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Original article

# Nanomedicine-based combination of gambogic acid and retinoic acid chlorochalcone for enhanced anticancer efficacy in osteosarcoma

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ABSTRACT

In this study, gambogic acid (GA) and retinoic acid chlorochalcone (RACC) co-loaded glycol chitosan nanoparticle was successfully developed and studied for its therapeutic efficacy against osteosarcoma cancer cells. The GA/RACC loaded glycol chitosan nanoparticles (RGNP) was nanosized and exhibited a controlled release of drug in either pH 7.4 and pH 5.0. Owing to the strong positive charge on the RGNP surface, efficiency cellular uptake was observed in cancer cells. Moreover, a synergistic combination of GA and RACC were effectively suppressed the tumor growth progression. The half maximal inhibitory concentration (IC50) values in MG63 cells were 0.89 μg/ml and 0.35 μg/ml for GA and RGNP after 24 h. The results clearly suggest the synergist effect of GA and RACC in effectively inhibiting the cancer cell proliferation. The RGNP as expected induced a remarkably higher apoptosis of cancer cells with ~28%. Overall, combination of GA and RACC encapsulated in a nanocarrier could be an effective strategy to treat osteosarcoma. Future studies will focus on the *in vivo* evaluation of GA/RACC-loaded polymeric nanoparticles.



## 1. Introduction

Osteosarcoma is one of the most frequent malignant bone cancers which constitute nearly 30% of all bone cancers [1]. It mainly affects the long bones of children and adults in the age between 10 and 25 years. The osteosarcoma is characterized by tumor metastasis that differentiates towards the cartilage and bones. Despite the significant advancement in the treatment of osteosarcoma, the 5-year survival rate is still around 60% and metastatic cancer constitutes to only 20% survival rate [2,3]. At present, surgery, radiation therapy, and adjuvant chemotherapy constitutes the standard treatment strategy [4]. Especially, chemotherapy which is the most preferred treatment method in the early stages suffers from drug resistance and severe dose-limiting side effects resulting in no substantial improvement in osteosarcoma [5]. Therefore, more effective chemotherapeutic agents and effective therapeutic formulations need to be investigated.

In this regard, Gambogic acid (GA) which is the main active ingredient of gamboge resin has been recently reported to possess effective anticancer effect. The gamboge resin has been extracted from the tree, *Garcinia hanburyi* in South Asia [6]. GA has been reported to possess wide biological properties including antioxidant, antiviral, anti-infectious, and anti-inflammatory. Especially, GA has shown wide spectrum anticancer effects against multiple cancers including lung cancer, breast cancer, gastric cancer, hepatoma cancer, and osteosarcoma [7]. GA can effectively induce the apoptosis of cancer cells and supress the proliferation of cancer cells by the downregulation of telomerase activity and interruption of steroid receptor coactivator-3 [8]. Recently, Wang et al. reported the synergistic effect of GA with 5-fluorouracil in inducing apoptosis in gastric cancer cells and showed marked tumor growth inhibition *in vivo* [9]. However, several physicochemical limitations such as poor solubility, high sensitivity to light and temperature, and rapid clearance from the blood circulation limit its further clinical use [10].

Nanoparticles-based drug delivery system is considered as one of the most promising strategies to improve the physicochemical property of GA [11]. Poorly soluble drugs can be made soluble within the hydrophobic core of the nanoparticles and effectively delivered [12]. Such nanoparticles exhibits important properties including excellent systemic stability, high loading capacity, and preserving the pharmacological property of encapsulated drugs

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[13]. Moreover, nanosized particles allow the high accumulation and delivery of anticancer drugs in the cancer cells *via* enhanced permeability and retention (EPR) effect [14,15]. Keeping all this facts in mind, we have selected a unique nanomaterial that would act to synergize the anticancer activity of encapsulated compound.

We have selected a unique retinoic acid chlorochalcone (RACC) derivative to combine with GA. Retinoic acid (RA) has been reported to inhibit cancer cell proliferation and induce cancer cell apoptosis [16]. Several authors demonstrated the anticancer effect of RA in colon, prostate, lung, and leukemia cancers. Recently, RA has been combined with paclitaxel and exhibited a synergistic anticancer effect. Moreover, RA can translocate into the nucleus by binding to specific cytosolic proteins such as cellular retinoic acid binding protein II [17,18]. Recurrent malignant cerebral gliomas have been treated with all-trans retinoic acid (ATRA) and 13-cisRA [19]. In the present study, we have used RACC in order to increase the biocompatibility profile and to avoid the side effects of parent compound RA.

We mainly aimed at increasing the anticancer efficacy of GA in osteosarcoma by incorporating in RACC-based glycol chitosan nanoparticles. We hypothesized that combinatory effect of RACC and GA will increase the chemotherapeutic efficacy in osteosarcoma. The physicochemical properties of GA/RACC loaded glycol chitosan nanoparticles (RGNP) have been evaluated in terms of particle size, particle shape and *in vitro* release kinetics. Intracellular distribution of RGNP has been studied in MG63 osteosarcoma cells by means of confocal microscopy. Finally, anticancer effect of free GA and RGNP has been studied by cytotoxicity assay and apoptosis assay in osteosarcoma cells (Fig. 1).

## 2. Materials and methods

### 2.1. Materials

Glycol chitosan (GC) and RACC were purchased from Sigma-Aldrich, China. GA was purchased from 2A PharmaChem (Lisle, IL). All other chemicals were reagent grade and used without further purifications.

### 2.2. Preparation of GA and RACC co-loaded GC nanoparticles

5 mg of RACC and 5 mg of GA was dissolved in 2 mL of DMF and stirred for 15 min. Separately, 50 mg of GC was dissolved in 10 mL of distilled water and stirred for 2 h. The organic solution was added into the aqueous phase in a drop-wise under continuous stirring. The solution mixture was allowed to stir for 3 h until all the organic solvent evaporated. Followed by, drug-loaded NPs were dialysed using a dialysis membrane (3500 MW Cut off) for 15 h to completely remove all the organic solvents. The GA/RACC-loaded nanoparticles were separated from the free molecules by centrifugation. The unbound drugs were removed by centrifugation using an Amicon centrifugal filter device (MWCO, 10000 Da; Millipore) by centrifuging at high speed of 5000 rpm for 20 min. The drug-loaded nanoparticles were washed two times and repeated the same process. The final concentrations of drugs loaded in the nanoparticles were determined by measuring filtrate. The RGNP is then stored at 4–8 °C until further analysis.

### 2.3. Particle size analysis

The particle size and size distributions of nanoparticles were evaluated by dynamic light scattering (DLS) method using Zetasizer Nano S (Malvern Instruments Ltd, Malvern, UK). For DLS analysis, samples were measured at fixed angle of 90° at 25 °C. The scattering intensity was adjusted in the range of 50–200 kcps by diluting the samples with PBS.

### 2.4. Morphology analysis

The morphology of nanoparticles was determined using transmission electron microscope (TEM) JEM-1000 Ultra High Voltage Electron Microscope (JEOL Ltd, Tokyo, Japan). Briefly, diluted samples were placed in a copper grid and negatively stained with phosphotungistic acid and air dried. The samples were then viewed under TEM microscope.

### 2.5. In vitro *drug release*

The release of GA from RGNP system was evaluated by dialysis method. Briefly, nanoparticles were lyophilized and diluted with 1 mL of distilled water and packed in a dialysis membrane (MW ~ 3500 cut off). The dialysis pack was placed in 10 mL of phosphate buffered saline (PBS) and acetate buffered saline (ABS) at 37 °C. At selected time intervals, 1 mL of release media was withdrawn and replaced with equal volume of fresh medium. The rate of GA released from the nanoparticles was determined by HPLC method. Agilent 1100 equipped with a G1311A pump, a G1314A programmable diode array detector (DAD) and a G1313A auto-injector was used. C18 (250 mm × 4.6 mm ID, 5 μm) analytical column was used. The mobile phase comprised methanol and 0.05% phosphoric acid (94:6, v/v) and detected at 360 nm.

% Drug release = Amount of drug released (mg)/Total amount of drug used × 100

### 2.6. Cytotoxicity assay

The cytotoxic potential of free GA and RGNP was evaluated by 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide

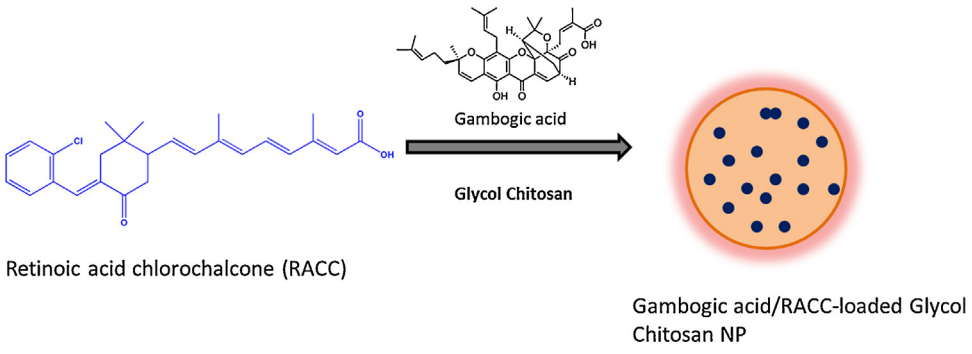


**Fig. 1.** Schematic presentation of preparation of gambogic acid (GA) and retinoic acid chlorochalcone (RACC) co-loaded glycol chitosan nanoparticle (RGNP).