

Effects of magnetic nanoparticles of Fe_3O_4 combined with gambogic acid on apoptosis of SMMC-7721 cells

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Objective: This study aims to investigate the potential benefit of combination therapy with magnetic nanoparticles of Fe_3O_4 (Fe_3O_4 -MNP) and gambogic acid (GA) on SMMC-7721 cells.

Methods: The inhibition of proliferation of SMMC-7721 cells was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Cell apoptosis was calculated and analyzed by flow cytometry, and the expressions of the apoptosis-related protein were detected by Western blot.

Results: GA enhanced the cytotoxicity of SMMC-7721 cells in a dose-dependent manner. The Fe_3O_4 -MNP itself had no obviously inhibitory effect, but it could enhance the effect of GA on proliferation of SMMC-7721 cells. The apoptotic rate of SMMC-7721 cells induced by combination of GA with Fe_3O_4 -MNP was higher than that by GA alone. The expression levels of caspase-3 and caspase-8 after co-treatment of GA and Fe_3O_4 -MNP were higher than that exposed to either GA or Fe_3O_4 -MNP alone, while the levels of bcl-2 were downregulated.

Conclusion: Fe_3O_4 -MNP can promote GA-induced apoptosis of SMMC-7721 cells, which may be related to the downregulation of Bcl-2 and upregulation of caspase-3.

Keywords: primary hepatocellular carcinoma, traditional Chinese medicine, anti-tumor activity, targeted-drug carrier

Introduction

Gambogic acid (GA; $\text{C}_{38}\text{H}_{44}\text{O}_8$) (Figure 1) is one of active components of gamboge, a traditional Chinese medicine, which is a dry resin secreted from *Garcinia hanburyi*.¹ It is reported in traditional Chinese medicine that GA is cold, acidic, acerbic, and poisonous. In addition, GA has a significant anticancer effect on a wide variety of solid tumors, including lymphoma, colorectal cancer, and glioblastoma.²⁻⁴ However, the molecular mechanisms of its antitumor activity and its effects on hepatocellular carcinoma (HCC) cells are still poorly understood and await further investigations.⁵

HCC is the most common type of liver cancer. It is the third leading cause of cancer-related death globally. Most cases of HCC are secondary to either a viral hepatitis infection (hepatitis B or C) or cirrhosis. The prognosis of patients with HCC remains poor despite the recent therapeutic advances in HCC treatment. Therefore, the identification of the new biomarkers for HCC will supply an arm for improving diagnosis and management of human HCC.

Magnetic nanoparticles of Fe_3O_4 (Fe_3O_4 -MNP), a kind of biocompatible nano-material with low toxicity, is widely used as a targeted-drug carrier with target and sustained-release properties.⁶

Our study aims to evaluate the potential benefit of combination therapy with GA and Fe_3O_4 -MNP for HCC and whether Fe_3O_4 -MNP could promote the GA-induced

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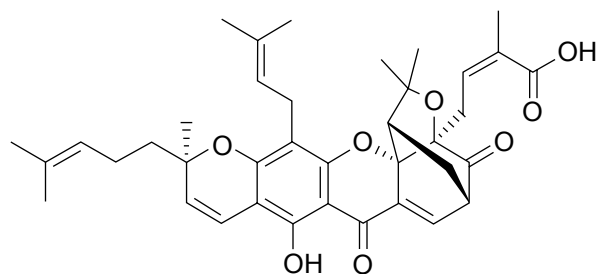


Figure 1 Molecular structure of GA.
Abbreviation: GA, gambogic acid.

apoptosis. To elucidate the mechanisms possibly involved, we also measured the expression of apoptosis-related proteins.

Materials and methods

Materials

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich Co. (St Louis, MO, USA). RPMI 1640 medium was from Gibco/BRL (Thermo Fisher Scientific, Waltham, MA, USA). Newborn calf serum was from Hangzhou Sijiqing Biological Engineering Materials Co., Ltd. (Hangzhou, People's Republic of China). Annexin V-Fluorescein Isothiocyanate Apoptosis Detection Kit and ECL (enhanced chemiluminescence) Western Blotting Detection Kit were from Nanjing KeyGen Biotech Co., Ltd. (Nanjing, People's Republic of China). Monoclonal antibodies, including caspase-3, caspase-8, survivin, and Bcl-2, were supplied by Santa Cruz Biotechnology Inc. (Dallas, TX, USA).

Gambogic acid

GA (Jiangsu Key Laboratory of Carcinogenesis and Intervention, China Pharmaceutical University, People's Republic of China) was dissolved in DMSO, stored at -20°C , and then diluted in RPMI 1640 medium.

Preparation of Fe_3O_4 -MNP

Fe_3O_4 -MNP was compounded and characterized by State Key Laboratory of Bioelectronics (Southeast University, People's Republic of China). The Fe_3O_4 -MNP was synthesized by electrochemical deposition under oxidizing conditions. Transmission electron microscopy indicated that the majority of Fe_3O_4 -MNPs were spherical in shape with particle sizes of approximately 30 nm (Figure 2).⁷ Before use, the Fe_3O_4 -MNPs were well dispersed in RPMI 1640 medium containing 10% (v/v) heat-inactivated newborn calf serum using ultrasound to obtain a colloidal suspension of Fe_3O_4 -MNP.

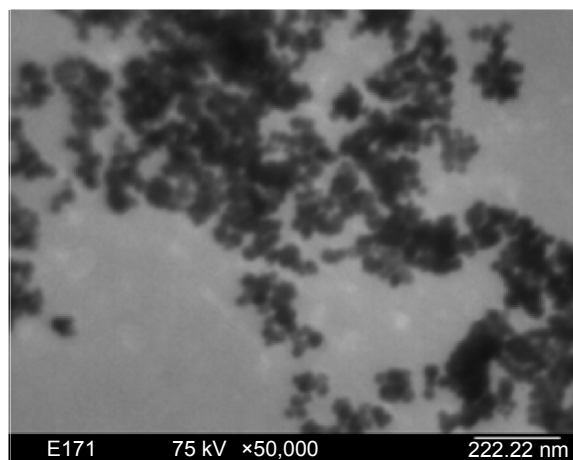


Figure 2 Image and mean size of magnetic nanoparticles of Fe_3O_4 under transmission electron microscope.

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Cell lines and culture conditions

Primary HCC SMMC-7721 cells were constantly preserved in our laboratory, which were cultured in RPMI 1640 medium containing 10% (v/v) heat-inactivated newborn calf serum at 37°C in a fully humidified incubator with 5% CO_2 .

Safe dose of Fe_3O_4 -MNP

SMMC-7721 cells were cultured with different concentrations of Fe_3O_4 -MNP (0–20 $\mu\text{g}/\text{mL}$) at 37°C for 24 hours, 48 hours, and 72 hours, respectively, and then the proliferation of cells was evaluated by MTT assay.

Inhibition of proliferation by MTT

As shown in our preliminary experiments, the inhibitory effects of the drugs were the most significant for 48 hours. So we chose 48 hours for the following experiments.

SMMC-7721 cells (5×10^4 cells per well) were incubated in a 96-well flat-bottomed plate (CoStar, Cambridge, MA, USA). Different concentrations of GA and 20 $\mu\text{g}/\text{mL}$ Fe_3O_4 -MNP were added separately and cultured at 37°C for 48 hours. A total of 20 μL of MTT solution (5 mg/mL) was added and incubated at 37°C for 4 hours, and then the optical density (OD) value was read at 540 nm using a plate reader. The inhibition rate of cells was determined as follows: $(1 - \text{OD of treated group} / \text{OD of control group}) \times 100\%$.

Apoptosis assay by flow cytometry

SMMC-7721 cells (5×10^5 cells per well) were incubated in a six-well flat-bottomed plate (CoStar). In all cells, 1 $\mu\text{mol}/\text{L}$

GA and 20 µg/mL Fe₃O₄-MNP were added separately and cultured at 37°C for 48 hours. The cells were collected and suspended in 500 µL of binding buffer, and 5 µL of Annexin-V–fluorescein isothiocyanate and 5 µL of propidium iodide were added, followed by incubation at room temperature for 15 minutes in the dark. Analyses were performed by using flow cytometry (BD, Franklin Lakes, NJ, USA).

Western blot analysis

After drug treatment, total protein was isolated from the SMMC-7721 cells (Whole Cell Lysis Assay, KGP250; KeyGen Biotech Co., Ltd.) and measured using BCA protein quantitation assay kit (KGPBCA; KeyGen Biotech Co., Ltd.). Then, it was subjected to SDS-PAGE and transferred to a polyvinylidene difluoride membrane. The blots were stained overnight with primary antibody (1:1,000–1,200) at 4°C and then with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1:5,000) for 1 hour at room temperature. The blots were visualized using the ECL system (Amersham, UK). Images were analyzed using ImageJ software from the National Institutes of Health (Bethesda MD, USA). β-Actin was used as an internal standard.

Statistical analysis

All experiments were repeated for at least three times. Data were expressed as mean ± SD and analyzed with the Statistical Package for the Social Sciences (SPSS Release 18.0, SPSS Inc., Chicago, IL, USA). Differences were evaluated using a two-way analysis of variance (ANOVA), followed by Bonferonni posttest. A *P*-value less than 0.05 was considered as statistically, significantly different.

Results

Fe₃O₄-MNP enhanced the effect of GA on SMMC-7721 cells

We noted that Fe₃O₄-MNP at less than 20 µg/mL concentration had no obviously effect on SMMC-7721 proliferation (*P*>0.05) (Figure 3). GA enhanced the inhibition of proliferation on SMMC-7721 cells in a dose manner. The IC₅₀ value after incubation with GA for 48 hours was 2.40±0.11 µmol/L (*P*<0.05). Then we investigated the combination of 20 µg/mL Fe₃O₄-MNP with different concentrations of GA to find the optimal combination to affect SMMC-7721 cells. It was observed that the addition of Fe₃O₄-MNP could indeed increase the inhibition of SMMC-7721 cells with GA, and 20 µg/mL Fe₃O₄-MNP could decrease the IC₅₀ value of GA to 1.29±0.07 µmol/L for 48 hours (*P*<0.05) (Figure 4).

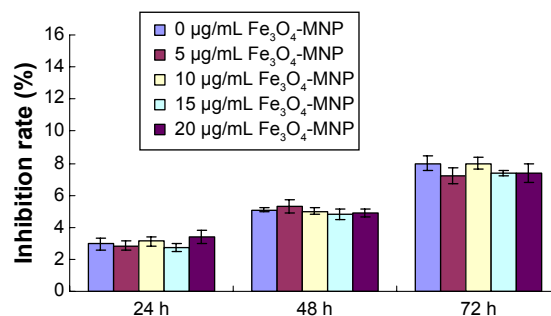


Figure 3 Inhibitory effects of the different concentrations of Fe₃O₄-MNP on growth of SMMC-7721 cells by MTT assay.

Abbreviations: Fe₃O₄-MNP, magnetic nanoparticles of Fe₃O₄; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; h, hours.

Enhancement of GA-induced apoptosis in SMMC-7721 cells

SMMC-7721 cells treated with 20 µg/mL Fe₃O₄-MNP (6.25%±0.58%) had no significant changes compared with the control group (6.57%±0.91%) (*P*>0.05). The apoptotic percentage of SMMC-7721 cells induced by 0.5 µmol/L GA for 48 hours was 20.05%±0.87% (*P*<0.05), while the combination of GA and Fe₃O₄-MNP (20 µg/mL) could obviously increase the percentage to 39.62%±2.10% (*P*<0.05) (Figures 5 and 6).

Effects of GA and GA + Fe₃O₄-MNP on the proteins of survivin, caspase-3, caspase-8, and Bcl-2

The protein level of caspase-3 and caspase-8 in cells treated with 0.5 µmol/L GA (lane C) was obviously elevated, compared with the control group (lane A) (*P*<0.05). Furthermore, it being upregulated by GA (0.5 µmol/L) with Fe₃O₄-MNP (20 µg/mL) in SMMC-7721 cells (lane D) was more than that exposed to GA alone (lane C) (*P*<0.05).

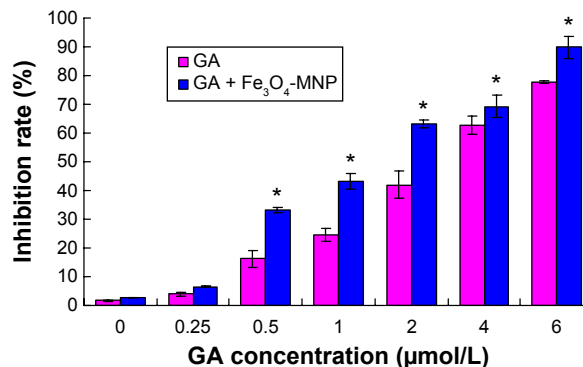


Figure 4 Inhibitory effects of the combination of 20 µg/mL Fe₃O₄-MNP with different concentrations of GA on growth of SMMC-7721 cells by MTT assay for 48 h.

Note: **P*<0.05.

Abbreviations: Fe₃O₄-MNP, magnetic nanoparticles of Fe₃O₄; GA, gambogic acid; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; h, hours.

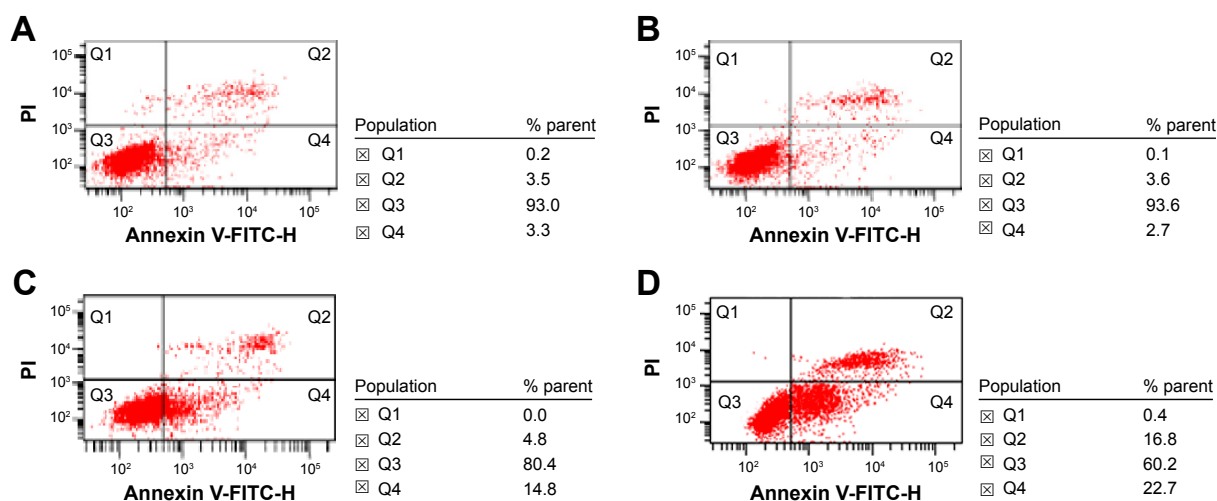


Figure 5 Effect of Fe₃O₄-MNP on GA-induced apoptosis in SMMC-7721 cells for 48 h.

Notes: (A) Control, (B) 20 µg/mL Fe₃O₄-MNP, (C) 0.5 µmol/L GA, and (D) 20 µg/mL Fe₃O₄-MNP + 0.5 µmol/L GA. Q1, mechanical injury cells; Q2, late apoptotic cells; Q3, live cells; Q4, early apoptotic cells.

Abbreviations: Fe₃O₄-MNP, magnetic nanoparticles of Fe₃O₄; GA, gambogic acid; h, hours; PI, propidium iodide; FITC, fluorescein isothiocyanate.

Reversely, compared with the control group (lane A), the protein expression of survivin and Bcl-2 was inhibited significantly in SMMC-7721 cells treated with 0.5 µmol/L GA (lane C) ($P < 0.05$). Moreover, the expression level of survivin and Bcl-2 in cells after co-treatment for 48 hours (lane D) was lower than that treated with GA alone (lane C) ($P < 0.05$). There was no statistical difference between the Fe₃O₄-MNP (20 µg/mL) group and the control group (Figure 7).

Discussion

Primary HCC is one of the most common malignant tumors, especially prevalent in Saharan Africa and Southeast Asia. Effective chemotherapy with standard agents in the treatment of HCC is often accompanied with serious adverse effects causing patients hard to be tolerated; this is one of the most important reasons leading to the failure of chemotherapy. Seeking HCC therapeutic agents with high effect

and low toxicity has become one of the main challenges for most pharmaceutical researchers to find or synthesize novel antitumor agents. GA, an apoptotic inducer, can selectively induce tumor cell death without toxicity on normal tissue, which offers a unique prospect for the development of new antitumor medicine.^{8,9}

Many polymer nanoparticles have been introduced as drug delivery systems.^{10,11} At present, Fe₃O₄ is the most common material of nanoparticles with low toxicity, magnetic properties, and metabolizable body.^{12–14} The present study is aimed to demonstrate the potential synergistic effects of Fe₃O₄-MNP and GA on apoptosis induction in SMMC-7721 cells. The data of MTT assay showed that Fe₃O₄-MNP at less than 20 µg/mL concentration had no obvious influence on the multiplication of SMMC-7721 cells but decreased the IC₅₀ value of GA. These results confirmed a good biocompatibility of Fe₃O₄-MNP and also demonstrated that the combination of GA with Fe₃O₄-MNP exerted a potent cytotoxic effect on SMMC-7721 cells. Our observations indicated that Fe₃O₄-MNP could promote GA-induced apoptosis in SMMC-7721 cells.

Anti-apoptosis could avoid cancer cell being killed by chemotherapy drugs. The Bcl-2 protein family plays a key role in the anti-apoptosis process.¹⁵ Caspase-3 and caspase-8 are the ultimate factors in the apoptotic process.¹⁶ The caspase-3 precursor could not be activated if the Bcl-2 is overexpressed. The caspase-3 expression decreases, and apoptosis pathway is blocked.¹⁷ GA combined with Fe₃O₄-MNP dramatically upregulated caspase-3 but downregulated the bcl-2 expression of SMMC-7721 cells, which supports the promotion of GA-induced apoptosis by Fe₃O₄-MNP with the increased expression level of proteins.

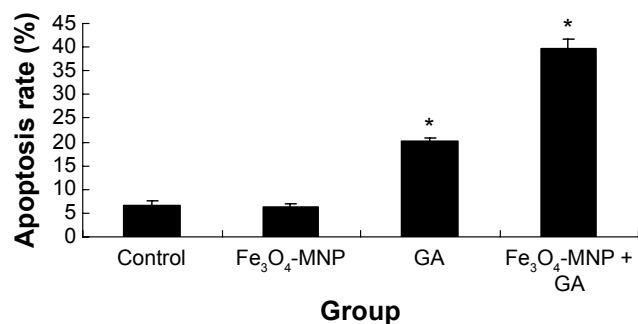


Figure 6 Bar chart of the apoptosis rate in different groups.

Notes: * $P < 0.05$. Apoptosis rate contains early apoptosis (Q4) and late apoptosis (Q2).

Abbreviations: Fe₃O₄-MNP, magnetic nanoparticles of Fe₃O₄; GA, gambogic acid.

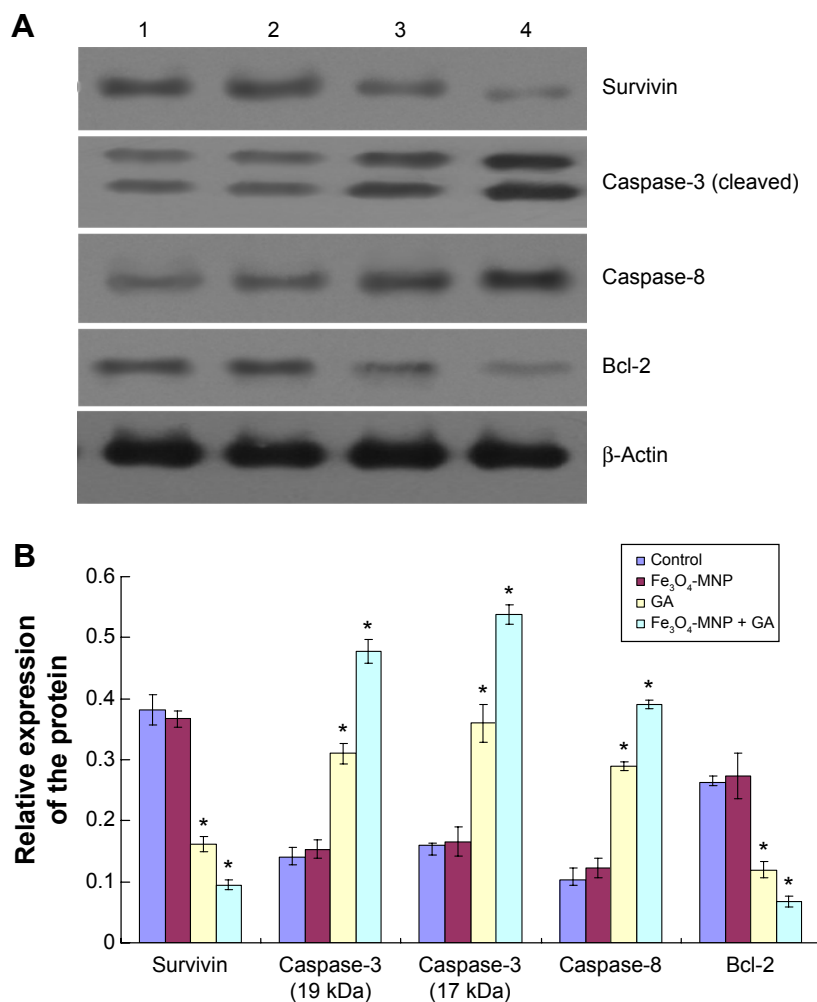


Figure 7 (A) Effect of GA and Fe₃O₄-MNP on the protein expression level of survivin, caspase-3, caspase-8 and Bcl-2 in SMMC-7721 cells for 48 h. (1) Control, (2) 20 µg/mL Fe₃O₄-MNP, (3) 0.5 µmol/L GA, and (4) 20 µg/mL Fe₃O₄-MNP + 0.5 µmol/L GA. **(B)** Bar graph showing Western blot analysis of survivin, caspase-3, caspase-8, and Bcl-2 in SMMC-7721 cells for 48 h.

Notes: Images were analyzed using Image J. β-Actin was used as an internal standard. The different levels of significance was indicated as **P*<0.05.

Abbreviations: GA, gambogic acid; Fe₃O₄-MNP, magnetic nanoparticles of Fe₃O₄; h, hours.

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Disclosure

The authors report no conflicts of interest in this work.

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