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Terrein Inhibits Aggressive Phenotype of A549 Human Lung Cancer Cell through Suppression of HIF-1α

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Abstract

Terrein is a fungal metabolite that has already been reported with anticancer properties. However, the effect on the aggressive phenotype of cancer cells has not been elucidated yet. In the present study, the cytotoxicity of terrein was first determined against lung cancer cells (A549) model and compared with several normal cell lines (Vero, L6, and H9C2 cells). The data demonstrated that terrein had a specific effect on A549 cells relative to normal cells with high selectivity index values. Then, the hypoxic model that recognized to induce aggressive abilities was established in A549 cells by cobalt chloride ($CoCl_2$) stimulation. With this model, terrein could reduce HIF-1α, a marker of hypoxia, and inhibit both migration and invasion of which the effect on invasion is more explicit. Our results demonstrated that terrein has a potential new role as the anti-aggressive phenotype by inhibiting cancer cell migration and invasion through HIF-1α reduction.

Keywords: Terrein, Lung cancer cells, Hypoxia, HIF-1α, Metastasis

Introduction

Lung cancer is one common cancer that has been specified as the leading cause of cancer death worldwide. In 2018, it was estimated that lung cancer caused mortality at 1.8 million deaths or 18.4 % of total cancer. In Thailand, lung cancer was reported at 23,957 (15.1 %) for new cases and 21,371 (20.1 %) as lung cancer deaths, which was ranked second after liver cancer [1]. The high level of mortality was from late diagnosis, in which the patients went through the advanced or metastatic stage [2]. Although standard therapies such as radiotherapy and chemotherapy are suitable for advanced lung cancer treatment, there are still several issues that need to be addressed such as side effects from the treatment, drug resistance, and the high expense of treatment. Therefore, the development of effective anti-lung cancer agents is one promising way to increase patient survival.

Nowadays, the world has turned more attention to use natural substances for cancer treatment. Terrein (4,5-dihydroxy-3-[(*E*)-1'-propenyl]-2-cyclopenten-l-one, $C_8H_{10}O_3$) is one potential natural compound that has been demonstrated in various biological activities. It is a secondary bioactive metabolite that was first isolated from the *Aspergillus terreus* by Raistrick and Smith in 1935 [3]. Numerous studies indicated that terrein has several potential functions, including anti-inflammation, melanogenesis inhibition, and anticancer [4-6]. The anticancer effect of terrein has shown in prostate cancer cells [7], breast cancer cell [8,9], cervical carcinoma cells [5], ovarian cancer cells [10], hepatoma cells [11], head and neck cancer cell [12] and esophageal cancer cells [13]. However, the anticancer effect of terrein on the aggressive phenotype of human lung cancer has not been elucidated yet.

The aggressive phenotypes of cancer cells, especially solid tumors, are typically presented as increasing abilities of cancer progression including migration, invasion, adhesion, and angiogenesis. Also, cancer resistance to chemotherapeutic agents is observed [14]. One factor that has been shown to enhance the aggressive cancer phenotype is the degree of hypoxia within the tumors [15]. A reduction of oxygen level induces stress to cancer cells, which can promote them to get through the aggressive stage or enhance their survival. In this condition, HIF-1α (hypoxia-inducible factors-1α) has been indicated as a critical regulator for cellular adaptation. It is a highly conserved transcription factor that is found in nearly all cell types that mediates the expression of hundreds of genes [16]. Concerning lung cancer, both small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC), the poor prognosis was found to be associated with highly expressed HIF-1α [17]. Therefore, the reduction of HIF-1α level can be a possible way to decrease the aggressive abilities of cancer.

To this aim, we investigated whether terrein, a potential anticancer agent, could inhibit the aggressive phenotypes of lung cancer. Human lung cancer cell (A549) was used as an experimental model, while different normal cell types were used to compare the level of cytotoxicity. We induced the hypoxic condition for aggressive phenotype by applying cobalt chloride ($CoCl_2$) to the A549 model. This chemical is commonly used and reported in numerous studies as an inducer of cellular hypoxia [18, 19]. Then, the cytotoxicity of terrein and its effect on the expression of HIF-1α was firstly investigated. Moreover, the effect of terrein on the aggressive phenotypes was monitored through the metastatic abilities of cancer cells, both migration and invasion.

Materials and methods

Extraction of terrein

The fungus Aspergillus terreus CRI301 was cultivated in sabouraud dextrose agar (30 grams SDB, 1-liter sea water-filtered) under room temperature for 34 days. Then, Aspergillus terreus CRI301 cells and broth culture were filtered to collect only broth. The culture broth was extracted three times with an equal volume of ethyl acetate (EtOAc), and then the EtOAc layers were combined and evaporated to dryness. The crude EtOAc extract was further purified by Sephadex LH-20 column chromatography (2 cm inner diameter and 125 cm long) and eluted with MeOH. The characteristics of terrein was detected by 1H NMR spectroscopic data.

Cell culture

The human NSCLC cell line (A549) (ATCC® CCL-185™) and normal African green monkey kidney (Vero) cell line (ATCC® CCL-81™) were purchased from ATCC. While skeletal muscle cell line (L6) and cardiomyoblast cell line (H9C2) were obtained from Professor Gary Sweeney, Department of Biology, York University, Toronto, Canada. A549 and H9C2 cells were maintained in DMEM containing 10 % fetal bovine serum (FBS) and 1 % penicillin/streptomycin. Vero cells were maintained in EMEM containing 10 % FBS and 1 % penicillin/streptomycin. L6 cells were maintained in AMEM containing 10 % FBS and 1 % antibiotic-antimycotic. All cell types were cultured at 37 °C with 5 % CO_2 and 95 % humidified atmosphere.

Cytotoxicity of terrein and cobalt chloride *($CoCl_2$)*

Cytotoxicity of terrein against lung cancer (A549) cells and different normal cell lines, including Vero, L6, and H9C2 cells were determined by MTT assay as previously described [2]. A549, Vero, L6 and H9C2 cells were harvested by 0.25 % trypsin containing 1 mM EDTA and plated on 96-well plate at 1×10^4, 1.8×10^4, 3×10^4, 2×10^4 cells/well, respectively. After overnight incubation, cell samples were treated with various concentrations of terrein of which A549 at 0 - 1 mM and all normal cell lines at 0 - 2 mM for 24 h. For vehicle control, 0.1 % dimethyl sulfoxide (DMSO) was employed. After incubation, the media were removed, and MTT solution at 0.5 mg/ml was added each well and incubated for 4 h. Next, the pellets were dissolved with DMSO and detected absorbance at 595 nm with a microplate reader (Synergy, BioTek, Winooski, VT, USA). To investigate the cytotoxicity of $CoCl_2$, A549 cells were treated with various concentrations of $CoCl_2$ for 24 h. and performed MTT assay, as mentioned.

HIF-1 alpha transcription factor assay

To induce hypoxia, A549 cells were seeded overnight on a 60-mm culture dish at a density of 8×10^5 cells/dish in DMEM containing 10 % FBS. Then, cells were treated with 600 μM of $CoCl_2$ for 24 h, following with terrein treatment at 0, 20, 40, and 80 μM for 24 h. After that, cellular nuclear proteins were extracted using a nuclear extraction kit (Abcam-ab113474) and further analyzed the level of HIF-1α with the HIF-1α transcription factor assay kit (Abcam-ab133104). By following the instruction, nuclear extracts were added to each well containing HIF-1α response element and incubated overnight at 4 °C. After removing the unbound reagents, HIF-1α primary antibody was added and incubated for 1 h at room temperature. Then, the HRP-conjugated secondary antibody was incubated for another 1 h. After that, a developing solution was added, following the stop solution measuring the absorbance at 450 nm.

Transwell migration and invasion assays

The effect of terrein on the aggressive A549 cell, both migration and invasion were performed as in the previously described method [2]. First, hypoxia was induced in A549 cells by adding 600 μM $CoCl_2$ for 24 h. Then, cells were harvested, resuspended at 5×10^4 in 200 μl of serum-free medium, and treated with terrein at 0, 20, 40, and 80 μM. After that, each reaction was plated on the upper chamber of transwell insert 8 μm pore size (BD Biosciences, New Jersey, USA), which were non-coated for migration assay and Matrigel-coated for invasion assay. For the lower chambers, 750 μl medium containing 10 % FBS were used as a chemoattractant for cell invasion and migration. The plates were incubated at 37 °C for 24 h. Then, transwell inserts were separated and washed twice with cold PBS. Each sample was fixed with 100 % ice-cold methanol for 20 min and washed twice with cold PBS. Then, the samples were stained with 0.5 % crystal violet for 15 min and washed with dH_2O for several times until dye stop coming off. The cells that remained on the upper chamber were removed using a cotton swab. The cells that invaded and migrated through the pore of the insert chamber into the lower chamber were photographed under an inverted microscope and quantified at least five randomly selected fields by Image J software. The percentage of cell invasion and migration were compared to the control vehicle group.

Statistical analysis

All experiments were performed at least triplicates in each group. The data were shown as Means ± SD and analyzed by GraphPad Prism version 5.01 software. Statistical significance was calculated using ANOVA with Dunnett's multiple comparisons posthoc test. At $p < 0.05$ was considered statistically significant.

Results and discussion

The effects of terrein on lung cancer cells viability

We firstly examined the cytotoxic effect of terrein on different cell lines, including A549, Vero, L6, and H9C2 cells by MTT assay. All cell lines were treated with various concentrations of terrein for 24 h and having DMSO at 0.1 % as vehicle control. The results demonstrated that terrein significantly inhibited cell viability of A549, Vero, L6 and H9C2 cells with an IC_{50} value at 229 ± 0.087 μM, 870 ± 0.093 μM, 1,240 ± 0.220 μM and 579 ± 0.080 μM, respectively as shown in **Figure 1** This data indicated that terrein was toxic to lung cancer cells more than all representative of normal cells. To be more specific, we calculated for the selective index (SI) of terrein on A549 cells and compared them with all normal cell lines in which data shown in **Table 1** The SI value was determined according to the following equation: SI = IC_{50} of normal cells/ IC_{50} of A549 cells. SI of terrein was 3.8, 5.4, and 2.5 for A549 cells compared to Vero, L6, and H9C2 cells, respectively. This data suggested that terrein has a specific cytotoxic effect on A549 lung cancer cells. As shown in other reports, terrein was cytotoxic to human cervical cancer cells (HeLa) with IC_{50} at 290 μM [5], human breast cancer cell lines, MCF-7 and MDA-MB-231 cells, were at 2,340 μM and 700 μM, respectively [8]. Our data indicated that the cytotoxicity of terrein on lung cancer cells has a much better effect than other cancer cell types. Importantly, terrein has less effect on normal cells, which indicates the value and potential use as anticancer agent.

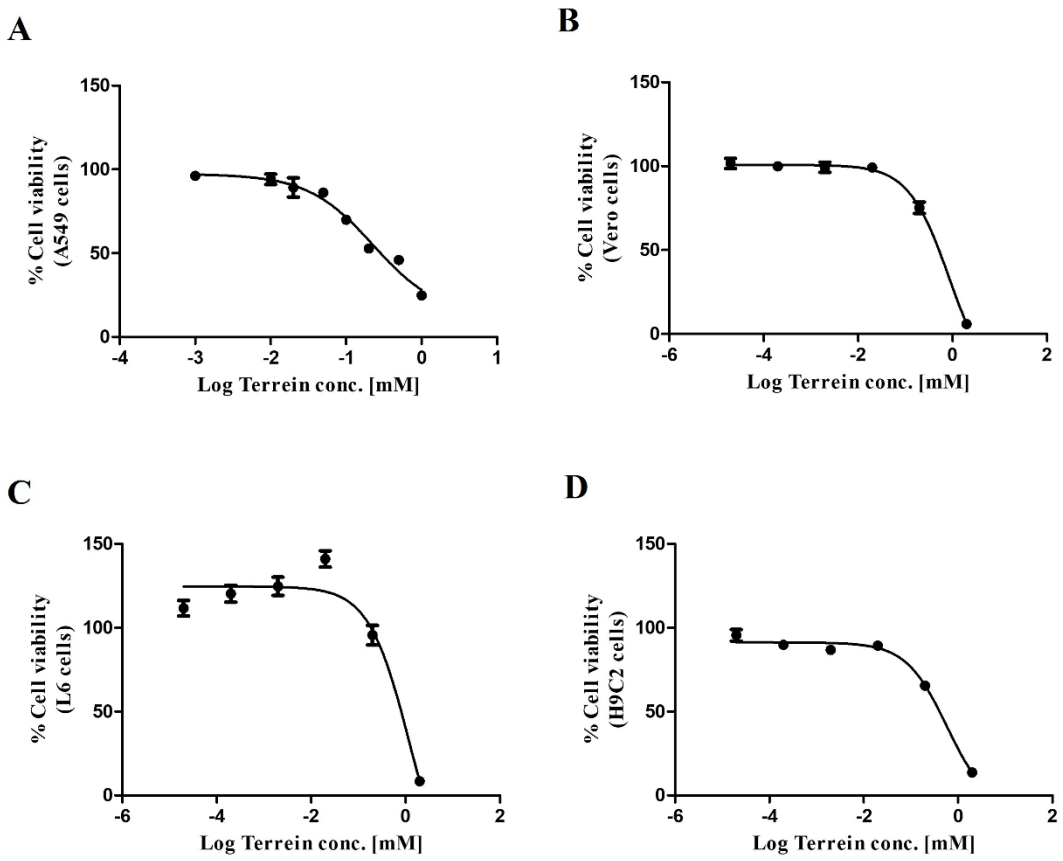


Figure 1 Terrein inhibits cell viability in different cell types. Cell viability was assessed by MTT assay, in which various concentrations of terrein were treated for 24 h. (A) A549 cells (B) Vero cells (C) L6 cells (D) H9C2 cells.

Table 1 The half-maximal inhibitory concentration (IC_{50}) and selective index (SI) of terrein.

Cell lines	IC_{50} (μM)	SI A549 cells
A549 cells	229 ± 0.087	-
Vero cells	870 ± 0.093	3.8
L6 cells	1,240 ± 0.220	5.4
H9C2 cells	579 ± 0.080	2.5

Selective index (SI) = IC_{50} of normal cells/ IC_{50} of A549 cells.

The hypoxic condition in lung cancer cells induced by $CoCl_2$

Hypoxia is one crucial factor that enhances the aggressive ability of cancer cells. Besides, this condition is related to tumor proliferation, clinical stages, and therapeutic efficacy. Then, the reduction of the hypoxic environment becomes another target for cancer treatment. To this aim, we further asked if terrein could affect the hypoxic condition of lung cancer cells, which sequentially inhibit the behavior of

the aggressive phenotype. The hypoxic model of lung cancer was then created by using $CoCl_2$ treatment. This chemical inhibits HIF-lα aryl hydrocarbon-hydroxylase activity which results in decreasing HIF-lα degradation [20]. This effect has been confirmed in several cancer cell types such as lung, breast, pancreas, cervical and neuronal cells [19,21-24]. To establish the hypoxic model, we had preliminarily tested the cytotoxicity of $CoCl_2$ itself against lung cancer cells by MTT assay. As indicated in **Figure 2**, A549 cells were treated with $CoCl_2$ from 1 - 1000 μM for 24 h. The cytotoxicity was observed only at 1,000 μM, in which cell viability of A549 cells reduced to 43.57 % compared with vehicle control. This data demonstrated that the concentration of $CoCl_2$ that not toxic and could be used to set up the model could vary up to 800 μM. To select the dose of $CoCl_2$, many reports indicated that it could be varied from 50 - 300 μM [20,25,26]. So, this range had been selected, and the level of HIF-1α was monitored. However, the stimulation of HIF-1α expression was not detected in A549 with this range of concentrations (data not shown). So, we evaluated further with higher doses of $CoCl_2$ and found that at 600 μM successfully induced the expression of HIF-1α marker. Thus, the time course of $CoCl_2$ was performed by treated cells at 4, 8, and 24 h. As shown in **Figure 3**, the level of HIF-1α increased as time-dependent, which 24 h. obtained the highest level. Therefore, we selected this condition to establish the hypoxic model.

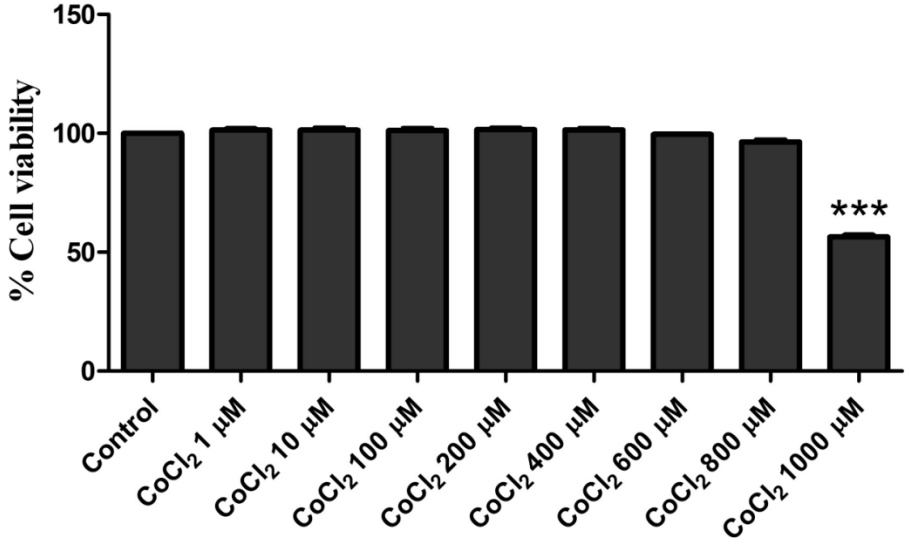


Figure 2 Cytotoxicity of $CoCl_2$ on A549 cells. Cell viability was assessed after 24 h of treatment with $CoCl_2$ at 1 - 1000 μM. ***$p < 0.001$, as compared with control.

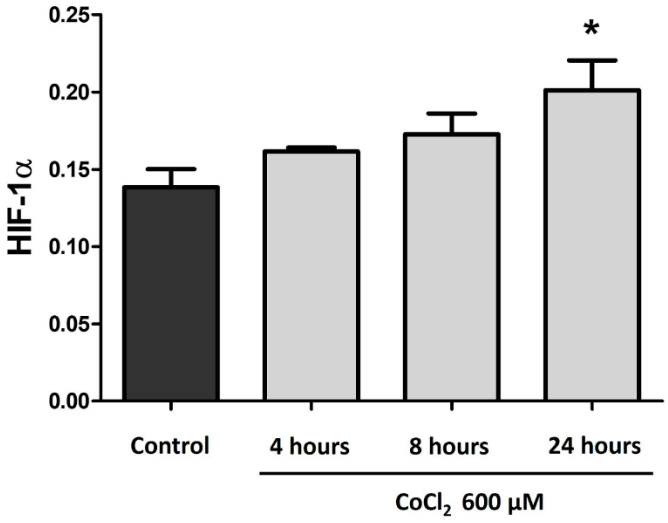


Figure 3 Cobalt chloride induced HIF-1α expression in A549. Cells were induced with 600 μM $CoCl_2$ for 0, 4, 8, and 24 h, and the level of HIF-1α expression was determined by colorimetric analysis at 450 nm. *$p < 0.05$, as compared with control.

Terrein suppressed HIF-1 alpha expression in $CoCl_2$ induced-A549 cells

To further determine whether terrein could suppress hypoxia in lung cancer cells, A549 cells were treated with 600 μM $CoCl_2$ for 24 h following the terrein treatment at 0, 20, 40, and 80 μM for another 24 h. The data indicated in **Figure 4** that $CoCl_2$ significantly stimulated the expression of HIF-1α in A549 cells when compared with control. For the reaction treated with terrein only, the level of HIF-1α did not increase and remained at the same level as a base-line of the untreated control. This result indicates that terrein itself does not activate HIF-1α expression. When the reactions pretreated with $CoCl_2$, terrein significantly decreased the expression of HIF-1α to the base-line level. This effect implies that terrein may interfere with some mediators upstream of HIF-1α expression, resulting to HIF-1α reduction. Previous reports indicated that there were several upstream signaling of HIF-1α expression, including PI3K/Akt and MAPK pathways [27]. In this aspect, terrein has been shown to downregulate Akt and its phosphorylated form in breast cancer model [9]. So, it is possible that terrein may interfere with the PI3K/Akt pathway and reduced the expression of HIF-1α.

Besides, the data showed that terrein has various activities including anti-inflammation, anti-oxidant, anticancer, anti-bacterial and anti-melanogenesis [4,6,8,28,29]. While hypoxia has been indicated to induce reactive oxygen species (ROS) accumulation, ROS demonstrated the activation of HIF1-α by inactivating its inhibitor, PHD (Prolyl Hydroxylase Domain) [30]. Then, it is also possible that the anti-oxidant effect of terrein may be responsible for HIF1-α reduction by reducing the level of ROS, which sequentially activates PHD, an inhibitor of HIF1-α.

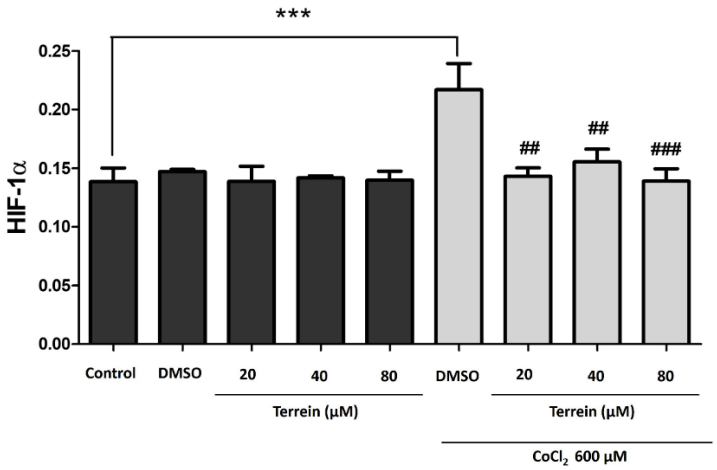
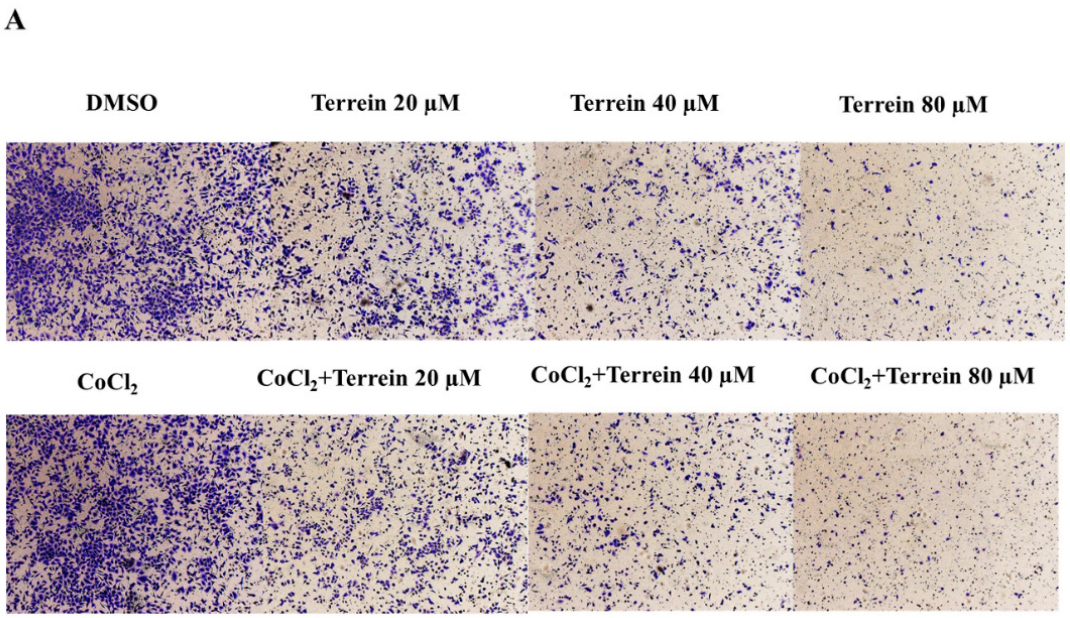


Figure 4 Terrein suppressed HIF-1α expression in A549. Cells were induced with 600 μM $CoCl_2$ for 24 h and further treated with terrein at 0, 20, 40, and 80 μM for 24 h. The level of HIF-1α expression was determined by colorimetric analysis at 450 nm. ***$p < 0.001$, as compared with control. $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, as compared with only $CoCl_2$ treated sample.

Terrein affected the metastatic process of A549 lung cancer cells

As terrein reduced the expression of HIF-1α and sequentially decreased the level of hypoxia, we asked if this effect would also reduce aggressive abilities. Thus, the migration and invasion properties were selected. The hypoxic condition was induced in A549 cells by treating with 600 μM $CoCl_2$ for 24 h and further added with terrein at 0, 20, 40, and 80 μM for 24 h. The ability of A549 cell migration was shown in **Figures 5A** and **5B**. From these results, $CoCl_2$ could induce migration of A549 cells but not significantly different when compared with cells in normal conditions. When observing the effect of terrein of both having $CoCl_2$ pretreated and un-pretreated, terrein demonstrated the inhibition of cancer cell migration in these two conditions. However, the number of cell migration in these two conditions was not much different. In other words, it could be said that terrein has a similar effect on the ability of cancer migration to either have $CoCl_2$ or not. When observing the density of migrated cells through the transwell, it also similar to either $CoCl_2$ pretreated or un-pretreated (**Figure 5A**). This data was not the case for cell invasion. As demonstrated in **Figures 5C** and **5D**, $CoCl_2$ could induce the invasion of A549 cells by as much as 51.82 %. The number of invading cells in hypoxic conditions after treated with terrein was significantly reduced, especially at 40 and 80 μM. Further, the density of cells that pass through the transwell was much higher when pretreated with $CoCl_2$. This was confirmed by the enhancement of aggressive ability as shown in **Figure 5C**.

Thus, these results demonstrated that $CoCl_2$ could better induce invasion than lung cancer cells' migration ability. The effect of terrein demonstrated the inhibition of both migration and invasion of lung cancer cells with $CoCl_2$ pretreated and un-pretreated. However, in the aggressive condition, terrein has a more explicit effect on cell invasion than migration. There are distinct differences between the transwell migration and invasion assays. The transwell migration assay measures the chemotactic capability of cells toward a chemoattractant. While the transwell invasion assay measures both cell chemotaxis and the invasion of cells through the extracellular matrix. This surface-coated insert for invasion assay contains the Matrigel, which consisted of extracellular matrix proteins and several growth factors [31]. Both of them have been recognized to play an essential role in promoting cell adhesion and cancer progression, especially cancer invasion [32]. Therefore, Matrigel-coated for invasion assay may enhance cell movement and invade through the transwell, making the results more apparent than the migration assay.


A
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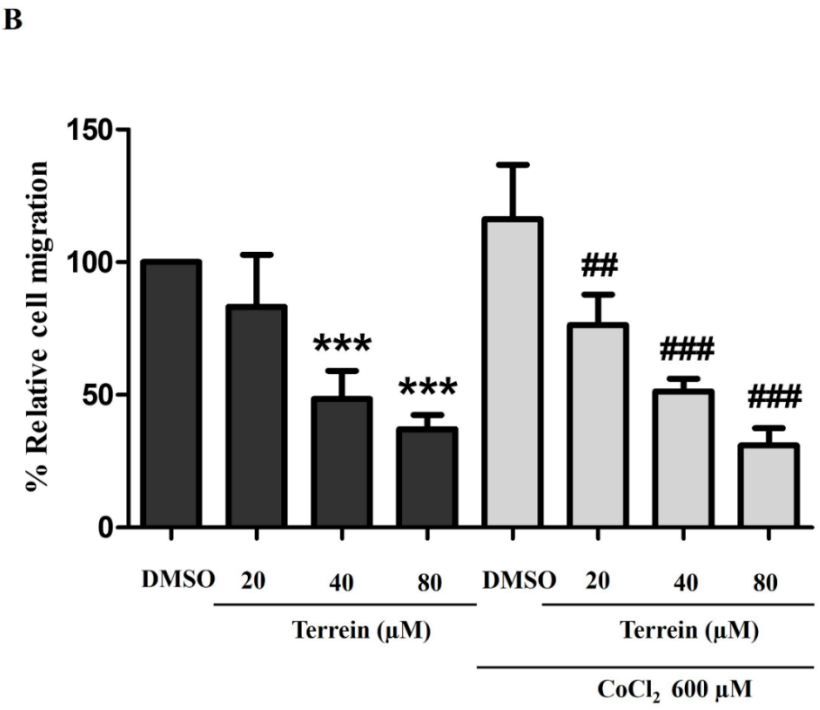
CoCl2+Terrein 40 µM
CoCl2+Terrein 80 µM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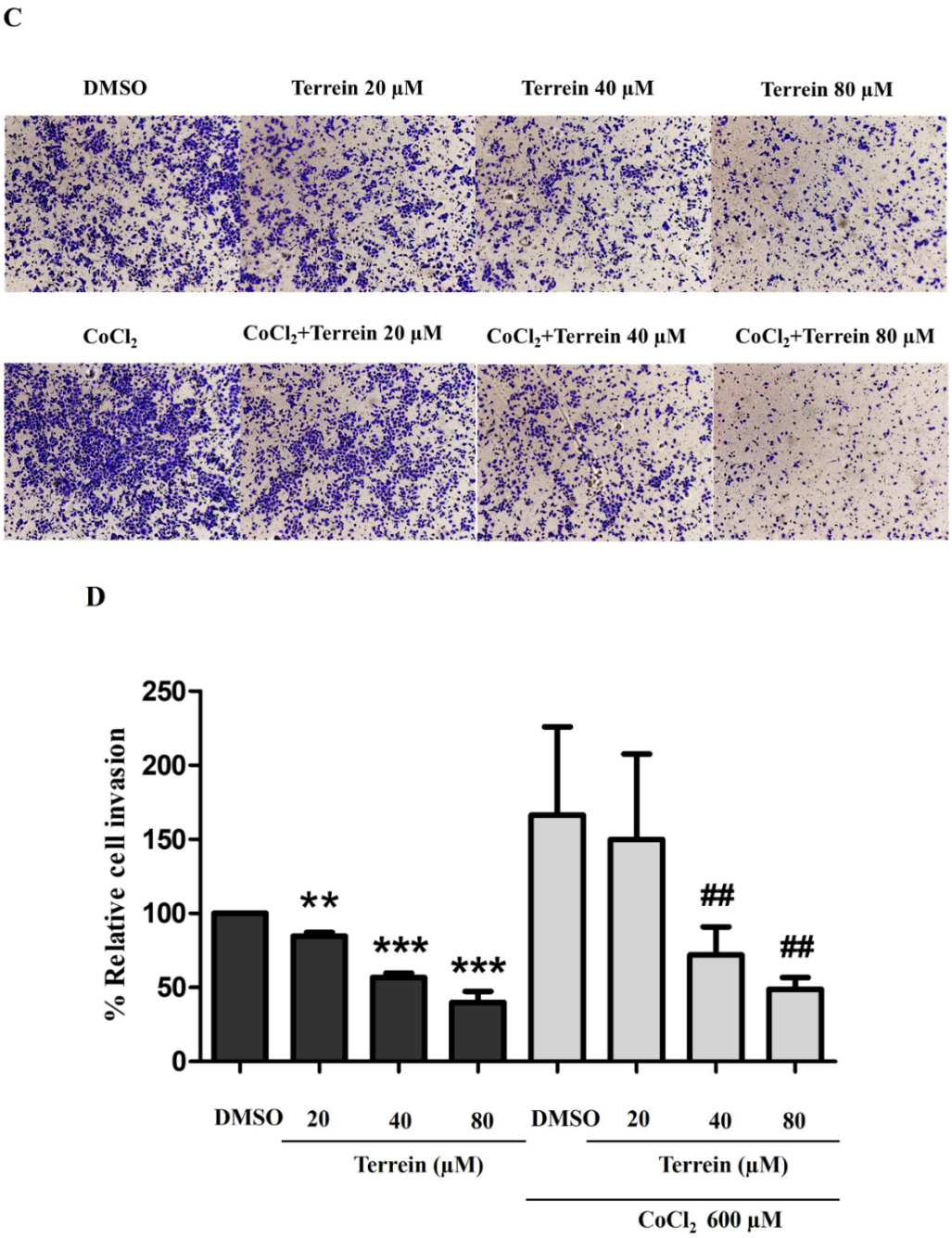

Figure 5 Terrein suppressed migration and invasion of A549 cells-induced hypoxia. Cells were induced HIF-1α with 600 µM $CoCl_2$ for 24 h and further treated with terrein at 0, 20, 40, and 80 µM for 24 h. (A) Effect of terrein on cell migration of A549 cells-induced hypoxia after crystal violet staining. (B) The migrated cells were quantified by using ImageJ. (C) Effect of terrein on cell invasion of A549 cells-induced hypoxia after crystal violet staining. (D) The invaded cells were quantified by using ImageJ. $^{**}p < 0.01$, $^{**}p < 0.001$, as compared with control. $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, as compared with $CoCl_2$ treated sample.

Conclusions

This study demonstrates a novel finding of the potency of terrein cytotoxicity to lung cancer cells, which has a specific activity comparing to the normal cells. Also, terrein suppressed both cancer cell migration and invasion, but the effect was more pronounced to the process of invasion in the aggressive condition. This suppression was probably from the reduction of transcription factor HIF-1α expression. Therefore, this study suggested that terrein is a potent compound that could apply as an anticancer agent in lung cancer.

Acknowledgements

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