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Original Research Article

Enhancement of the antitumor effect of cisplatin and ginsenoside Rg3 by encapsulation in polylactic-co-glycolic acid nanoparticles

Wen Xu[1], Qingping Zhang[1], Leiming Sun[2]*
[1]Internal Department, the Second Affiliated Hospital of Zhejiang University School of Medicine, Hangzhou, [2]Department of Critical Care Medicine, Hangzhou Red Cross Hospital, Hangzhou, Zhejiang 310003, PR China

For correspondence:* ***Email:* *nightsun16@sina.com*

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Abstract

Purpose: *To develop dual-loaded polylactic-co-glycolic acid (PLGA) nanoparticles (NPs) with two chemotherapeutic agents, i.e., cisplatin (DDP) and 20(R)-ginsenoside Rg3(Rg3), and to evaluate the drug release profiles and synergistic inhibitory effects of the nanoparticles on lung cancer A549 cells.*
Methods: *The dual-laden PLGA NPs were synthesized using modified emulsion and solvent vaporization procedures. Drug loading (DL) and efficacy of co-encapsulation (EE) were determined with a modification of column elution technique. The cytotoxic and inhibitory effects of individual and combined drugs on A549 lung cancer cells were evaluated using MTT assay.*
Results: *A sustained pattern of drug release was shown by PLGA/DDP, PLGA/Rg3, and PLGA/DDP+Rg3. The formulated PLGA and unbound drugs exerted dose- and time-reliant cytotoxic effects on A549 cells. At all concentrations tested, drugs encapsulated in PLGA nanoparticles were more effective in killing the cells than the free drugs ($p < 0.05$). In particular, PLGA/DDP + Rg3 formulation produced a synergistic inhibitory effect on the proliferation of A549 cells.*
Conclusion: *Single- and dual-load PLGA groups display variabilities in profiles of drug release and in vitro cytotoxic effect. Furthermore, dual-load PLGA group produces significantly enhanced treatment effect. These results suggest that PLGA formulations hold great potential for future drug delivery.*

Keywords: *Polylactic-co-glycolic acid (PLGA), Drug delivery, Cisplatin (DDP), 20(R)-ginsenoside Rg3(Rg3), Combination therapy*

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INTRODUCTION

Lung carcinoma is a major contributor to cancer-associated mortalities all over the world. In the year 2020, the GLOBOCAN project reported a projected 2.2 million newly diagnosed lung carcinoma cases likely to result in about 1.8 million fatalities [1]. Cisplatin (DDP) is a well-known chemotherapeutic agent with excellent clinical effectiveness against a wide variety of cancers, including lung cancer. However, the use of cisplatin is associated with drug resistance which results in clinical treatment failure and reduced drug efficiency [2]. Ginsenoside Rg3, one of the most abundant ginsenosides in Traditional Chinese Medicine (TCM), plays a

unique therapeutic role in cancer [3]. However, the poor water solubility and low bioavailability of Rg3 [4] limit both its clinical application and its basic use in research. Thus, there is need to boost the therapeutic effectiveness of Rg3 through enhancement of its aqueous dissolubility.

The polymer PLGA is a new and safe material for drug encapsulation which is officially recognized in USP as a biocompatible drug adjuvant [5]. It is a fabricable polymer used not only for encapsulating a hydrophobic drug such as gentiopicroside in an organic core but also for entrapping a high proportion of a hydrophilic therapeutic agent like epigallocatechin-3-gallate in an aqueous core [6]. The interest in fabrication of PLGA is due to the nature of its central core, which might be organic or aqueous, thereby allowing hydrophobic or hydrophilic substances to be entrapped. Indeed, PLGA is preferred for developing rational theragnostic systems because of its desirable features such as biocompatibility, controllability of drug release kinetics, protection of drugs or genes from decomposition, and capacity for surface modification with targeting agents [7]. These characteristics make it possible to attain the same level of treatment effect with lower drug doses and frequencies of administration, thereby reducing possible side effects. Moreover, PLGA has an appreciable knack for indissoluble and poorly-dissoluble medications. These qualities make it an appropriate polymer for encapsulating DDP and Rg3.

Platinum-based combination chemotherapy remains the best choice for non-small cell lung cancer. A recent study demonstrated that a ginseng-containing Chinese herbal formulation reversed cisplatin resistance in lung cancer patients [8]. This study, aimed to prepare PLGA-encapsulated DDP + Rg3 nanoparticles and evaluate their drug release profiles.

EXPERIMENTAL

Materials

Polylactic-co-glycolic acid (PLGA) was produced by Macklin Biochemical Corp., Shanghai; DDP was supplied by MedChem Express (Monmouth Junction, NJ, USA), while Rg3 was bought from Spring & Autumn Biological Engineering Corporation (Nanjing, China). Human lung adenocarcinoma cell line A549 was obtained from AtaGenix Laboratories (Wuhan, China). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was product of Sigma-Aldrich (St Louis, MO, USA). All other reagents were of topmost possible purity.

Synthesis of PLGA/DDP+Rg3, PLGA/DDP, PLGA/Rg3, and PLGA nanoparticles

The PLGA/DDP+Rg3 nanoparticles were prepared using a modified emulsion solvent evaporation method. In the first step, DDP and Rg3 were solubilized in CH_2Cl_2 to form a clear solution which was added to 1% (w/v) polyvinyl alcohol. The mixture was subjected to probe sonication using a probe sonicator (Scientz-IID; Scientz, Zhejiang, China) to form an oil-in-water emulsion. Then, isopropanol (2 % (w/v)) was added, and the emulsion was stirred for 3 h at room temperature to evaporate the organic phase. The resultant nanoparticles were recovered via centrifugation for 30 min at 10,000 rpm, washed with double-distilled water, and freeze-dried. In the preparation of PLGA/DDP nanoparticles, Rg3 was omitted, while DDP was omitted in the preparation of PLGA/Rg3 nanoparticles. Moreover, PLGA nanoparticles lacking both drugs were prepared. The final drug loading (DL) of each formulation was measured with HPLC.

In vitro release of DDP and Rg3 from PLGA nanoparticles

In vitro drug-release profiles were determined as described previously [9]. The free drugs and PLGA nanoparticles were dispersed in solution in the dark. At defined intervals, 1 mL samples were collected and replaced with 1 mL of fresh medium. The drug contents of the samples were analyzed with HPLC. Absorbance values were measured at 254 nm and 203 nm which correspond to the wavelengths of maximum absorption for DDP and Rg3, respectively.

In vitro cytotoxicity of PLGA nanoparticles

Cytotoxicity was measured using the 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. The assay is hinged on the enzyme-catalyzed reduction of the faintly-colored tetrazolium salt to deep-blue formazan crystals, the absorbance of which is quantified spectrophotometrically. The A549 lung carcinoma cells were incubated in 96-well plates overnight for attachment prior to treatment. Then, the cells were exposed to blank PLGA, free DDP, free Rg3, free DDP+Rg3, PLGA/DDP, PLGA/Rg3, and PLGA/DDP+Rg3 at various levels for 24, 48, and 72 h. Thereafter, the culture medium was replaced with 20 µL of MTT solution (5 mg/mL), followed by incubation for 4 h. The resultant formazan crystals were

solubilized in 150 µL of DMSO over 10 min, and the absorbance of formazan solution in each well was read at 490 nm in a spectrophotometer (Envision; PerkinElmer, Waltham, MA, USA). All experiments were repeated three times.

Statistics

Results are displayed as mean ± standard deviation. Two-group differences were evaluated using unpaired *t*-test. The SPSS ver. 17.0 software package was employed for statistics. Statistical significance was assumed at $p < 0.05$.

RESULTS

Drug-loaded PLGA

Based on the data reported in a previous study [9], the higher co-encapsulation efficiency (EE) of Rg3 (relative to DDP) is attributable to the hydrophobic nature of the former. The EE was calculated using Eq 1.

$$EE\ (\%) = (W_{encapsulated\ drug}/W_{total\ drug})100 \quad \ldots\ldots\ (1)$$

As shown in Chen DN's preliminary sustained-release experiments (Figure 1), no initial burst release was observed for either drug (i.e., for either single or dual-loaded PLGA). *In vitro*, free DDP, free Rg3, PLGA/DDP, PLGA/Rg3, and PLGA/DDP + Rg3 differed in terms of release rates. Free drug release peaked within 24 h. No initial burst release of encapsulated drugs was apparent. Drug releases from PLGA/DDP, PLGA/Rg3 and PLGA/DDP + Rg3 occurred in a sustained manner for up to 120 h. However, DDP and Rg3 exhibited different release patterns from PLGA/DDP + Rg3 nanoparticles: DDP was released in a sustained manner from the dual-loaded nanoparticles for 200 h, while Rg3 was released for 72 h. The rate of release of Rg3 from dual-loaded PLGA within the first 24 h was faster than that of DDP from dual-loaded PLGA, but the rate gradually decreased after 48 h. For example, after 24 h, approximately 78.18 % of Rg3 was released, when compared to 73.21 % of DDP from PLGA/DDP + Rg3. After 72 h, 84.19 % of Rg3 was released, while the release level of DDP was 86.66 %.

In vitro cytotoxicity

Both the free and nano-encapsulated drugs exhibited dose-dependent cytotoxicity on A549 cells, but free PLGA produced no obvious effect. After 72 h, the viabilities of cells treated with PLGA/DDP+Rg3 nanoparticles were 66.28 ± 2.11% and 1.10 ± 0.09% at nanoparticle concentrations of 0.5 and 200 µg/mL, respectively (Figure 2).

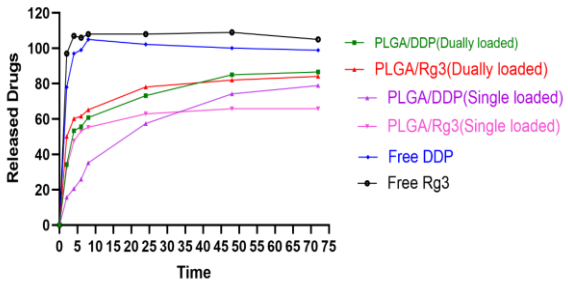

Figure 1: Drug release profiles of free DDP, free Rg3 and DDP from singly or dually loaded PLGA.

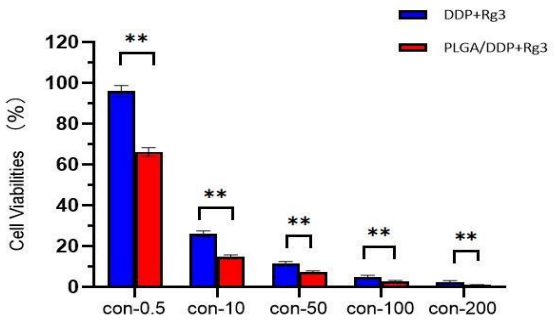

Figure 2: Cell viabilities of PLGA/DDP+Rg3 and free DDP+Rg3 at different concentrations after 72 h of treatment. $^{**}P < 0.01$ vs. control

Cytotoxicity was time-dependent. From 24 to 72 h, the effects of PLGA/DDP, PLGA/Rg3 and PLGA/DDP + Rg3 on A549 cells were gradually increased, particularly those of PLGA/DDP + Rg3. These results are shown in Figure 3. As expected, drugs encapsulated in PLGA nanoparticles were more effective at killing A549 cells than the free drugs at all concentrations tested ($p < 0.05$). At 72 h, cell viability was significantly lower in PLGA/DDP+Rg3 group than in DDP + Rg3 group (Figure 4).

Synergistic effect of PLGA/DDP+Rg3 on A549 cell suppression at 72 h were revealed using CompuSyn (Figure 5). The data on dose *(D)* and effect *(fa)* were entered into CompuSyn for the individual and combined drugs. The program outputted combination index (CI) at various *fa* (in sec) based on the algorithm for CI, and Chou-Talalay theorem was used to define the additive effect (CI = 1), synergism (CI < 1), and antagonism (CI > 1) of drug combinations. The combination index (CI) was <1 for the dual-loaded PLGA (PLGA/DDP + Rg3), indicating a synergistic suppressive effect on A549 cells by the combined nano-formulated drugs.

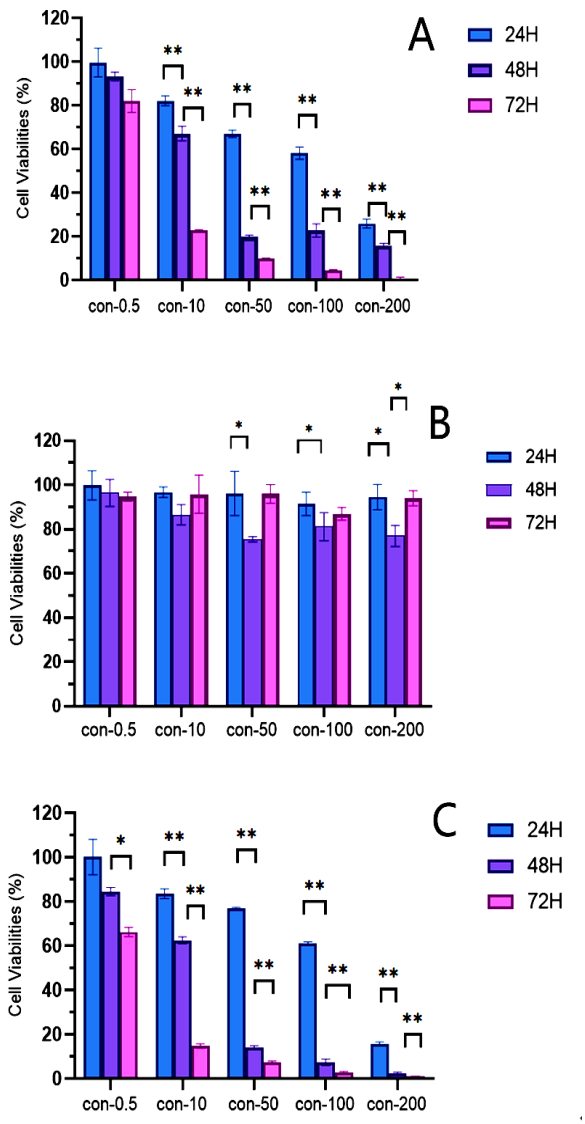

Figure 3: Viabilities of A549 cells exposed to PLGA/DDP(A), PLGA/Rg3 (B) and PLGA/DDP+Rg3 (C) from 24 to 72 h, as revealed in MTT assay. *P < 0.05 ; **p < 0.01

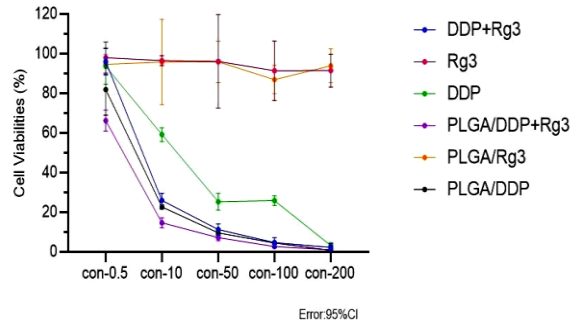

Figure 4: *In vitro* cytotoxicity in A549 cells treated with different concentrations of free DDP, free Rg3, free DDP+Rg3, PLGA/DDP, PLGA/Rg3, and PLGA/DDP+Rg3 for 72 h

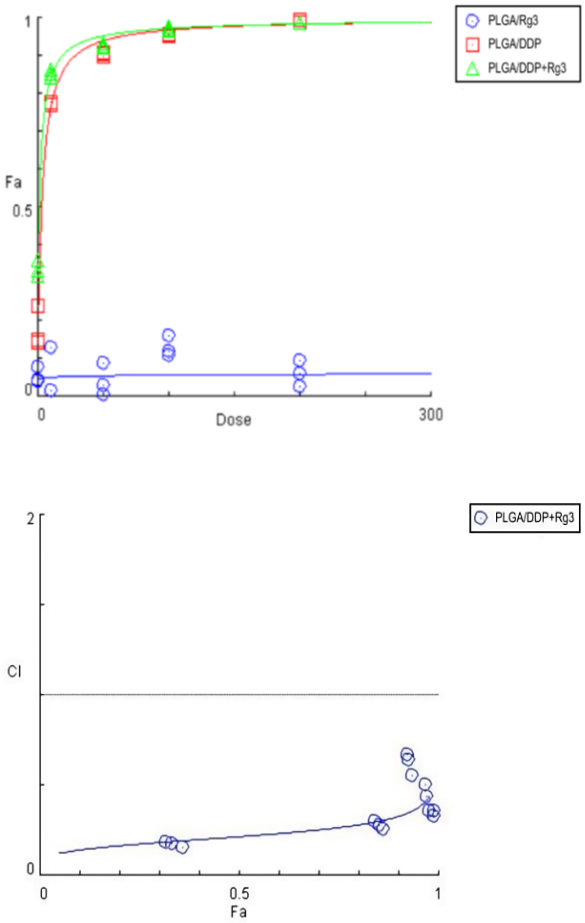

Figure 5: Synergistic effect of PLGA/DDP + Rg3 on A549 cells. (1) The CI was <1 for the dual-loaded PLGA (PLGA/DDP + Rg3), (2) program outputted CI at various *fa* (in sec)

DISCUSSION

Globally, lung cancer is the most common cancer and the leading cause of cancer death in men and women. In China, approximately 631,000 cancer-related deaths are reported yearly [10]. Cisplatin (DDP) is a first-line cancer drug. It has been reported that DDP-based combination chemotherapy provides a small but definite survival benefit in a significant proportion of individuals with advanced cancer of the lungs. However, the development of resistance to DDP and the severe side effects of the drug pose major problems [11]. Ginsenoside Rg3, which is extracted from the roots of ginseng, is active against various tumors. The possible mechanisms of action of ginsenoside Rg3 include induction of apoptosis, inhibition of tumor proliferation, inhibition of angiogenesis and metastasis, suppression of multidrug resistance, increased chemo-sensitivity, and enhancement of immunity [12].

It has been reported that the combined use of Rg3 and first-line chemotherapy provided better objective response and disease control rates, as well as improved Karnofsky performance status (KPS) and 1-2-year survival rates [13]. Moreover, the combined treatment resulted in more weight gain and greater reduction in side effects, when compared to chemotherapy alone [13]. Therefore, Rg3 is an invaluable anticancer drug which is safe and efficacious. However, the clinical utility of Rg3 is limited due to its poor water-solubility and low bioavailability [8]. Thus, it is necessary to enhance its water solubility to improve its efficacy.

Polylactic-co-glycolic acid (PLGA) used for drug delivery, is biodegradable, biocompatible, and drug-compatible, and it exhibits good mechanical properties. It has been used to transport proteins, peptides, bacterial and viral DNAs, and various anticancer drugs [14]. *In vivo,* PLGA is hydrolyzed to lactic acid and glycolic acid. These acids are further metabolized to water and carbon dioxide via the tricarboxylic acid cycle, and the end-products are excreted by the lungs. The use of PLGA as a pharmaceutical excipient has received approval from USFDA and European Medicines Agency [15]. In the present study, DDP and Rg3 were encapsulated in PLGA. The drug release profiles and the antitumor effects of the formulations were determined. Regulated release of Rg3 and DDP from PLGA is crucial for the success of cancer therapy. The different release rates of DDP and Rg3 probably reflect differences in their hydrophobicity. Hydrophobic drugs exhibit a zero-order release rate, while hydrophilic drugs show a triphasic pattern, i.e., an initial burst, followed by steady/controlled release and, finally, bulk erosion [16]. Since Rg3 is hydrophobic, it exhibits zero-order release kinetics. In contrast, DDP, being water-soluble, exhibits a triphasic release pattern. This may minimize any initial toxicity associated with a high drug dose. However, more precise (i.e., real-time) monitoring of drug release is required to predict the *in vivo* effects [17].

Results from *in vitro* cytotoxicity assay revealed that both free and PLGA-encapsulated drugs reduced the viability of A549 cells. The effects were more obvious in the PLGA/DDP and PLGA/DDP+Rg3 groups. This may be attributed to controlled drug release. It may be speculated that the free drugs easily pass through the cell membrane to reach peak concentrations. The micellar drugs entered the cells in a sustained and controlled manner. The efficacies of DDP and DDP+Rg3 when coated with PLGA were significantly greater than those of the corresponding free drugs.

The Chou-Talalay median-impact relationship serves as the basis of the CI-isobologram equation which allows for quantitative measurement of drug-drug interactions. Values of CI < 1, = 1, and >1 indicate synergistic, additive, and antagonistic effects, respectively [18]. The value of CI was <1 for the PLGA/DDP + Rg3 treatment at 72 h, indicating a synergistic effect. Thus, the drug combination was better than the two individual drugs when used alone. This method significantly improved the efficacy of combination treatments.

It was found that drugs encapsulated (singly or together) in PLGA nanoparticles exerted greater inhibitory effects on A549 cancer cells than free drugs. The inhibitory effect was dose- and time-dependent. The PLGA formulations were highly stable and produced sustained drug release. In nanoparticles, DDP and Rg3 acted synergistically. This will improve the efficacy of Chinese medicines. Other drug combinations should also be tested.

CONCLUSION

A nanoparticle strategy has been developed for co-delivery of DDP and Rg3 for synergistic anticancer effect *in vitro*. Dual-load formulation PLGA/DDP+Rg3 nanoparticles display an increase in anticancer effect in lung cancer A549 cells. These results suggest that PLGA formulations hold potentials for use in clinical practice.

DECLARATIONS

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Ethical approval

None provided.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them. Wen Xu designed the study, supervised the data collection, and analyzed the data. Qingping Zhang interpreted the data and prepared the manuscript for publication. Leiming Sun supervised the data collection, analyzed the data and reviewed the draft of the manuscript.

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