

Terrein performs antitumor functions on esophageal cancer cells by inhibiting cell proliferation and synergistic interaction with cisplatin

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Abstract. Terrein is a bioactive fungal metabolite isolated from *Aspergillus terreus*. Besides being a melanogenesis inhibitor, previous studies have revealed that terrein has antiproliferative effects on a number of types of cancer tumors. In the present study, the inhibitory effect of terrein on esophageal cancer was evaluated and the possible underlying mechanisms were investigated. The results revealed that terrein inhibited the proliferation of Eca109 esophageal cancer cells in a dose- and time-dependent manner. Mechanistically, terrein treatment led to the G₂/M phase arrest of Eca109 cells by indirectly regulating *cyclin B1* and phosphorylating the cell division cycle protein 2 genes. Notably, terrein exhibited a synergistic effect on Eca109 cells when combined with cisplatin, which is a commonly used chemotherapeutic drug. Taken together, these findings indicate that terrein suppresses the proliferation of esophageal cancer cells, and may prove to be a novel therapeutic

approach for the treatment of esophageal cancer via inhibiting the proliferation of cancer cells.

Introduction

Esophageal cancer is one of the most common and malignant types of tumor (1). During 2013, ~17,990 new cases of esophageal cancer and ~15,210 esophageal cancer-associated mortalities were estimated to occur in the United States (2). In China, the incidence rate of esophageal cancer during 2007, and the mortality rate associated with esophageal cancer during 2008 increased annually; the mortality rate was higher for males than for females. In addition, the mortality rate increased when the esophageal cancer patients were aged 45 years or older (3). Risk factors for esophageal cancer include columnar metaplasia (Barrett's esophagus) (4), obesity (5) and smoking (6,7). A recent study demonstrated that dietary behavior is also associated with esophageal cancer (8). Despite recent advances in surgical techniques and treatment strategies for esophageal cancer, the five-year survival rate post-surgery is only 20-30% (9). Furthermore, conventional chemotherapeutic drugs used in esophageal cancer treatment, including cisplatin, often have severe side effects that limit their efficacy (10). Therefore, exploration of novel drugs to minimize toxicity and maximize efficacy is required.

Cellular reproduction is carefully regulated to prevent uncontrolled proliferation of cells (11). Carcinogenesis occurs when a cell migrates from its dormant inactive state and enters the cell cycle without stimulation from external growth factors, and by avoiding growth-inhibitory signals (12). Deregulation of the cell cycle and cell cycle-associated factors was revealed in the majority of patients with esophageal cancer. Multiple strategies have been proposed in previous years to impair tumor cell proliferation in esophageal cancer (13). A number of tumors avoid inhibition signaling pathways and escape chemotherapy-induced apoptosis (7); therefore, targeted cell cycle therapy for esophageal cancer presents an alternate treatment strategy.

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Natural products are potential sources of novel anticancer drugs (14-16). Terrein is a bioactive fungal metabolite isolated from *Aspergillus terreus* (17). Terrein has a simple structure and may be easily synthesized (18). Mel-Ab is a mouse derived spontaneously-immortalized melanocyte cell line, which produces large amounts of melanin (19). It has previously been revealed that terrein is able to decrease melanin content, but has no cytotoxic effect, in Mel-Ab cells (20). In addition, terrein was reported to suppress the proliferation of keratinocytes via extracellular signal-related kinase (ERK) signaling pathway inactivation and G₂/M cell cycle arrest (21). Terrein demonstrated cytotoxicity against the MCF-7 breast cancer cell line, comparable to that of paclitaxel (22). Terrein may also inhibit cell proliferation and induce cell cycle arrest in human ovarian cancer cells (23). More recently, it has been revealed that terrein inhibited Bel-7402 human hepatoma cell proliferation through cell cycle arrest (24).

The role of terrein in esophageal cancer remains unclear. In the present study, the possibility that terrein may have an antitumor effect on esophageal cancer was investigated. The Eca109 human esophageal cancer cell line was used to determine the growth inhibitory effect of terrein and its possible underlying mechanisms.

Materials and methods

Cell culture. The, Eca109 human esophageal cancer cell line (provided by Professor Libing Song, Sun Yat-Sen University Cancer Center, Guangzhou, China) was cultured in Dulbecco's modified Eagle's high-glucose medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (Biological Industries, Beit Haemek, Israel) and incubated at 37°C in a humid atmosphere with 5% CO₂.

Antibodies and chemicals. Terrein (T5705; Sigma Aldrich; Merck Millipore, Darmstadt, Germany) was resolved in ethanol at 38.25 mmol/l and stored in the dark at -20°C. MTT (catalog no. #M5655) and dimethyl sulfoxide (DMSO; catalog no. #D8418) were provided by Sigma-Aldrich (Merck Millipore). MTT was resolved in PBS at 5 mg/ml and stored at -20°C. For western blot analysis, antibodies recognizing cell division control 2 (CDC2; catalog no. #9116), phosphorylated (p)-CDC2 (Tyr 15; catalog no. #9111), CDC25C (catalog no. #4688) and cyclin B1 (catalog no., #4138), were obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA). Antibodies recognizing β-actin were purchased from ProteinTech Group, Inc. (Wuhan, China; catalog no. #20536-1-AP). Peroxidase-conjugated anti-mouse IgG (catalog no. #A0168) and anti-rabbit IgG (catalog no., #A0545) were purchased from Sigma-Aldrich (Merck Millipore).

Cell viability assay. Cells were plated in 96-well plates at densities of (1-5) x10³ cells/well and were treated with a variety of terrain (0-40 μmol/l) and/or cisplatin (0-10 μmol/l) concentrations. Following 72 h of drug exposure, cells were treated with MTT solution (5 mg/ml) for an additional 4 h at 37°C. The formazan generated by living cells was dissolved with 150 μl/well DMSO, and absorbance was detected at 490 nm (OD₄₉₀) using an ELx800 Strip Reader (BioTek Instruments,

Inc., Winooski, VT, USA). The percentage of cytotoxicity was calculated as follows: Cytotoxicity (%)=(1-OD₄₉₀ of experimental well)/OD₄₉₀ of control well. The median inhibitory concentration (IC₅₀) was expressed as the drug concentration at which cell growth was inhibited by 50%.

Analysis of in vitro drug interaction. The coefficient of drug interaction (CDI) was used to analyze the synergistic inhibitory effect of drug combination. CDI was calculated as follows: CDI=AB/(AxB). AB is the ratio of the two-drug combination group to the control group in OD490; A or B is the ratio of the single drug group to the control group in OD490. Therefore, CDI <1 indicates synergism, CDI <0.7 indicates a significantly synergistic effect, CDI=1 indicates additivity and CDI >1 indicates antagonism.

Cell cycle analysis by flow cytometry. Cell cycle analysis was performed as previously described (25). Briefly, Eca109 cells were treated with 0/20/40 μM of terrein for 48 h under normal culture conditions, following which they were trypsinized with 0.25% trypsin, washed twice with PBS and fixed with 70% ice cold ethanol at 4°C overnight. Subsequently, the cells were resuspended in PBS supplemented with 1% Triton 100, 0.1 mg/ml RNase (catalog no. #ST576; Beyotime Institute of Biotechnology, Shanghai, China) and 6 μg/ml propidium iodide (PI) (catalog no. #ST511; Beyotime Institute of Biotechnology). Following this, the cell suspensions were incubated at 37°C for 30 min in the dark and analyzed on a BD Accuri C6 flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA).

Apoptosis assay. Eca109 cells were treated with 0/20/40 μM of terrein for 48 h at normal culture conditions, prior to being trypsinized with 0.25% trypsin, washed twice with PBS. The cells were then subject to apoptosis assay using Annexin V-FITC/PI kits (catalog no. #KGA106; Nanjing KeyGen Biotech Co., Ltd., Nanjing, China) according to the manufacturer's protocol. Briefly, cells were suspended with 300 μl binding buffer. Subsequently, 3 μl fluorescein isothiocyanate and PI were added to each cell sample. Following incubation at room temperature for 5 min in the dark, the cell samples were analyzed with a BD Accuri C6 flow cytometer.

Western blotting. The procedure was conducted as previously described (26). The cells were washed three times with PBS and lysed in RIPA buffer (50 mM Tris-HCl, 5 mM EDTA and 150 mM NaCl) with protein inhibitor, in the presence of a protease inhibitor cocktail (catalog no. #78439B; Thermo Fisher Scientific, Inc.). The protein concentration was determined using a bicinchoninic acid assay. Aliquots (25 mg) were separated by 10% SDS-PAGE, transferred to a polyvinylidene difluoride membrane and probed with antibodies against CDC2 (dilution, 1:1,000), p-CDC2 (dilution, 1:1,000), CDC25C (dilution, 1:1,000), cyclin B1 (dilution, 1:1,000) and β-actin (dilution, 1:2,000) at 4°C overnight. Peroxidase conjugated secondary antibodies anti-mouse IgG (dilution, 1:2,000) and anti-rabbit IgG (dilution, 1:2,000) were used at room temperature for 1 h, and the antigen-antibody reaction was visualized using an enhanced chemiluminescence assay (catalog no. #WBKLS0500; Merck Millipore).

Statistical analysis. Statistical analyses were carried out using SPSS version 11.5 software package (SPSS, Inc., Chicago, IL, USA). All evaluations were performed in triplicate and the results are expressed as the mean \pm standard deviation. One-way analysis of variance was used for evaluating the differences between groups. Spearman's rho correlation coefficient was used to analyze continuous independent and dependent variables. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Terrein inhibits the proliferation of Eca109 esophageal cancer cell line in a dose- and time-dependent manner. Previous studies revealed that terrein had anti-proliferation effects on a number of types of cancer (20-23). To determine the terrein antitumor effects of terrein on esophageal cancer, Eca109 esophageal cancer cells were treated with terrein at concentration of 0, 2.5, 5, 10, 20 and 40 μM for various time periods. Cell viability was evaluated by an MTT assay. As presented in Fig. 1, terrein inhibits Eca109 cell proliferation in a dose (Fig. 1A) and time (Fig. 1B) dependent manner. The IC_{50} was $\sim 25 \mu\text{mol/l}$ (Fig. 1). In addition, the tablet clone forming experiment demonstrated that terrein repressed colony formation (Fig. 1C).

Terrein inhibits the proliferation of Eca109 cells by arresting the cell cycle at the G_2/M phase and not promoting cell apoptosis. Inhibition of cell proliferation may be a consequence of the induction of cell apoptosis, necrosis or cell cycle arrest (27). To determine the potential mechanism by which terrein suppresses the growth of Eca109 cells, the cell cycle profile and apoptosis rate of Eca109 cells, following terrein treatment, was investigated. As presented in Fig. 2, treatment with 20 and 40 μM terrein for 48 h increased the proportion of cells in G_2/M phase from 16.2 to 21.3 and 28.0%, respectively (Fig. 2A). However, terrein had little effect on the apoptosis of Eca109 cells (Fig. 2B). These results indicated that terrein suppresses the cell growth of Eca109 cells by inducing G_2/M phase arrest.

Effects of terrein on cell cycle associated proteins. Cell cycle progression from G_2 to M phase requires the activation of M-phase promoting factor (MPF) (28). *Cyclin B1* and *CDC2* was reported to serve a crucial role in regulating G_2/M transition (28). In the present study, to investigate the mechanisms involved in the regulation of G_2/M arrest in Eca109 cells, the expression of G_2/M associated regulators was examined by western blotting. As presented in Fig. 3A, the expression levels of *p-CDC2* (Tyr-15) and *cyclin B1* were elevated in Eca109 cells following treatment with terrein. Compared with *p-CDC2* expression, *CDC2* was unaffected by treatment with terrein. Consistently, the levels of *CDC25C*, a dephosphorylation mediator of *CDC2*, were revealed to decrease subsequent to treatment with terrein (Fig. 3B).

Synergistic inhibitory effect of terrein combined with cisplatin on Eca109 cells. Chemotherapy is one of the most commonly used treatments for esophageal cancer, particularly with unresectable tumors (10). Cisplatin is a conventional

chemotherapeutic drug (29). Previous studies on increasing esophageal cancer cell sensitivity to cisplatin have revealed positive results (30). Therefore, the possible synergistic effect of terrein on Eca109 cells when combined with cisplatin was investigated in the present study. The results revealed that terrein increased the cytotoxicity of cisplatin in Eca109 cells (Fig. 4A). CDI was used to evaluate the synergistic effect. As shown in Fig. 4B, 5 μM cisplatin had the most significant synergistic effect on Eca109 cells when combined with 40 μM of terrein ($\text{CDI} < 0.7$).

Discussion

Esophageal cancer remains one of the most fatal types of cancer worldwide, with an incidence on the rise (9). Despite clinical advances, esophageal cancer remains one of the leading causes of cancer-associated mortality (31). The overall five-year survival rate for patients with esophageal cancer is $\sim 20\%$ (32). Therefore, exploration of novel strategies to treat patients with esophageal cancer is required.

Natural products are potential sources of novel anticancer drugs (33). Previous studies have demonstrated that terrein has antiproliferative activity in a number of types of cancer (20-22,24). Cancer is an uncontrolled cell proliferation and death (12); therefore, inhibiting specific proteins that drive the cell cycle may be an efficient strategy to develop novel anticancer drugs. In the present study, it was revealed that terrein may inhibit the proliferation of Eca109 esophageal cancer cells in a dose- and time-dependent manner, by causing G_2/M phase cell cycle arrest.

Cell cycle progression in mammalian cells is regulated by numerous mechanisms, including cell cycle dependent kinase (CDK) phosphorylation, regulation of cyclin expression and CDK inhibitor (CKI) activity (34). In terms of G_2/M cell cycle regulation, *cyclin B1/CDC2* complexes serve an important role (35). During the G_2 phase the level of *cyclin B1* is increased and, consequently, *cyclin B1/CDC2* complexes are accumulated (35). Subsequently, *cyclin B1* must be degraded by proteolysis prior to the end of the M phase (35). *CDC2* is phosphorylated at Thr-14 and Tyr-15 and held in an inactive state (36). Dephosphorylation of *CDC2* by *CDC25* on Thr-14 and Tyr-15 serves a major role in the activation of *CDC2-cyclin B1* (37). Therefore, phosphorylation of Thr-14 and Tyr-15 are crucial for G_2/M checkpoints (35). The results presented in Fig. 3 demonstrate that terrein treatment induced the accumulation of *p-CDC2* (Tyr15). In addition, *cyclin B1* expression levels were elevated following terrein treatment. Thus, it is reasonable to assume that terrein-exerted G_2/M arrest may be mediated by the upregulation of *p-CDC2* and *cyclin B1* in Eca109 cells. However, considering the accumulation of *cyclin B1*, it may be attributed to an increasing number of cells arrested in the G_2/M phase, the possibility that G_2/M arrest is caused by terrein and mediated by *cyclin B1* accumulation requires further study.

Kim *et al* (21) reported that terrein causes G_2/M phase cell cycle arrest of keratinocytes through the downregulation of *cyclin B1* and *CDC2*, without *CDC2* phosphorylation and upregulation of tumor protein p27 (KIP1). In addition, Liao *et al* (22) reported that terrein demonstrates high cytotoxicity against MCF-7 breast cancer cells, by inducing apoptosis

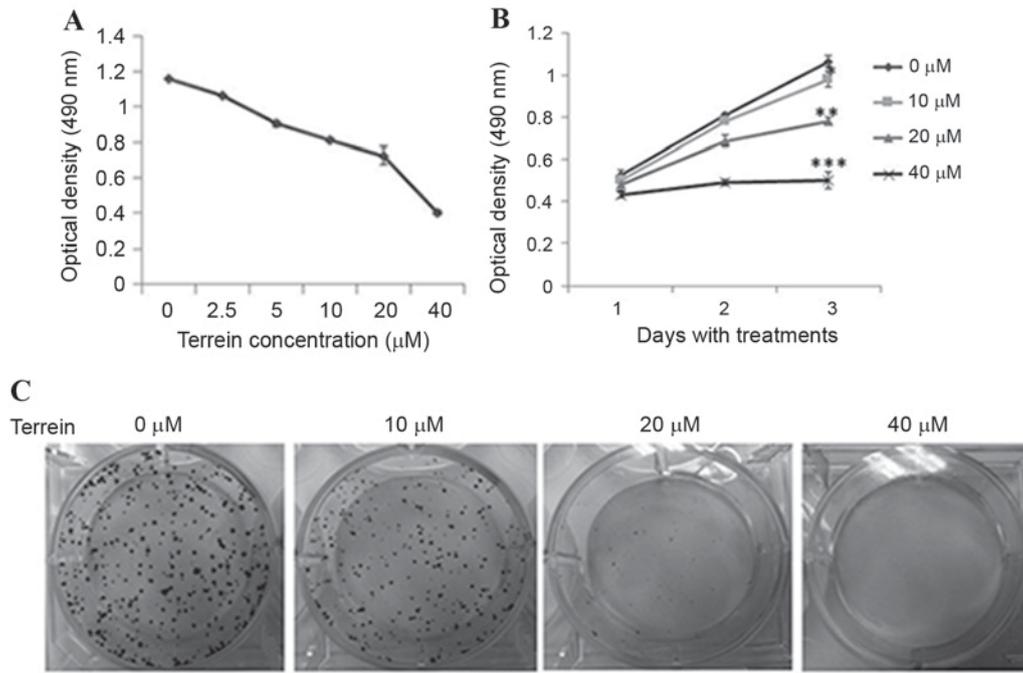


Figure 1. Terrein inhibits Eca109 esophageal cancer cell proliferation in a dose- and time-dependent manner. (A) Eca109 cells treated with 0-40 μ M terrein for three days were treated with MTT solution for 4 h and the absorbance was quantified. Data are presented as the mean of three independent experiments. (B) Eca109 cells were treated with 0-40 μ M terrein for three days and the absorbance was quantified daily. Data are presented as the mean of three independent experiments. ** $P < 0.01$; *** $P < 0.001$. (C) Eca109 cells were plated in 6-well plates (2,000 cells/well) and medium containing 0-40 μ M terrein was changed every four days. Following two weeks, cell colonies were fixed with 4% paraformaldehyde and stained with crystal violet.

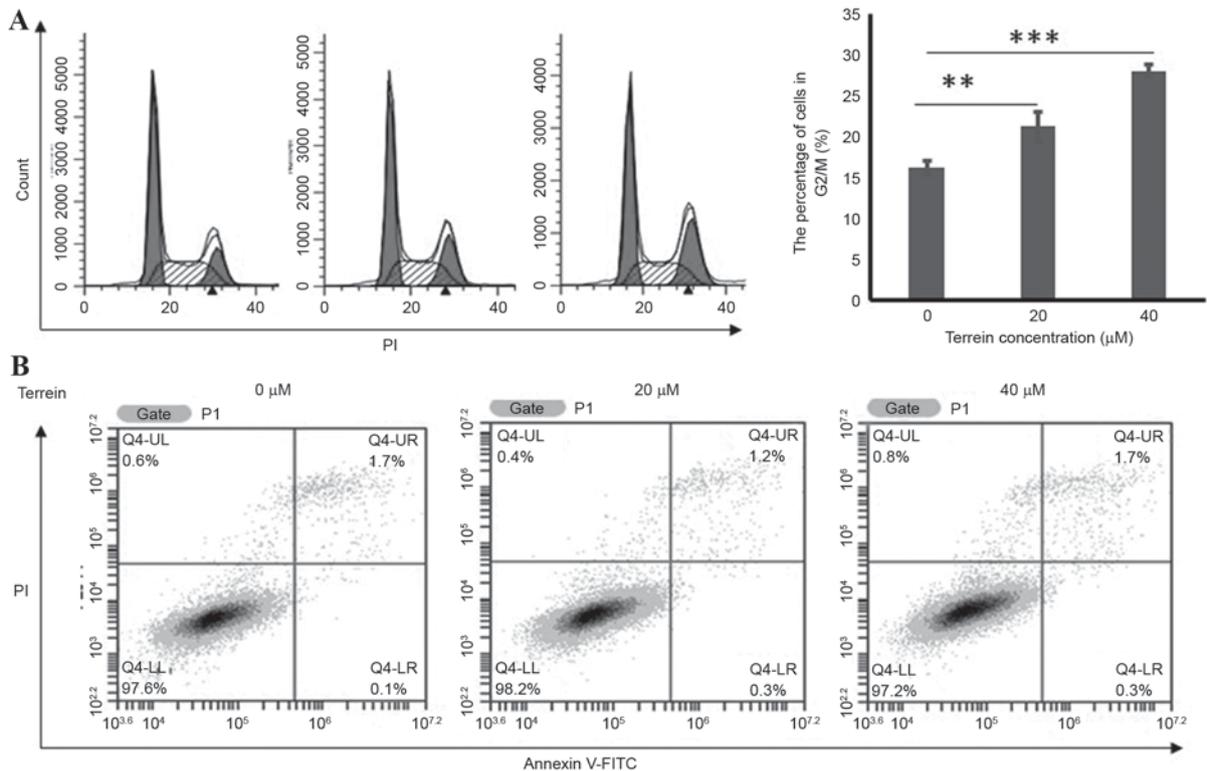


Figure 2. Terrein inhibits Eca109 cell proliferation by causing G₂/M phase cell cycle arrest and not promoting cell apoptosis. Eca109 cells treated with 0/20/40 μ M terrein for 48 h were collected for (A) cell cycle and (B) apoptosis analysis. n=3. ** $P < 0.01$; *** $P < 0.001$. PI, propidium iodide; Annexin V-FITC, Annexin V-fluorescein isothiocyanate.

via activating the caspase-7 signaling pathway and inhibiting the protein kinase B signaling pathway. A previous study

has revealed that terrein induces apoptosis in HeLa human cervical carcinoma cells through p53 and ERK signaling

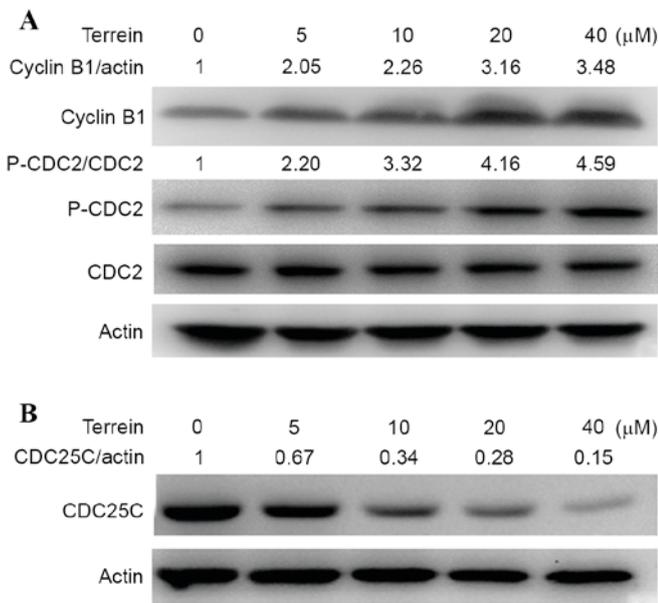


Figure 3. Effects of terrein on cell cycle associated proteins. (A) Terrein treatment upregulated *p-CDC2* and *cyclin B1* expression. Eca109 cells were treated with 0-40 μM terrein for 48 h were examined by western blotting with the indicated antibodies. (B) Terrein suppresses *CDC25C* expression. P-CDC2, phosphorylated cell division cycle protein 2.

pathway regulation (38). However, in the present study terrein demonstrated anticancer effects on Eca109 cells by causing G₂/M phase arrest through the upregulation of *p-CDC2*. The contradiction of terrein's effect on keratinocytes and esophageal cancer cells may be as a result of various cell types and the cellular context. Keratinocytes are normal cells whereas Eca109 are malignant cells, indicating that they have distinct growth mechanisms (21). In addition, the reason terrein demonstrated various effects on MCF7, HeLa and Eca109 cells, may be attributed to various types of cancer.

Chemotherapy is one of the most common treatment methods of esophageal cancer, especially for patients unable to receive resection surgery (30). Conventional chemotherapeutic drugs in esophageal cancer treatment, including cisplatin, often have severe side effects which limits their efficacy (10). Combination therapy may achieve greater therapeutic efficacy and fewer side effects (39). The present study demonstrated that terrein has a synergistic inhibitory effect on Eca109 cells when combined with cisplatin, which indicates that the combination of cisplatin and terrein may be a novel strategy for esophageal cancer treatment.

The biological properties of cisplatin were revealed over 40 years previously, and since then it has had a major impact on the field of cancer treatment, changing the methods of therapeutic tumor management, including for ovarian, testicular and head and neck cancer, and is still widely used today (40). The underlying mechanism by which cisplatin kills the tumor cells involves the formation of intra-strand and inter-strand crosslinks, resulting in the activation of p53-mediated apoptosis (29). Cisplatin-induced DNA damage activates a number of pathways, which temporally induce a transient S-phase arrest, followed by inhibition of the *CDC2-cyclin B1* kinase to effect a durable G₂/M phase

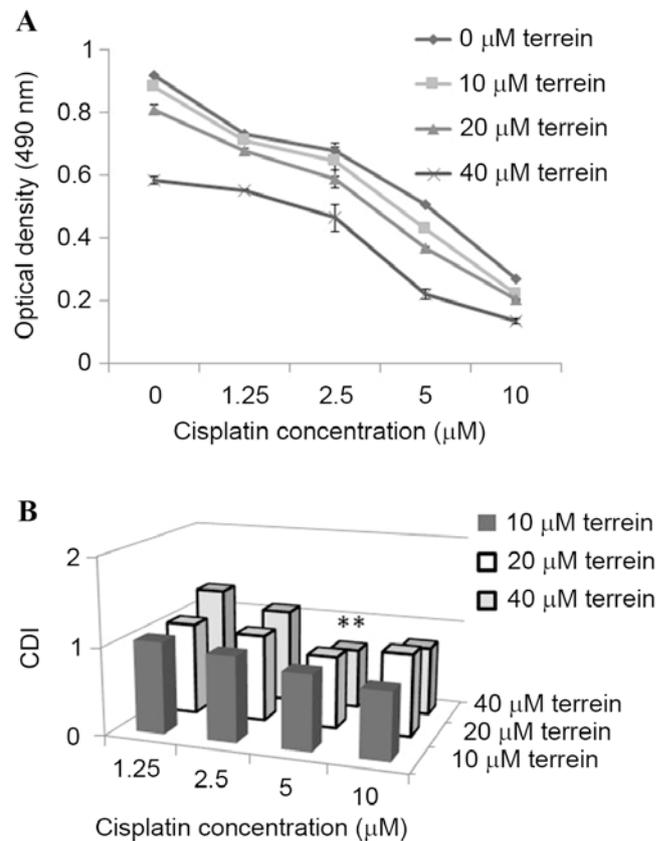


Figure 4. Synergistic inhibitory effect of terrein and cisplatin on Eca109 cells. (A) Eca109 cells were treated with 0-10 μM cisplatin alone or combined with 10/20/40 μM terrein. Three days following treatment, cell viability was examined using an MTT assay. (B) The CDI was used to analyze the synergistic inhibitory effect of various concentrations of cisplatin and terrein combined. n=3. **P<0.01. CDI, coefficient of drug interaction.

arrest (41). The present study revealed that terrein treatment caused Eca109 cell arrest in the G₂/M phase. It was demonstrated that terrein and cisplatin are able to cause G₂/M arrest, and this may be an alternative mechanism by which terrein synergistically interacts with cisplatin to inhibit the proliferation of Eca109 cells. However, the exact mechanism underlying this synergistic effect of terrein and cisplatin needs to be studied further.

In conclusion, the role of terrein on esophageal cancer cells was investigated in the current study. The results suggest that terrein suppresses the proliferation of Eca109 esophageal cancer cells through the induction of G₂/M phase cell cycle arrest. More importantly, combination assays indicate that terrein has synergistic effects on Eca109 cells when combined with addition to cisplatin.

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