Targeting Delivery Nanocarriers for (+)-Terrein to Enhance Its Anticancer Effects

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1. INTRODUCTION

Cancer is now one of the most serious diseases all over the world. The search for new effective chemotherapeutic drugs for cancer therapy has always been in focus. Natural products have been proven to be rich sources of structurally novel and biologically active compounds, and they have become one of the major chemical entities for drug discovery. As a compound from marine fungi, (+)-terrein showed significant anticancer activity. In this study, (+)-terrein was extracted from the marine-derived fungus and showed significant cytotoxicity against cancer cells, especially in A549 cells. To enhance its anticancer effects, redox-responsive nanocarriers based on folic acid-chitosan decorating the mesoporous silica nanoparticles were designed to control (+)-terrein target delivery into cancer cells. (+)-Terrein was loaded in the holes, and folic acid-chitosan worked as a gatekeeper by disulfide linkage controlling (+)-terrein release in the tumor microenvironment. The (+)-terrein drug delivery systems exhibited cytotoxicity toward A549 cells through induction of apoptosis. The apoptosis effect was confirmed by the increase in the expression of cleaved caspase-3, caspase-9, and PARP. Taken together, this work evaluates for the first time the (+)-terrein delivery system and provides a promising nanomedicine platform for (+)-terrein.

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responsive DDS to control and enhance (+)-terrein targeting delivery. In the tumor microenvironment, the disulfide bonds can be cleaved and chitosan is deshielded to control the (+)-terrein release in tumor cells. The growth inhibition and cytotoxicity of (+)-terrein DDS were investigated, and the possible apoptotic mechanisms were evaluated in A549 human cancer cell line.

2. RESULTS AND DISCUSSION

2.1. Cytotoxic Activity of (+)-Terrein. The cytotoxicity of (+)-terrein was evaluated against different cancer cells. As shown in Table 1, (+)-terrein exhibited cytotoxicity against H1975, K562, DU145, A549, H441, and MCF-7 cell lines. Compared with the other cancer cells, A549 cell line was more sensitive to (+)-terrein, and the representative IC_{50} curve is shown in Figure 1. A549 cell line is known to harbor KRAS mutation and LKB1 inactivating mutations, which could accelerate energy consumption, destroy intracellular components, and ultimately induce rapid cell apoptosis, leading to accelerated energy consumption, destroy intracellular components, and ultimately induce rapid cell apoptosis, leading to

Table 1. Cytotoxic Activity of (+)-Terrein

| cell lines      | type                                | IC_{50} (μg/mL) |
|-----------------|-------------------------------------|----------------|
| H1975           | human adenocarcinoma non-small cell lung cancer | 7.55           |
| K562            | human chronic myelogenous leukemia cell | 7.11           |
| DU145           | human prostate cancer cell           | 4.63           |
| A549            | human adenocarcinoma alveolar basal epithelial cells | 3.32           |
| H441            | human distal lung epithelial cell    | 7.55           |
| MCF-7           | human breast cancer                  | 7.11           |

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![Figure 1](image1.png)

Figure 1. Inhibition rate (%) of (+)-terrein in A549 cells. A549 cells were treated with various concentrations of (+)-terrein for 72 h and then subjected to the MTT assay. Values were expressed as mean ± SD of three independent experiments.

Figure 2. TEM images and particle size analysis of MSN-ss-COOH (A) and MSN-ss-FCS (B).

![Figure 2](image2.png)

± 23 nm (n = 300) (Figure 2B1). Compared to unmodified MSNs, MSNs modified by chitosan can disperse in water and form stable colloid solution. Dynamic light scattering was used to test the particle dispersity in water. The results showed that the polidispersity index was decreased from 0.359 to 0.182 after being covered by chitosan, indicating that the polymer decoration of nanoparticles helped to disperse in the aqueous solution (Figure 2A2, B2).

The Fourier transform infrared (FTIR) spectra of the MSN-ss-COOH and MSN-ss-FCS are shown in Figure 3A. Compared with MSN-ss-COOH, 1555 and 1660 cm\(^{-1}\) new peaks appeared in MSN-ss-FCS, which were attributed to N\(=\)H asymmetric bending vibration and acylamino groups in the chitosan, respectively. The results of zeta potential also indicated that the chitosan chains were grafted in the surface of nanoparticles. The zeta potential (Figure 3B) was changed from \(-18.12 ± 1.96\) to \(26.71 ± 2.12\) mV after being decorated by chitosan.\(^{25,26}\) Thermogravimetric analysis (TGA) was performed to investigate the amount of grafted chitosan derivatives. As shown in Figure 3C, the weight losses of MSN-ss-COOH and MSN-ss-FCS were 23.6 and 62.6% at 700 °C, respectively. The amount of grafted chitosan was about 36.6%. Moreover, the results of nitrogen physisorption (Figure 3D) showed that MSN-ss-COOH exhibit the typical IV behavior for a well-developed meso-structure with a sharp adsorption step at the 0.1–0.3 relative pressure (P/P_{0}) range.\(^{27}\)

Compared with MSN-ss-COOH, the adsorption step was not so steep compared with MSN seeds, and the inflection point of the step shifted to somewhat lower P/P_{0} in the case of MSN-ss-FCS, indicating that the pore channels were blocked by chitosan chains. Through the above results and analyses, chitosan chains have been successfully conjugated in the surface of nanoparticles by disulfide bonds. Moreover, the retained mesoporosity can potentially provide space for further functionalization.

2.2. Preparation and Characterization of (+)-Terrein Nanocarriers. To effectively control the (+)-terrein release in cancer cells, chitosan was used to decorate the nanocarriers. Chitosan chains were anchored on the surface of nanoparticles via disulfide linkers and fabricated an effective gatekeeper to encapsulate the (+)-terrein molecules inside the holes. The morphology and distribution of nanocarriers were studied by transmission electron microscopy (TEM) images and particle size analysis. As seen in Figure 2A1, the MSN-ss-COOH particles have an average diameter of ~100 nm with uniform pore size. After being decorated by chitosan, the nanoshell was appeared around the MSN-ss-folic acid-functionalized chitosan (FCS) nanoparticles, and the particle size was increased to 110 ± 1 nm.
ment) and 10 μM (blood microenvironment) of GSH were used to trigger the (+)-terrein release. It showed that (+)-terrein release was fast in the beginning of 6 h in the presence of 10 mM of GSH. After 24 h of an in vitro assay, more than 80% (+)-terrein was released. In contrast, the rate of (+)-terrein release was remarkably slowed in the presence of 10 μM of GSH. The cumulative release of the total amount of (+)-terrein was much lower than the cumulative release in the presence of 10 mM of GSH. Compared to Terrein@MSN-ss-FCS nanocarriers, the release behavior of (+)-terrein from Terrein@MSN-FCS nanocarriers was not obviously different between 10 μM and 10 mM of GSH solution. As shown in the results of the redox-sensitivity study, GSH can rapidly cleave the disulfide bonds among MSNs and chitosan chains and trigger the (+)-terrein release from nanocarriers.28

We also evaluated the intracellular release behaviors of (+)-terrein-loaded MSNs in A549 cells by confocal laser scanning microscopy (CLSM). Because of the lack of own fluorescence, fluorescein isothiocyanate (FITC)-replaced (+)-terrein was used as a tracer to monitor cellular accumulation quantitatively in MSN-ss-FCS and MSN-CS nanoparticles. As shown in Figure 5A, green fluorescence was obviously observed in the surroundings of the cell nucleus (blue fluorescence). The fluorescence intensity of MSN-FCS nanoparticles was also evaluated by CLSM. As shown in Figure 5B, weak green fluorescence intensity was observed in the cells, illustrating that MSN-ss-FCS nanoparticles were efficiently internalized and released FITC inside cells. FITC could not be effectively released from MSN-CP nanoparticles.
These results indicated that GSH could rapidly cleave the disulfide bonds among MSNs and chitosan chains and trigger the (+)-terrein release from nanocarriers. In blood circulation, the nanocarriers could remain stable and avoid the premature (+)-terrein release. Once the nanocarriers enter the tumor tissue, the high concentration of GSH (over 10 mM) could cleave the disulfide bonds and trigger the (+)-terrein release in cancer cells.29,30 These results were also consistent with the results of (+)-terrein release in vitro.

2.4. Cytotoxic Activity of (+)-Terrein DDS. The in vitro cytotoxic effect of Terrein@MSN-ss-FCS and other formulations was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method. As shown in Figure 6A, after 24 h incubation, Terrein@MSN-ss-FCS showed obvious cytotoxicity to A549 cells. The obtained inhibition rates of Terrein@MSN-ss-FCS nanocarriers were 13.14, 29.08, and 57.03% at the (+)-terrein concentration of 3, 6, and 12 μg/mL, respectively. The IC50 value was 10.84 μg/mL. These results approached or briefly exceeded the cytotoxicity of free (+)-terrein (IC50 = 9.97 μg/mL), suggesting that the decorated polymer chains were able to break away from the particle surface in response to the cancer microenvironment, and the loaded (+)-terrein was released in cancer cells. In comparison, the inhibition rates of Terrein@...
MSN-FCS nanocarriers (without disulfide bonds) were 5.84, 22.88, and 41.60% at the (+)-terrein concentration of 3, 6, and 12 μg/mL, respectively, which were significantly lower than the cytotoxicity of Terrein@MSN-ss-FCS and free (+)-terrein, indicating that the decorated polymer chains were tightly anchored at the particle surface and prevented the intracellular release of (+)-terrein. To further confirm the cytotoxicity of Terrein@MSN-ss-FCS, the clone formation test was employed. A549 cells were incubated with Terrein@MSN-ss-FCS for 7 days, and the clone formation of A549 cells were effectively inhibited at the 2.5 and 5 μg/mL of (+)-terrein (Figure 6B).

Annexin V-FITC/PI double staining was used to assess the apoptosis activity of Terrein@MSN-ss-FCS in A549 cells. As shown in Figure 7, after 36 h incubation, the cells treated with blank MSN-FCS carriers showed negligible apoptosis. With the increase of the concentration of Terrein@MSN-ss-FCS, the percentage of total apoptotic cells was obviously increased and reached 14.7%, and the percentage of necrotic cells increased to 11.7% at the concentration of 6 μg/mL. These results demonstrated that Terrein@MSN-ss-FCS could induce apoptosis in A549 cells.

The apoptosis-related proteins in A549 cells were further detected by western blot analysis. After incubation with Terrein@MSN-ss-FCS for 36 h, the cleavage of caspase-3 and caspase-9 which were the important apoptotic proteins increased significantly, confirming the occurrence of apoptosis. PARP is an important DNA repair enzyme, which may play an important role in the tumor growth and metastasis. As shown in Figure 8, the cleavage of PARP (C-PARP) increased obviously when the cells were treated with 8 μg/mL (+)-terrein DDS. These results confirmed that (+)-terrein loaded in nanocarriers was able to effectively induce the apoptosis of A549 cells. Therefore, the redox-stimulated MSNs could act as effective carriers to deliver anticancer drugs and enhance cancer therapy.

3. CONCLUSIONS

The hydrophilic (+)-terrein was extracted from marine-derived fungus, and its chemical structure was identified by NMR and X-ray single crystal diffraction methods. In this study, (+)-terrein was loaded in the holes of MSNs, and chitosan chains were conjugated on the surface of nanoparticles by disulfide bonds. The loading capacity of (+)-terrein reached 13.6%, and the obtained Terrein@MSN-ss-FCS nanocarriers formed a stable dispersion system in aqueous solution. The results of CLSM clearly indicated that the fluorescence molecules were released from nanocarriers and diffused to the nucleus in A549 cells. Moreover, the (+)-terrein delivery systems exhibited cytotoxicity toward A549 cells through induction of apoptosis. The apoptotic pathway was confirmed by the increased expression of cleaved caspase-3, caspase-9, and PARP. Taken together, this work provided an effective strategy of DDSs for (+)-terrein.

4. EXPERIMENTAL SECTION

4.1. Materials and Reagents. Chitosan (Mw = 10 kDa, deacetylation degree = 85.3%), folic acid, 1-[3-(dimethylamino)propyl]-3-ethylcarbamidomethyl hydrochloride (EDC), 3,3′-dithiobispropionic acid, and 3-aminopropyltriethoxysilane were purchased from TCI. Tetraethyl orthosilicate, cetyltrimethyl ammonium bromide, succinic acid, and sodium ascorbate were purchased from Energy Chemical (Shanghai, China).

H1975, K562, DU145, A549, H411, and MCF-7 cell lines were provided by Shanghai Cell Bank, Chinese Academy of Science. A549 and H411 cell lines were cultured in F-12K medium with 10% fetal bovine serum (FBS). MCF-7 cell line was cultured in modified Eagle’s medium with 10% FBS containing 10 μg/mL insulin, and the others were cultured in RPMI 1640 medium with 10% FBS. Cells were cultured at 37 °C in a humidified incubator with 5% CO₂. The Annexin V-FITC/PI apoptosis detection kit was provided by KeyGEN BioTECH, Co., Ltd, China. Antibodies against cleaved caspase-3 (C-Cas3), cleaved caspase-9 (C-Cas9), and cleaved PARP (C-PARP) were purchased from Cell Signaling Technology (Boston, MA, USA). Other agents and kits were the products of Beyotime Biotechnology, Shanghai, China.

4.2. Extraction and Separation of (+)-Terrein. (+)-Terrein (Figure 9) was extracted and separated, as previously reported. The fungal strain was collected from Rumphella aggregata gathered from Nansha Islands of China in 2015. Briefly, an ethyl acetate (EtOAc) extract from the culture broth of R. aggregata was concentrated in vacuo, fractioned, and purified by silica gel VLC with a stepwise gradient of petroleum ether-EtOAc to afford five fractions (Fr.1-Fr.5). The compound was further purified by semipreparative high-performance liquid chromatography using a C18 (Kromasil) column at a flow rate of 2.0 mL/min (MeOH/H₂O 9:1) to obtain (+)-terrein.

4.2.1. (+)-Terrein (1). White crystal; ESI-MS m/z 153 [M – H]⁻; 1H NMR (CD₂COCD₂): δ, ppm, J/Hz): δH 6.81 (1H, dq, J = 15.6, 6.9 Hz, H-7), 6.42 (1H, d, J = 15.6 Hz, H-6), 5.96 (1H, s, H-5), 4.73 (1H, d, J = 2.5 Hz, H-3), 4.08 (1H, d, J = 2.5 Hz, H-2), 1.90 (3H, d, J = 6.8 Hz, 8-CH₃). 13C NMR (CD₂COCD₂): δC: 202.8 (C, C-1), 81.4 (CH, C-2), 76.9 (CH, C-3), 168.3 (C, C-4), 124.9 (CH, C-5), 125.4 (CH, C-6), 139.3 (CH, C-7), 18.5 (CH₃, C-8).

4.3. Preparation of (+)-Terrein Nanocarrier. MSNs containing carboxyl (MSN-ss-COOH) was prepared according to our previous work. (+)-terrein (5 mg) was dissolved in 10 mL of tetrahydrofuran. Subsequently, 30 mg of MSN-ss-COOH was added and the mixture was stirred for 24 h. The suspension was centrifuged and washed with ethanol to remove unabsorbed (+)-terrein. Then, the sample was vacuum dried overnight and the product Terrein@MSN-ss-COOH was obtained.

Chitosan (100 mg) and EDC (10.0 mg) were dissolved in 1% acetic acid solution. Folic acid (5.0 mg) was dissolved in 5 mL dimethyl sulfoxide (DMSO) and slowly dropped into chitosan solution. The mixture solution was stirred for 24 h at room temperature. Subsequently, the mixture solution was dialyzed [molecular weight cut-off (MWCO) 3.5 kDa] in distilled water for 3 days and lyophilized, obtaining the FCS.
FCS (10 mg) was dissolved in 2 mL of 0.5% acetic acid solution. Terrein@MSN-ss-COOH (20 mg) and EDC (5.0 mg) were added, and the mixture was stirred for 24 h at room temperature. The suspension was centrifuged, washed three times with water to remove the unreacted chitosan, and dried in vacuum overnight to give the product Terrein@MSN-ss-FCS. As a control sample, MSN-FCS (without disulfide bonds) nanocarriers for (+)-terrein were prepared from MSN-COOH to study the drug release by a similar synthetic procedure. The obtained Terrein@MSN-FCs and Terrein@MSN-ss-FCS samples were sealed in plastic tubes and stored in the fridge at 4 °C. The amount of loaded (+)-terrein was determined by UV–vis at 276 nm. The (+)-terrein loading content was 12.36% according to the following equations:

4.4. (+)-Terrein Release In Vitro. Terrein@MSN-ss-FCS (10 mg) and Terrein@MSN-FCS (10 mg) were suspended in 5 mL of phosphate-buffered saline (PBS) with 10 μM or 10 mM GSH and loaded in a dialysis bag (MWCO = 3 kDa), respectively. The dialysis bag was immersed and dialyzed against 20 mL of PBS buffer (pH 7.4) at 37 °C. The samples were kept at 37 °C in a thermostated incubator with a shaking speed of 100 cycles/min. At predetermined time intervals, 1.0 mL of incubation solution was taken out for analysis and replaced by an equal volume of fresh PBS. The released (+)-terrein was analyzed using a UV–vis spectrophotometer at 276 nm. All measurements were performed in triplicate.

4.5. Cellular Uptake Studies. The intracellular release from nanoparticles was observed by CLSM. A549 cells were seeded into a 24-well plate (5 × 10⁴ cells/well). FITC was loaded in MSN-ss-FCS, and MSN-FCS nanoparticles replaced (+)-terrein. After being cultured for 24 h, FITC-loaded nanoparticles were added and treated for 2 h, respectively. Subsequently, the cells were washed with PBS, fixed with fresh 2.5% glutaraldehyde for 30 min, and then stained with DAPI for cell nucleus. CLSM was used to observe the cell uptake and intracellular FITC release.

4.6. Cytotoxicity Assays. The cytotoxicity against A549 cells was evaluated using the MTT method. Cells (3000 cells/well) in their log phase of growth were seeded into 96-well plates, followed by treating with different amounts of free (+)-terrein, Terrein@MSN-FCS, and Terrein@MSN-ss-FCS (3, 6, and 12 μg/mL). The amount of Terrein@MSN-FCS and Terrein@MSN-ss-FCS was converted into loaded (+)-terrein according to the loading content) for 72 h. MTT (20 μL, 0.5 mg/mL) was added and incubated for another 4 h, and then the supernatant was removed and the formazan product was dissolved in 200 μL DMSO. Absorbance was measured at 490 nm using a microplate reader (BioTek, USA).

4.7. Colony Formation Assay. A549 cells were seeded into 6-well plates (800 cells/well). After 24 h, different concentrations (0–5 μg/mL) of Terrein@MSN-ss-FCS were added and incubated for 7 days. Then, supernatant was removed, and cells were washed with ice-cold PBS, fixed in methanol for 20 min, and stained with Giemsa. Finally, colonies were scored and photographed. Blank MSN-ss-FCS nanoparticles were used as a control.

4.8. Annexin V-FITC/PI Double Staining Assay. The apoptosis was assayed using the Annexin V-FITC/PI apoptosis detection kit, according to the manufactures’ protocol. Briefly, A549 cells (3 × 10⁵ cells/well) were seeded into 6-well plates, and then, cells were incubated with Terrein@MSN-ss-FCS (0–6 μg/mL) for 36 h. Cells without treatment were used as control. At the end of incubation, cells were collected and washed with PBS and then stained with Annexin V-FITC and PI for 10 min at room temperature in the dark. Cells were gated, and 3 × 10⁵ cells were collected by the flow cytometry system (Beckman Coulter MoFlo XDP, Fullerton, CA, USA).

4.9. Western Blotting Assay. After treatment with Terrein@MSN-ss-FCS (0–8 μg/mL) for 36 h, A549 cells were collected, washed with PBS, and lysed with radio-immunoprecipitation assay buffer. C-cas3 was separated by 12% SDS-polyacrylamide gels, and C-cas9, C-PARP, and tubulin were separated by 8% SDS-polyacrylamide gels and then transferred to nitrocellulose membranes. The proteins were probed with C-cas3, C-cas9, C-PARP, and β-tubulin primary antibodies, respectively, and then, the membranes were incubated with HRP-secondary antibodies. Bands corresponding to the antibodies were determined by enhanced chemiluminescence kits and detected by FluorChem E (ProteinSimple, USA).

4.10. Statistical Analyses. Statistical analyses were performed by one-way ANOVA with Tukey’s post hoc test on the data, and the value was expressed as mean ± SD. Differences of P < 0.05 were considered as statistically significant.

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Notes
The authors declare no competing financial interest.

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