terrein at 10 μM and 10 mM inhibited early apoptosis. As indicated in Table III, the mean percentage ± SD (n=3) of late apoptosis and necrosis for the Bel-7402 and A549 control cells was 1.77±0.61 and 5.43±2.55, respectively, but that for the Bel-7402 and A549 cells following treatment with (+)-terrein for 48 h was 0.83±0.23 and 1.93±1.07, respectively (Table III). Based on these results, (+)-terrein inhibited late cell apoptosis and necrosis (Fig. 4). Standard deviation was larger than the average deviation; however, for every independent experiment, the percentage value (%) of apoptosis and necrosis in the treated Bel-7402 and A549 cells was lower than that in the control cells.

Effect of (+)-terrein on Bel-7402 cell cycle. Cell proliferation depends on the specific progression of the cell cycle (21). Thus, the cell cycle was analyzed to investigate the anti-proliferative mechanism of (+)-terrein against Bel-7402 cells. Provided the influence of (+)-terrein on A549 occurs at an exceedingly high concentration (>mmol), the anti-proliferative mechanism of (+)-terrein against A549 was not examined. When the Bel-7402 cells were treated with 10 μM (+)-terrein for 48 h, the proportion of the cells in the G_0/G_1 and S phases was reduced, whereas the proportion of cells in the G_2/M phase was increased (Table IV). This result suggested that the cell cycle was arrested by (+)-terrein. Thus, (+)-terrein might decrease Bel-7402 cell growth by inducing cell cycle arrest.

Effect of (+)-terrein on cell-cycle regulators. In this study, high-throughput gene expression analysis of 73 genes was performed (Table I). Cell cycle-related gene expression was performed by comparing the gene expression between cDNA samples of (+)-terrein-treated Bel-7402 cells and control cells. Melting curve analysis was used to assess the specificity of the array. A single product peak observed from each reaction without secondary products indicated a high specificity of PCR assay (data not shown). A subset of differentially expressed genes involved in the cell cycle was selected from all the microarray data by performing initial filtration of the P-value (P≤0.05) and expression level (>2-fold) of the 10 μM (+)-terrein-treated cells. The cell-cycle scheme from the KEGG database (http://www.kegg.jp/kegg/pathway.html) was presented. Downregulated genes were labeled in green, while no upregulated genes were overexpressed (Fig. 5). Compared with the control group, the average expression values of *CCND2*, *CCNE2*, *CDKN1C*, *CDKN2B*, *ANAPC5*, *PKMYT1*, *CHEK2*, and *PCNA* genes in the 10 μM (+)-terrein-treated group were significantly decreased by 5.52-, 3.30-, 5.32-, 2.24-, 2.52-, 2.52-, 2.31-, and 2.10-fold (>2-fold; P≤0.05), respectively (Table V), and the expression level of the remaining 65 genes was evidently unchanged (data not shown). Eight obviously downregulated genes were visualized in the cell-cycle pathway (Fig. 5). Moreover, the results of flow cytometry (Table IV) indicated that (+)-terrein arrested the cell cycle.

Discussion

Numerous drugs affect tumorigenesis and tumor growth through several mechanisms, including signal transduction, cell-cycle regulation, apoptosis, telomere biology, angiogenesis and cell senescence (11,13). Most available cancer drugs are antimitotic and act by interfering with the basic mechanism of DNA synthesis and cell division (20). (+)-Terrein inhibited cell growth at various concentrations in various human tumor cell strains (8-10). The IC_{50} value of (+)-terrein against human breast cancer MCF-7 cells was 1.1 nM (9). The IC_{50} value was 0.29 mM for HeLa cells (10) and 0.3 mM for NCI-H292 (8). Strese *et al* (24) observed that the chemosensitivity of various cell lines was differentially expressed, indicating that distinct cell types with distinct genetic backgrounds exhibit distinct responses to drug treatment (15,25).

Since the adhesion and distribution of anchorage-dependent cells are prerequisites for cell viability and proliferation, cell growth and survival also depend on cell morphology (26). Changes in the synthesis and structure of actin induce changes in cell morphology. FN is a ubiquitous extracellular matrix glycoprotein assembled into an FN matrix in all tissues and

Table V. Downregulated cell-cycle genes in the Bel-7402 cells treated with (+)-terrein.

Gene no.	Gene name	Function in cell cycle	n-fold
1	*CCND2*	G_1 phase and G_1/S transition	-5.52
2	*CCNE2*	G_1/S checkpoint	-3.30
3	*CDKN1C*	Causes arrest of the cell cycle in G_1 phase	-5.32
4	*CDKN2B*	Cell-cycle checkpoint and cell-cycle arrest	-2.24
5	*ANAPC5*	G_2 phase and G_2/M transition	-2.52
6	*PKMYT1*	Protein kinase, membrane-associated tyrosine/threonine 1	-2.52
7	*CHEK2*	Checkpoint kinase 2	-2.31
8	*PCNA*	Proliferating cell nuclear antigen	-2.10

Bel-7402 cells treated with 10 μM (+)-terrein for 48 h and cells not treated with (+)-terrein served as controls. Each value is the mean of three independent experiments.

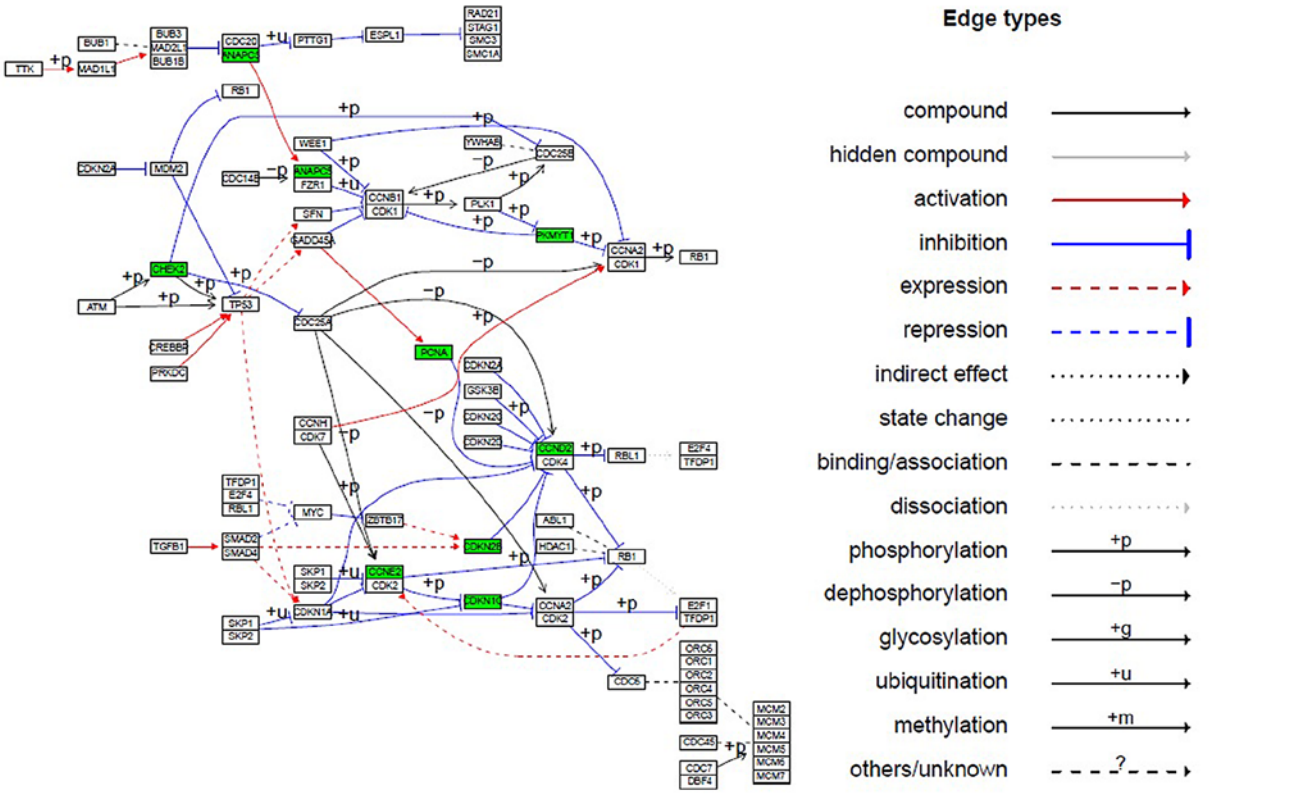

Figure 5. Graphviz view of a cell cycle canonical signaling pathway with gene data. Each box is one gene and the green boxes show the downregulated gene expression of mRNA after the Bel-7402 cells were treated with 10 μM (+)-terrein (>2-fold).

throughout all stages of life. Loss of an FN matrix causes changes in cell morphology, cell signaling, proliferation, and cell-cycle progression (27,28). The aberrant expression of N-cadherin by cancer cells contributes to invasiveness and metastasis by making the cells more motile (29). Vimentin is responsible for maintaining cell shape, adhesion and motility (30).

The inhibitory mechanism of (+)-terrein against the Bel-7402 cell differed from the apoptosis of breast cancer and pulmonary tumor cell lines (9,10). Liao *et al* (9) and Porameesanaporn *et al* (10) reported that (+)-terrein suppressed the growth of breast cancer and HeLa cancer cell lines by inducing apoptosis. Demasi *et al* (8) determined that various ranges of (+)-terrein induced pulmonary tumor cell apoptosis through protease inhibitors.

Kim *et al* (31) determined that (±)-terrein inhibited human epidermal keratinocyte proliferation through extracellular signal-regulated protein kinase inactivation and G_2/M cell-cycle arrest. The fundamental task of the cell cycle is to ensure that DNA is successfully replicated in the S phase and that the identical chromosomal copies are equally distributed between two daughter cells in the M phase (32,33). Cell proliferation depends on the progression of the cell cycle through the G_0/G_1 phase to the S phase (34). In some biological systems, cell-cycle delay and long-term arrest in the G_2 phase

are well documented, but most variation in cell-cycle duration among tissues is due to variability in the length of the G_1 phase (32,33). We determined that (+)-terrein decreased the proportion of the Bel-7402 cells in the G_0/G_1 and S phases (Table IV). The data suggested that (+)-terrein delayed the progression of the cell cycle. The results indicated that 10 μM (+)-terrein inhibited Bel-7402 cell growth (Fig. 2) by arresting the cell cycle in the G_2/M phase (Table IV). The sensitivity of the drug-induced DNA damage was, not only associated with the interaction between drug and target, but also depended on the dose, time (15) and cellular status (34). The results of this study confirmed that various strains (Bel-7402 and A549) exhibit a distinct response to (+)-terrein (Fig. 2). Therefore, the anti-proliferative mechanism of (+)-terrein based on distinct drug doses, time and cellular status remains to be investigated.

Cell-cycle progression in mammalian cells is regulated by various proteins (35). Cell proliferation is closely controlled by positive and negative regulators that determine cell progress throughout the cell cycle (36). The cyclin/cyclin-dependent kinase (Cdk) complexes and Cdk inhibitors (CDKIs) are crucial regulators of cell-cycle progression (37).

Eight genes are related to the cell cycle (Fig. 5). The *CCND2* (38), *CCNE2* (39), *CDKN1C* (40) and *CDKN2B* (41) genes, positively regulated the G_1 and G_1/S phases of the cell cycle, whereas the *APC* gene negatively regulated the G_2/M transition in the cell cycle (42). Distinct cyclins exhibited distinct expression and degradation patterns, which contribute to the temporal coordination of each mitotic event (38). Cyclin D encoded by the *CCND2* gene forms a complex regulatory subunit of CDK4 or CDK6, the activity of which is required for cell-cycle G_1/S transition (38). Cyclin E encoded by the *CCNE2* gene controls the G_1- to S-phase transition in the cell cycle (39). Cyclin-dependent kinase inhibitor 1C (p57, Kip2), also known as CDKN1C, causes cell cycle arrest in the G_1 phase (40). Cyclin-dependent kinase 4 inhibitor B (ink4b) encoded by the *CDKN28B* gene is a potential factor of cell cycle arrest in the G_1 phase (41). In addition, anaphase-promoting complex subunit 5 (ANAPC5) consists of at least eight protein subunits, including APC5, CDC27 (APC3; MIM 116946), CDC16 (APC6; MIM 603461), and CDC23 (APC8; MIM 603462). The APC/C targets the mitotic cyclins for degradation, resulting in the inactivation of mitotic cyclin-dependent kinase (M-CdK) complexes, promoting exit from mitosis and cytokinesis (42). Although distinct processes are responsible for this inhibition, a crucial process is the activation of the APC/C by Cdh1. This continued activation prevents the accumulation of cyclin, which triggers another round of mitosis instead of exiting (42). The results from the multiple mRNA analysis further proved that the Bel-7402 cells were arrested in the G_2/M phase by 10 μM (+)-terrein.

Membrane-associated tyrosine- and threonine-specific CDC2-inhibitory kinase encoded by the *PKMYT1* gene negatively regulates cell-cycle G_2/M transition (43). A decrease in the expression of *PKMYT1* (Table V) and *ANAPC* (Fig. 5 and Table V) genes was conducive to arresting the cells in the G_2/M phase of the cell cycle. The *CHEK2* gene provides instructions for producing checkpoint kinase 2 (CHK2). CHEK2 activation in response to DNA damage prevents the cell from entering mitosis (44). Proliferating cell nuclear antigen (PCNA) is a nuclear protein that acts as a processivity factor for DNA polymerase ε in eukaryotic cells (45). The expression levels of PCNA varied throughout the cell-cycle progression, and the maximal expression was observed in the G_1 and S phases (46). Furthermore, the PCNA expression level was downregulated as the cells exited the cell cycle and differentiated (47). Based on the PCNA expression level characteristics (46,47), we obtained consistent results indicating that 10 μM (+)-terrein increased the proportion of treated cells in the G_2/M phase (Table IV) and decreased the PCNA expression 2.10-fold, compared with the control group (Table V). Thus, (+)-terrein induced cell cycle arrest by breaking down the balance between multiple gene expressions of the cell cycle.

In conclusion, (+)-terrein exhibited cytotoxicity against the Bel-7402 human hepatoma cell line, yielding an IC_{50} value of 11.63 μM ±0.02. In addition, (+)-terrein induced round-cell morphology of Bel-7402 and A549 cells but did not induce cell apoptosis. Furthermore, (+)-terrein inhibited Bel-7402 human hepatoma cell proliferation and arrested the cell cycle in the G_2/M phase by breaking down the expression of the *CCND2*, *CCNE2*, *CDKN1C*, *CDKN2B*, *ANAPC5*, *PKMYT1*, *CHEK2* and *PCNA* cell cycle-related genes. The results suggest that (+)-terrein inhibited human tumor cell growth through various strategies. The potential application of (+)-terrein remains to be investigated in future studies.

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