Triptolide Inhibits MCF-7 and HepG2 Cells Invasion and Migration by Inhibiting the Synthesis of Polylactosamine Chains

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Abstract: Triptolide is a bioactive natural products isolated from Tripterygium wilfordii, a traditional Chinese herbal medicine. Clinical studies reveal that triptolide can be used in autoimmune disorders, such as rheumatoid arthritis, kidney disease and systemic lupus erythematosus. Recently, some studies revealed that triptolide has anti-tumor effects, which attracts more and more attention. This experiment aimed to explore the relationship between anti-tumor effects of triptolide and N-type polylactosamine. With increasing the concentration of triptolide, the viability of MCF-7 and HepG2 cells was reduced significantly and the polylactosamine expression on these cells declined as well. In addition, the expression of β1, 3-N-acetylglucosamine transferase (β3GnT8) participated in catalyzing the synthesis of N-type polylactosamine was also decreased and the expression of genes and proteins of downstream signaling was altered consequently. Finally, triptolide weakened the cancer cells invasion and migration. All of these indicate that triptolide can impair MCF-7 and HepG2 cells invasion and migration through downregulating the expression of polylactosamine chains. These studies establish that triptolide is a potential novel therapy in breast cancer and hepatic carcinoma.

Keywords: Triptolide, polylactosamine, β1, 3-N-acetylglucosamine transferase, tumor, invasion, migration.

INTRODUCTION

Triptolide is a traditional Chinese medicine, which is the major active principle of Tripterygium wilfordii Hook f. Triptolide is very effective in anti-infection, immune suppression and anti-tumor [1-3]. It has been confirmed that triptolide has anti-tumor effect on solid tumors *in vitro* experiments [4]. In addition, several experiments show that triptolide has significant cytotoxicity to lung cancer, colon cancer, leukemia [5-8]. Triptolide could downregulate the nucleus NF-κB expression and active the Caspase-3, and then the activation of caspases 3 cascades will promote apoptosis, which make tumor more sensitive to chemotherapy drugs [9]. Triptolide were reported to accelerate the degradation of CDK7-dependent RNA polymerase II, and then lead to the overall transcription suppression, resulting in the death of cancer cells [10-11].

It's known that glycan of glycoprotein, proteoglycan and glycolipid plays an important role in cell recognition, cell-cell interaction, inflammation and disease progression [12, 13]. Abnormal glycosylation is closely related to a variety of human diseases, particularly cancer [14]. Polylactosamine [(Galβ1-4GlcNAcβ1-3) n] is a basic structure of sugar compound, composed by repeated N-acetyllactosamine (LacNAc) sequences. The expression of polylactosamine in high metastatic gastric cancer and colon cancer cells is higher than that in low metastatic gastric cancer and colon cancer cells.

β1, 3-N- acetylglucosamine transferase family (β3GnTs) catalyze the initiation and elongation of β1, 6-branched polylactosamine chains [15]. Akira Seko's study shows that polylactosamine chains of three antennas or four antenna β1, 6-branched N-glycan participate in malignant phenotype of tumor, thereby affecting the proliferation and migration of cancer cells [16]. β3GnT8 and β3GnT2 were co-transfected to cells, greatly enhancing the synthesis of N-type polylactosamine, which suggest that the two glycosyltransferases influence and complement each other in the cell [17]. β3GnT2 and β3GnT8 both have ability to synthesis polylactosamine, and the latter can significantly activate the enzymatic activity of the former at the protein level [18].

Interestingly, CD147 is a kind of glycoprotein carrying poly-GalNAc β1, 6 branch chains. In many tumors, CD147 regulates the expression of MMPs which influence the invasion and migration of tumor [19-22]. Depending on the degree of glycosylation,

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CD147 has two forms—low-glycosylation CD147 (LG-CD147) and high-glycosylation CD147 (HG-CD147). In addition, some studies demonstrated that only HG-CD147 (55kDa) is able to self-cross on the cell surface and induce the synthesis of MMPs [23, 24]. *In vitro* experiments, after triptolide treated, the invasion ability of lung cancer cells is inhibited, and MMP14 expression is also downregulated [5].

This study aims to explore the relationship of anti-tumor mechanism of triptolide and N-type polylactosamine. MCF-7 and HepG2 were treated with different doses of triptolide which significantly promoted the apoptosis of the cancer cells (Figure **2**). At the same time, N- glycans on the cell surface were downregulated (Figure **3**). Triptolide inhibited the transcription of β3GnT8 and other glycosyltransferase genes, which blocked the synthesis of N-type polylactosamine, thus affecting the glycosylation of CD147 (Figure **4**). CD147 with abnormal glycosylation changed its regulation of MMPs, and ultimately weakened the invasion and migration of tumor cells (Figure **5**).

RESULTS AND DISCUSSION

Triptolide Inhibits MCF-7 and HepG2 Cells Growth in a Dose-Dependent Manner

In order to determine whether the triptolide affects the activity of the two cell lines, cells were treated with various doses of triptolide for 48 h using a MTT assay. Results were shown in Figure **1**, triptolide significantly inhibited MCF-7 and HepG2 cells proliferation, and the inhibition was dose-dependent. The IC50 of MCF-7 was 360nM and the IC50 of HepG2 was 30nM. The concentrations of the triptolide used in the subsequent experiments were chosen in the reference of IC50.

Triptolide Induces MCF-7 and HepG2 Cell Apoptosis

To figure out whether the inhibition of cell activity by triptolide was associated with apoptosis, Hoechst 33258 staining was chosen to detect apoptotic bodies in cells after being treated with triptolide. In Figure **2A**, the number of apoptotic bodies in MCF-7 cells was gradually increased with increasing the concentration of triptolide. In Figure **2B**, apoptotic bodies in HepG2 cells were significantly increased at high concentration of triptolide. By counting the number of apoptotic bodies, we got the histogram (Figure **2C**) which reflected that high concentration of triptolide could significantly improve the apoptosis rate of MCF-7 and HepG2 cells ($*P < 0.05$, $**P < 0.01$). These results suggest that triptolide induces apoptosis.

Triptolide Inhibits the Expression of N- Type Polylactosamine in MCF-7 and HepG2 Cells

In order to confirm that oligosaccharide chains on the cell surface play an important role in apoptosis and tumor metastasis, the research group try to explore the anti-tumor mechanism of triptolide from the perspective of N-type polylactosamine chains. In Figure **3A** and **D**, flow cytometry was used to detect the polylactosamine chains binded by tomato lectin labeled with biotin on the cell surface. X-Mean value indicates the fluorescence signal strength, reflecting the expression level of polygalactosamine chains. As shown in

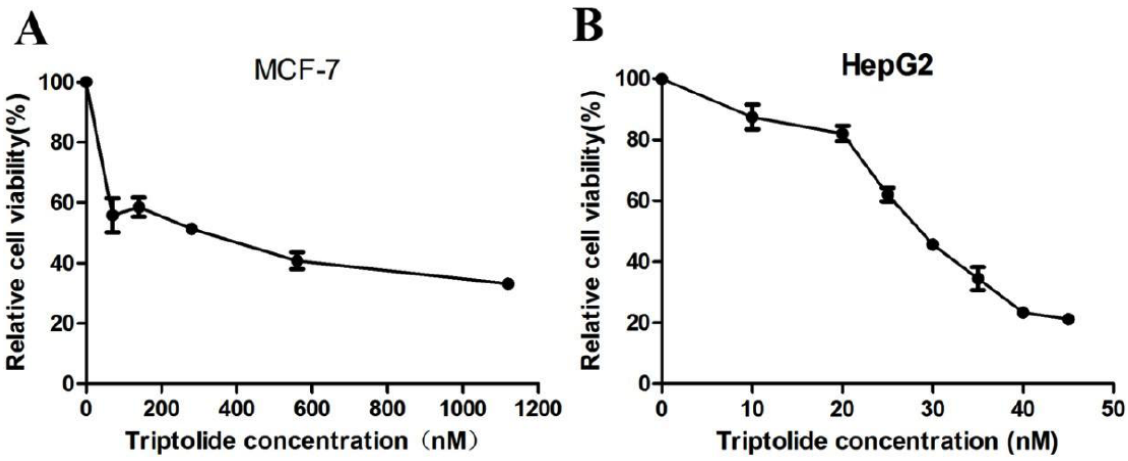

Figure 1: Effects of triptolide on the viability of MCF-7 and HepG2 cells. (**A**) MCF-7 cells were treated with triptolide at different concentrations (0, 70, 140, 280, 560 and 1120 nM) for 48 h and then supplemented with MTT to detect the cell viability. (**B**) HepG2 cells were treated with triptolide at the concentrations (0, 25, 50, 70, 80, 95, 100, 125 nM) for 48 h. The data are showed as the mean ± standard deviation of the mean of three independent experiments.

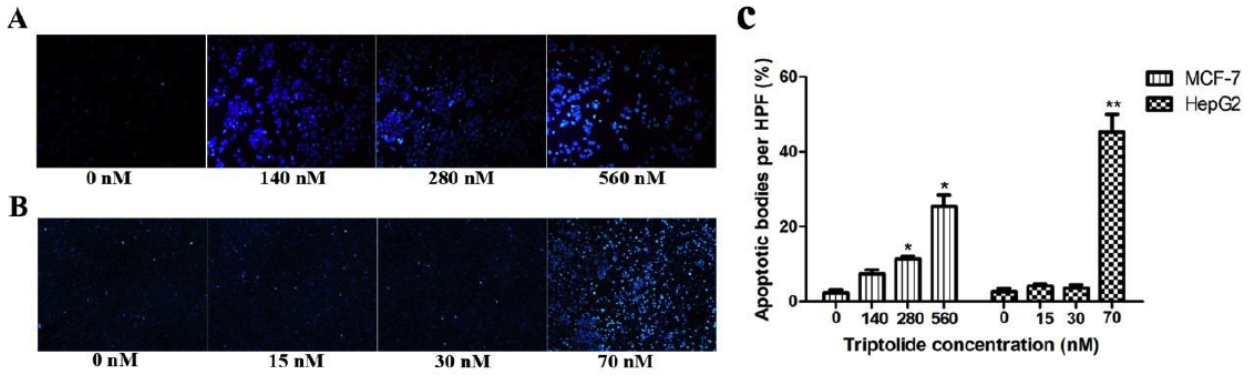

Figure 2: Triptolide induces apoptosis in MCF-7 and HepG2 cells. (**A**) MCF-7 and (**B**) HepG2 cells were incubated with triptolide at indicated doses for 48 h. Then, Hoechest 33258 staining was performed to detect apoptosis cells (cyan; magnification, ×100). (**C**) Statistics analysis of apoptotic cells with triptolide. The histogram represents the percentage of apoptotic cells of MCF-7 and HepG2 cells in the horizon within a high-power field. The data are showed as the mean ± standard deviation of the mean of three independent experiments (*P < 0.05 and **P < 0.01 vs 0 ng/ml).

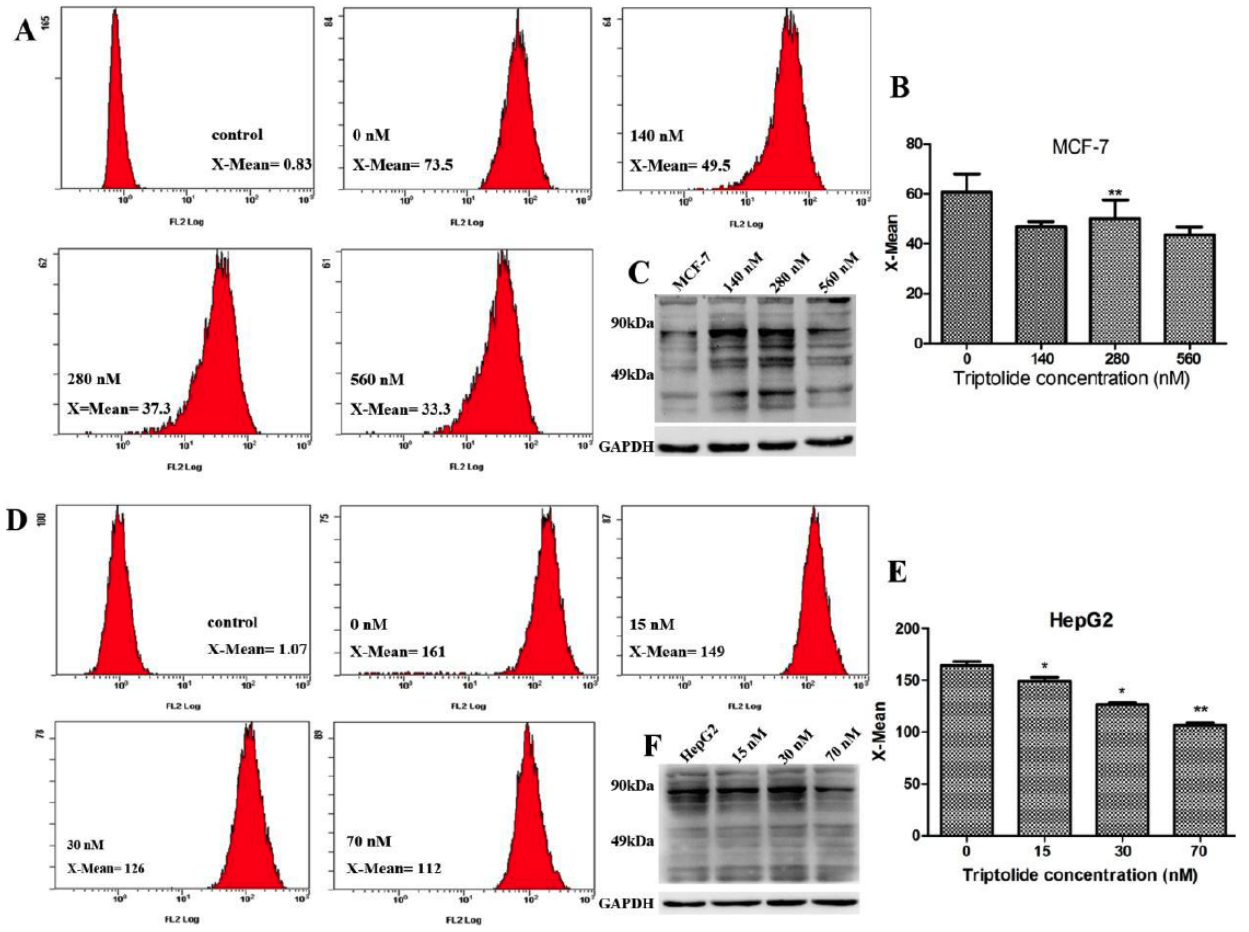

Figure 3: Triptolide reduces the expression of N-type polylactosamine in MCF-7 and HepG2 cells. (**A** and **D**) Flow cytometric to test polylactosamine chains on MCF-7 and HepG2 cell surface. The relative expression of polylactosamine was showed in (**B**) and (**E**). (**C** and **F**) Lectin blot was chosen to examine level of polylactosamine (49~90kDa) on glycoprotein in MCF-7 and HepG2 cells. The data are showed as the mean ± standard deviation of the mean of three independent experiments (*P < 0.05 and **P < 0.01 vs 0 ng/ml).

Figure **3B** and **E**, with increasing dose of triptolide, N-glycans on MCF-7 and HepG2 cell surface declined. In Figure **3C** and **F**, the experimental method we used was Lectin blot, which is an immunoblot detecting level of polylactosamine on glycoprotein. In MCF-7, low concentration of triptolide made cells' polygalacto-samine increased, while if the concentration continued to rise, it would significantly decrease cells'

polylactosamine (Figure **3C**). In Figure **3F**, expression of polygalactosamine in HepG2 decreased with increasing concentration of triptolide. It was consistent with the results detected by flow cytometry. These results showed that triptolide-induced apoptosis was related to N-type polylactosamin.

Triptolide Inhibits the Expression of β3GnT8 and the other Genes and Proteins Involved in the Synthesis of Polygalactosamine Chains

In Figure **4A**, triptolide resulted in a significant decline of mRNA expression of β3GnT8 and β3GnT2 in MCF-7 cells. Figure **4B** reflected the protein expression of the two genes in MCF-7. Compared with the control group, the high concentration of triptolide significantly restrained the protein expression of β3GnT8 and β3GnT2. In Figure **4C** and **D**, high concentration of triptolide lowered the transcription and expression of β3GnT8 and β3GnT2 in HepG2.

In Figure **4E**, low concentration of triptolide resulted in a significant increase of HG-CD147 in MCF-7 cells, while at high concentration, the expression of HG-CD147 was almost blocked. In HepG2 cells, high concentration of triptolide also significantly inhibited the expression of HG-CD147 (Figur **4F**). In Figure **4G** and **H**, we further tested mRNA level of HG-CD147 in the two cell lines after dealing with triptolide, and we found that the trend of mRNA and protein of HG-CD147 is consistent. In Figure **4I** and **J**, we detected the expression of mRNA of MMP14 in two cell lines which

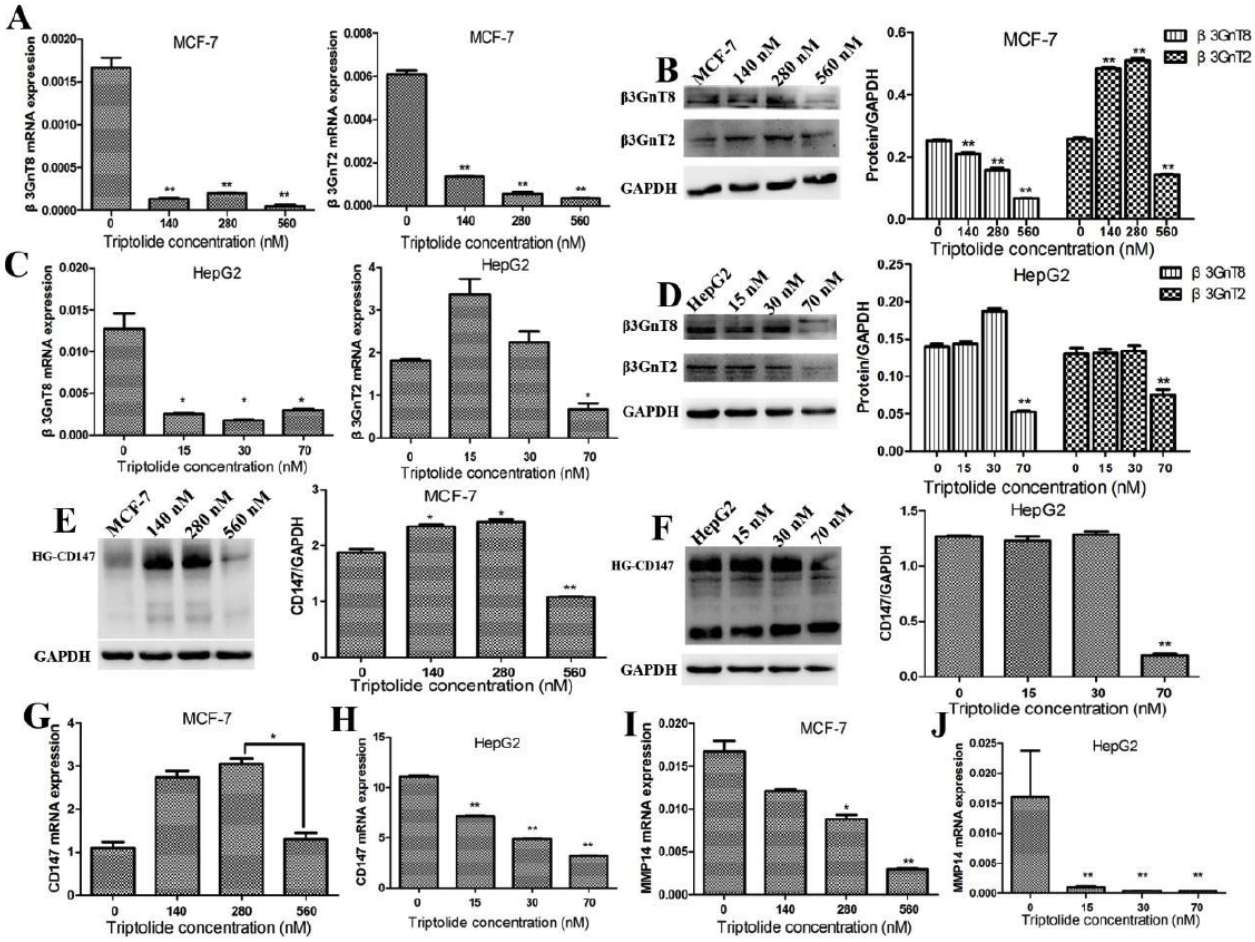

Figure 4: Changes of mRNA and protein expression in MCF-7 and HepG2 following treated with increasing concentration of triptolide. (**A**) Real-time PCR results of β3GnT8 and β3GnT2 in MCF-7cells. (**B**) Western blot analysis was performed to detect the expression level of β3GnT8 (71kDa) and β3GnT2 (50kDa) in MCF-7 cells. The blots were stripped and reprobed with a human GAPDH (36kDa) probe to confirm equal loading. In total, 30 μg total protein was loaded in each lane. (**C**) Real-time PCR results of β3GnT8 and β3GnT2 in HepG2 cells. (**D**) Western blot analysis was performed to detect the expression level of β3GnT8 and β3GnT2 in HepG2 cells. Western blot analysis was performed to examine the expression level of HG-CD147 (55kDa) in (**E**) MCF-7 cells and (**F**) HepG2 cells, and the corresponding grey values were showed beside them. Real-time PCR results of CD147 in (**G**) MCF-7 cells and (**H**) HepG2 cells. MMP14 mRNA expression of MCF-7 and HepG2 cells was respectivly exhibited on (**I**) and (**J**). The data are showed as the mean ± standard deviation of the mean of three independent experiments (*$P < 0.05$ and **$P < 0.01$ vs 0 ng/ml).

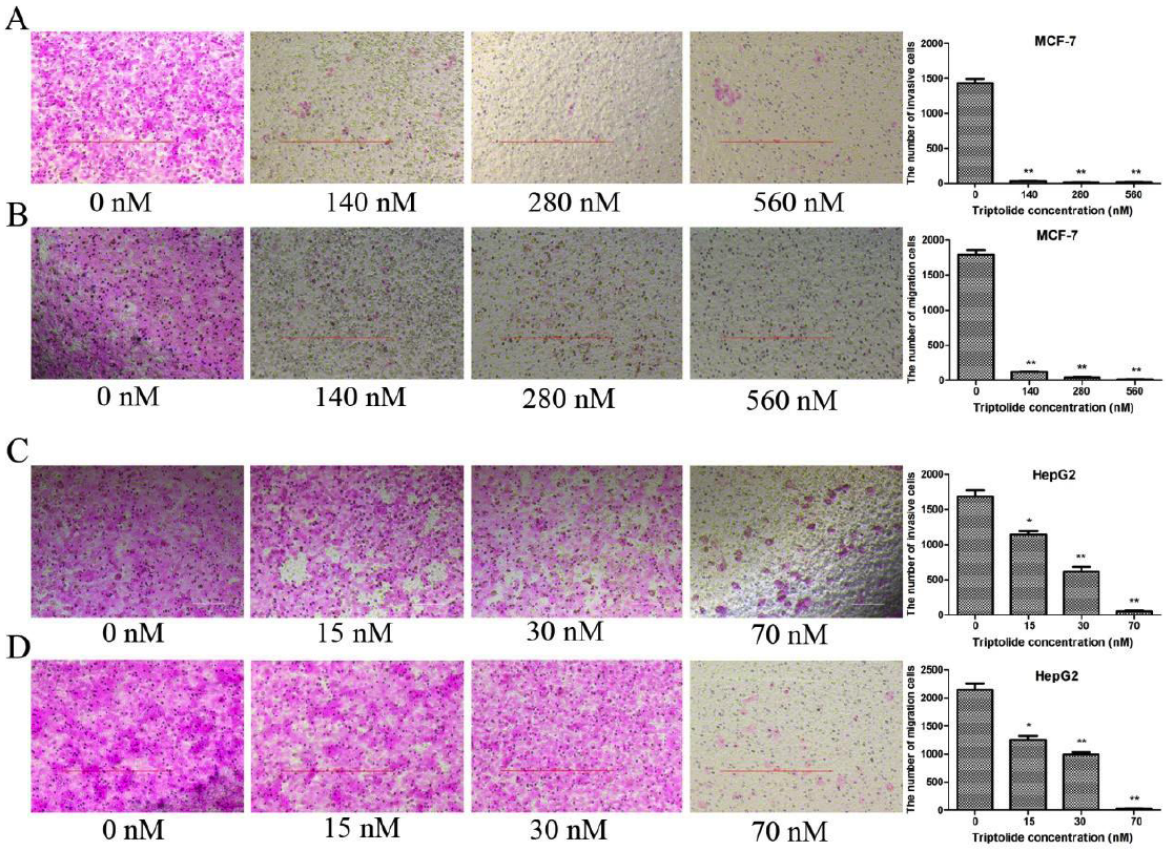

Figure 5: Triptolide inhibits the invasion and migration of MCF-7 and HepG2 cells. Images (eosin stain; magnification, ×100) and corresponding quantitative analysis of the invasion capacity of (**A**) MCF-7 and (**C**) HepG2 cells analyzed by Transwell assays. Images (eosin stain; magnification, ×100) and corresponding quantitative analysis of the migration capacity of (**B**) MCF-7 and (**D**) HepG2 cells analyzed by Transwell assays. The data are showed as the mean ± standard deviation of the mean of three independent experiments (*P < 0.05 and **P < 0.01 vs 0 ng/ml).

had been treated with triptolide. The results demonstrated that mRNA of MMP14 in MCF-7 and HepG2 cells was significantly downregulated with the drug concentration increasing.

Triptolide Significantly Inhibits the Invasion and Migration of MCF-7 and HepG2 Cells

Figure **5A** showed the results of invasion experiments. Low concentrations of triptolide had significantly inhibited the invasion ability of MCF-7. In Figure **5B**, the experimental results of migration were consistent with that of the invasion. Therefore, triptolide could significantly weaken the invasion and migration of MCF-7 cells. After treating with triptolide, we observed that invasion and migration of HepG2 cells were also fading (Figure **5C** and **D**).

MATERIALS AND METHODS

Materials

Breast cancer cells MCF-7 and hepatocellular carcinoma cells HepG2 were purchased from ATCC (Manassas, VA, USA). Triptolide was handseled by Suzhou Hospital of Traditional Chinese Medicine. Triptolide solution is formulated by dissolving the drug powder into PBS. RPMI-1640 and Dulbecco's modified Eagle's medium were obtained from Gibco Life Technologies (Carlsbad, CA, USA). MTT was purchased from Solarbio Life Sciences (Beijing, China). RNA reverse transcription diagnostic kit and SYRB green were purchased from Toyobo (osaka, Japan). MicroAmp Optical 96-Well Reaction Plate was purchased from Applied Biosystems (Foster City, CA, USA). Rabbit anti-human β1, 3-N-acetylglucosaminyl-transferase-8 (β3GnT8) polyclonal antibody was purified by our laboratory. Goat-anti-human cluster of differentiation 147 (CD147) antibody and goat-anti-human β3GnT2 antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Hoechst 33258 detection kit was obtained from KeyGEN BioTECH (Nanjing, China). Biotin-labled Lectin from Lycopersiconesculentum was purchased from Sigma-Aldrich (Louis, MO, USA). Transwell 24 well plate was purchased from Corning Life Science (Shanghai, China).

Cell Culture

MCF-7 was cultured in RPMI-1640 (Gibco Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Gibco Life Technologies), and HepG2 was cultured in DMEM (Gibco Life Technologies) supplemented with 10% fetal bovine serum (FBS). The cells were cultured in a humidified atmosphere with 5% CO2 at 37℃.

Cell Viability Assay

Cell viability was detected by MTT assay. Cells were seeded at 5×10^3/well in 96-well plate and incubated for 6-24 h. Then we added increasing doses of triptolide to MCF-7 (0, 25, 50, 100, 200 and 400 ng/ml) and HepG2 (0, 10, 20, 25, 30, 35, 40, 45 nmol/l) for 48 h. Remove the supernatant and add 90 μl fresh medium and 10 μl MTT solution (5 mg/ml) to the wells. The cells were incubated for 4 h at 37℃. The cell culture medium was removed and 110 μl Formazan solution was added into the wells. The absorbance was read using a microplate reader (Ultraviolet Spectrophotometer AquaMate-Plus; Thermo Fisher Science, Waltham, MA, USA) at 490 nm.

Quantitative RT-PCR

Total RNA was extracted from cells with TriZOL (Invitrogen). cDNA was reversed using an RNA reverse transcription diagnostic kit (Toyobo). PCR reaction system was carried out on an ABI 7500 Sequence Detection System (Applied Biosystems) using the SYBR-Green real-time PCR Master Mix kit (Toyobo). PCR conditions and experimental concrete operations can refer to the article [25]. Primers (designed by Sangon Biotech, Shanghai, China) of all genes are listed in Table **1**.

Table 1: Sequences of the Primers Used for Quantitative RT-PCR.

Gene	Oligonucleotide sequence (5'-3')
β3GnT8	F: GTCGCTACAGTGACCTGCTG
	R:GTCTTTGAGCGTCTGGTTGA
β3GnT2	F:ATACTGGAACCGAGAGCAAG
	R:TCAGGTTCGCAGTAGTTCAG
CD147	F:ACCGTAGAAGACCTTGGCTC
	R:CGTCGGAGTCCACCTTGAAC
MMP14	F:AAACATCAAAGTCTGGGAAGG
	R:ACTTGGGATACCCTGGCTCT
GAPDH	F:AGAAGGCTGGGGCTCATTTG
	R:AGGGGCCATCCACAGTCTTC

Western Blot Analysis

Extract protein from the cells and measure its concentration using BCA. 30μg protein was run on 10% SDS-PAGA and transferred onto Pure Nitrocellulose Blotting Membrane (PALL, Mexico). After blocking with 5% fat-free milk to prevent non-specific background binding, the membrane was incubated with suitable antibodies. Then the proteins were visualized by using an ECL detection kit (CWBIO, Beijing, China). Detailed experimental procedures can be found in the reference [26].

Lectin Blot

SDS-PAGE and electrophoretic transferring were carried out as same as western blot analysis. After blocking with 5% fat-free milk, the membrane was incubated with 2 μg/ml of biotin-labled Lycopersicon esculentum agglutinin (LEA) (Sigma) overnight at 4˚C. Reactive bands were detected with a diluted HRP-conjugated streptavidin (Sigma), and the glycoproteins were visualized by using ECL (CWBIO).

Hoechst 33258 Staining

In apoptotic cells, the capacity of membrane to take in Hoechst 33258 is strengthened. Also, due to chromosomes are highly concentrated, the combination between Hoechst 33258 and chromosomes is enhanced and the staining emits strong blue fluorescence. Dead cells will not be stained. The two cell lines were treated with different doses of triptolide for 48 h. The concrete operations about fixing and staining the cells can be found in reference [26]. The stained cells were observed under a fluorescence microscope at UV 340 nm.

Flow Cytometric Analysis of Cellular Glycosylation

Collected cells were stained with 20μg/ml LEA (Sigma) at 37˚C for 2 h and next incubated with Streptavidin-R-Phycoerythrin from Streptomyces avidinii (Sigma) at 37˚C for 1 h. The fluorescence intensity of the stained cells were detected by flow cytometry (Becton-Dickinson, Mountain View, CA, USA), with cells only incubated with avidinii serving as controls. Details can be found in the reference [26].

Transwell Invasion and Migration Assays

Cells treated with different doses of triptolide were collected and resuspended in serum-free medium. 5×10^4 cells were added to the upper chamber, and the

bottom chamber was filled with 500μL medium with 10% FBS. After 24-48 h, cells of upper chamber were removed with a cotton swab, and cells on bottom of the filter were fixed using 40% paraformaldehyde and stained using eosin dye (Beyotime). Stained cells could be observed under a microscope (magnification, ×100). Detailed steps can be seen in reference [27].

Statistical Analysis

All quantified data was presented as the Mean ± standard deviation (S.D.). Statistical analysis was performed using GraphPad Prism software (La Jolla, CA, USA). Statistically significant differences were determined using paired-samples T test in SPSS 16.0 software (IBM, Armonk, NY, USA). $P < 0.05$ was considered to a statistically significant difference. The data was obtained from at least three independent experiments.

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