Chinese Chemical Letters 31 (2020) 3168–3172

Contents lists available at ScienceDirect

Chinese Chemical Letters

journal homepage: www.elsevier.com/locate/cclet

Communication

In vivo formation of $Cu(DDC)_2$ complex induced by nanomedicine for mesothelioma chemotherapy

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ARTICLE INFO

Article history:
Received 24 March 2020
Received in revised form 24 April 2020
Accepted 27 April 2020
Available online 7 May 2020

Keywords:
Mesothelioma
Nanoparticles
In vivo formation
PLGA
$Cu(DDC)_2$ complex

ABSTRACT

The copper(II) diethyldithiocarbamate ($Cu(DDC)_2$) complex exhibited excellent inhibition to cancer cells. The usual administration is intravenous injection for disulfram and oral for copper. A new strategy was reported to improve the administration efficiency of the $Cu(DDC)_2$ drug. Poly(lactide-*co*-glycolide) (PLGA) nanoparticles were used to trap disulfram and copper gluconate separately, the two types of drug loaded nanoparticles were injected in mesothelioma-bearing nude mice *via* intraperitoneal injection. The *in vivo* formation of $Cu(DDC)_2$ complex was induced by disulfiram and Cu^{2+} released from PLGA nanoparticles. This strategy avoided many obstacles in the use of $Cu(DDC)_2$ complex as chemotherapeutic and exhibited excellent anticancer activity to mesothelioma.

Mesothelioma is a malignant tumor occurred at mesothelial lining of the pleura, peritoneum, pericardium and tunica vaginalis. It is rapidly progressing with high morality and gets constantly growing attention as its peak is expected to reach in the next few years. The pathopoiesia of mesothelioma is overexposure to asbestos which was consumed excessively around the world, especially in Asia, in the past couple of decades [1–3]. Recent research reveals that carbon nanotubes would lead to mesothelioma [4]. As mesothelioma has a very long latency, even over 40 years, and the patients with mesothelioma have only approximately 10 months once diagnosed, if the treatment cannot be implemented in the early stage [5]. Current treatment of mesothelioma is inefficient and local recurrence is common. Antifolate combined with platinum is the only established chemotherapy for mesothelioma but benefits are usually modest at best and prognosis is poor. Some other clinical schemes were reported, however, no optimistic results were received at present [6].

The drawbacks of chemotherapy such as the toxicity of drug, multidrug resistance (MDR) and huge R&D cost of a new drug are particularly obvious [7–10]. "Drug repurposing" is an effective strategy that could greatly reduce time and cash consumption. Disulfiram (DS) as one example of "drug repurposing" is attracted more and more interests in cancer treatment. Studies have explored that DS is highly specific to cancer cells and could inhibit drug resistance [11]. The anticancer mechanism of DS is that DS could decompose into diethyldithiocarbamate (DDC), which easily chelates with copper(II) to form $Cu(DDC)_2$ complex to generate reactive oxygen species (ROS) and kill cancer cells [12]. The $Cu(DDC)_2$ complex is easily precipitated if DS and Cu^{2+} are complexed directly, the $Cu(DDC)_2$ precipitations could not be dispersed into nano-scale particles for encapsulation. In applications, DS and Cu^{2+} were administrated in different routes. DS was injected *via* intravenous injection, copper-containing preparations were administrated orally to increase the content of copper ions in tumor cells to generate more ROS intracellularly [13,14]. However, the utilization of oral copper ions was low, taking large doses of copper ions would cause serious poisoning [15].

Free DS is very unstable in the blood transportation [16], encapsulation of DS in nanoparticles could elongate the metabolic time, liposomes and polymeric micelles were reported for DS delivery [17,18]. In order to avoid the precipitation of $Cu(DDC)_2$ complex, an *in situ* complexation was carried out to encapsulate DS and Cu^{2+} in a same nanoparticle [19]. A new poly(ethylene glycol)-*b*-poly(ester-carbonate) block copolymer with carboxyl pendant groups was synthesized to chelate Cu^{2+}, DS was encapsulated in

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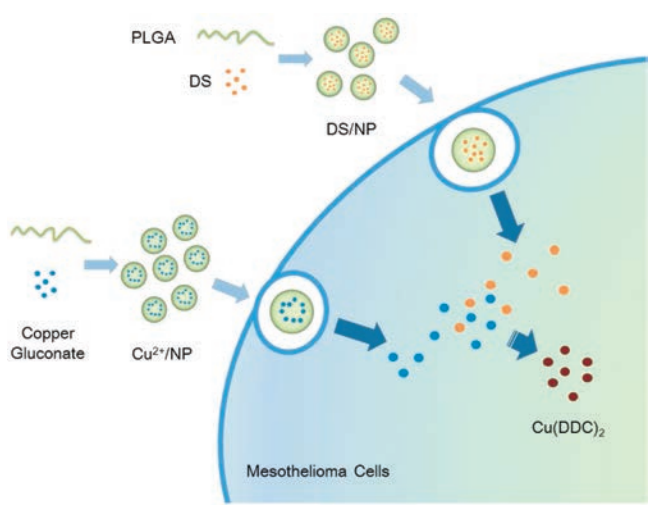
https://doi.org/10.1016/j.cclet.2020.04.051

the self-assembly polymeric micelles, as the chelating ability between Cu^{2+} and DS was much stronger than that between Cu^{2+} and carboxyl groups, the $Cu(DDC)_2$ complex was formed in the micelles and the drug loaded micelles were intravenously injected for non-small cell lung cancer (NSCLC) treatment. As the formation of $Cu(DDC)_2$ was too fast, it was hard to control the formation process. Other than encapsulation, a ROS responsive DDC prodrug with an aryl boronic ester modification was presented to overcome the rapid metabolism of DS in blood. The bond between DDC and aryl boronic ester in the prodrug was cleaved to return free DDC in the presence of high concentrated H_2O_2 in tumors, the complexation of DDC and oral administrated Cu^{2+} exerted efficient anticancer activity in breast cancer bearing mice model [20].

The mesotheliomas are composed of many nodules, which is very different from NSCLC and/or breast cancer with a solid tumor tissue in subcutaneous cancer bearing mice models. Nanomedicine is convenient for sustaining or controlled release and drug protection with syringe ability [21–23]. The *in situ* injection of nanomedicine facilitates local drug depot to maintain effective drug concentration over a long period of time to broaden the therapeutic window. In this study, a new strategy was used to be aimed at dispersive mesothelioma. Poly(lactide-*co*-glycolide) (PLGA75/25) was synthesized and fabricated nanoparticles (NPs) to load both hydrophobic DS and hydrophilic copper gluconate separately. The two kinds of nanoparticles were injected to the mesothelioma-bearing nude mice *via* intraperitoneal injection. The released DS and Cu^{2+} from the nanoparticles formed $Cu(DDC)_2$ *in situ* to kill mesothelioma cells. This *in situ* formation strategy of $Cu(DDC)_2$ complex could not only increase the Cu^{2+} utilization, but also avoid the precipitation and reduce systemic toxicity (Scheme 1).

The experimental section was presented in Supporting information. The ^1H NMR spectrum of PLGA75/25 was exhibited in Fig. S1 (Supporting information), and the molar ratio between lactide and glycolide was 77:23 according to the integrals calculation. The intrinsic viscosity of the copolymer was 1.10. The morphology of PLGA75/25 nanoparticles loaded with DS and copper gluconate was shown in Figs. 1A and B. It demonstrated that most of the nanoparticles were spherical with the size ranged from 90 nm to 240 nm. The drug loading contents of the two nanoparticles were presented in Fig. 1C. They were 3.70% and 0.40% for DS and copper gluconate loaded nanoparticles. The drug loading content of DS was much higher than that of copper gluconate due to the hydrophobicity of DS. As a water soluble molecule, copper gluconate was hard to be trapped in PLGA75/25 nanoparticles *via* double emulsion method. In order to promote the loading capacity of hydrophilic copper salt, copper gluconate was added to external phase to enhance the osmotic concentration [24]. Although the drug loading content of Cu^{2+} in PLGA75/25 nanoparticles was only 0.40%, it was high enough comparing to the dose of Cu^{2+} absorbed from oral administration. The drug release profiles of DS and Cu^{2+} in Fig. 1D revealed the release rate of Cu^{2+} was much faster than that of DS due to its hydrophilic essence, whose diffusion rate from nanoparticles was much higher than that of DS. Both accumulative release rates of DS and Cu^{2+} were around 60%, however, the reaching time for DS and Cu^{2+} were about 26 h and 13 h, respectively. The fast release of Cu^{2+} could also make up for deficiency of low drug loading content.

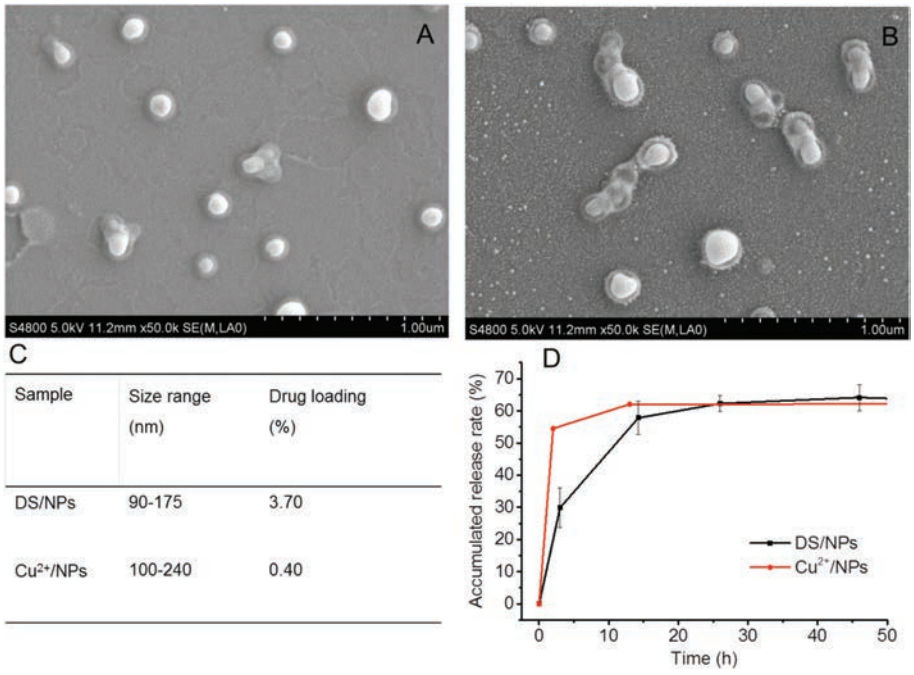


Scheme 1. Schematic formation of $Cu(DDC)_2$ complex *in vivo* for mesothelioma chemotherapy.

The cytotoxicity of blank PLGA75/25 nanoparticles was investigated and the results were demonstrated in Fig. 2A. The blank nanoparticles in different concentrations were incubated with mesothelioma cells for three days, the cell viabilities were tested for 24, 48 and 72 h incubation, interestingly, and the cell viabilities for 48 h incubation were the lowest no matter what the concentration was, they were in the range of 80%–90%. The cell viabilities in other conditions were all higher than 90%. It was probably because that the intracellular concentration of blank NPs reached the maximum when the incubation time was 48 h. The cell viabilities of all nanoparticles samples were higher than 80%, which could be considered as non-toxic according to the evaluation standard of biomaterials. Other than cancer cells, normal L929 cells were also incubated with blank NPs for 48 h to evaluate the cytotoxicity, the NPs were non-toxic as all the cell viabilities were higher than 80% (Fig. S2 in Supporting information).

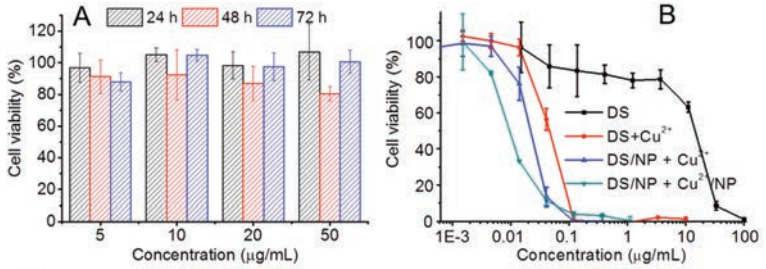
Four formulations of free DS, free DS with free copper gluconate ($DS + Cu^{2+}$), DS loaded NPs with free copper gluconate ($DS/NP + Cu^{2+}$) and DS loaded NPs with copper gluconate loaded NPs ($DS/NP + Cu^{2+}/NP$) were used to study the *in vitro* anticancer activity (Fig. 2B). The formulations with different concentrations were incubated with mesothelioma cells to explore the values of half maximal inhibitory concentration (IC_{50}), which were 16.0, 0.0436, 0.0217 and 0.00915 μg/mL calculated by Graph Pad Prism 5 (Fig. 2C). The results clearly demonstrated that only DS without Cu^{2+} would not kill mesothelioma cells efficiently (IC_{50} = 16.0 μg/mL) while the *in situ* formation of $Cu(DDC)_2$ complex with DS and Cu^{2+} released from PLGA75/25 NPs exhibited absolutely the best *in vitro* anticancer activity as the IC_{50} was as low as 0.00915 μg/mL. These results exhibited the superiority of nanomedicine [25–27].

Different from the classic application of DS nanomedicine with oral Cu^{2+} administration [28], two nanomedicine groups of DS loaded NPs plus oral copper gluconate ($DS/NP + Cu^{2+}$) and DS loaded NPs plus copper gluconate loaded NPs ($DS/NP + Cu^{2+}/NP$) were utilized to investigate the *in vivo* anticancer activity. Two groups of saline and blank NPs were used as controls. The nanomedicines were administrated *via* intraperitoneal injection under regulations approved by the Laboratory Animal Center of Sichuan University. As mesothelioma was composed of nodules, the nodules volumes and numbers were used to evaluate the *in vivo* anticancer activity of the nanomedicine in mesothelioma-bearing nude mice. The *in vivo* therapeutic effects of nanomedicines were showed in Fig. 3. As shown in Figs. 3A and B, the values of average volumes and numbers of $DS/NP + Cu^{2+}/NP$ group were 0.41 mm^3 and 0.33, however, those average volumes and numbers of the other three groups were from 16 mm^3 to 69 mm^3 and 5.67–8.33, respectively. Both numbers and volumes of $DS/NP + Cu^{2+}/NP$ group were much lower than those of the other three groups. The mesothelioma-bearing nude mice were dissected to observe the tumors in the mice (Fig. 3C) and their amplified



Sample	Size range (nm)	Drug loading (%)
DS/NPs	90-175	3.70
Cu^{2+}/NPs	100-240	0.40

Fig. 1. The morphology, size and drug release profiles of nanoparticles loaded with DS and Cu^{2+}. The SEM images of DS/NPs (A) and Cu^{2+}/NPs (B), the size and drug loading (C) and drug release profiles (D) of DS/NPs and Cu^{2+}/NPs.



C

Samples	DS	DS+Cu^{2+}	DS/NP+Cu^{2+}	DS/NP+Cu^{2+}/NP
IC_{50} (μg/mL)	16.0	0.0436	0.0217	0.00915

Fig. 2. The *in vitro* toxicity of blank NPs (A), different formulations (B) and the calculated IC_{50} of different formulations (C).

images were shown in Fig. S3 (Supporting information). There were many nodules observed in the peritoneum of saline and blank NP groups mice. Several nodules were found in the peritoneum of DS/NP + Cu^{2+} group, nearly no nodule was found in the peritoneum of DS/NP + Cu^{2+}/NP group. All these results revealed that the therapeutic efficacy of DS/NP + Cu^{2+}/NP formulation was excellent.

To most nanomedicines administrated by intravenous injection, the side effects of chemotherapeutics could be reduced greatly due to the utilization of carriers as well as the enhanced permeation and retention (EPR) effect. Body weight variation is an important parameter to show the toxicity of nanomedicine. The body weight variations of the nude mice administrated with the four formulations were presented in Fig. S4 (Supporting information). Different from other nanomedicines, the body weight variation of DS/NP + Cu^{2+}/NP formulation was undulated around the initial weight, however, the weight variations of the other three formulations were directly increased with time elongated.

After the mice were sacrificed, the organs and tumor nodules were stained. The H&E staining images were showed in Fig. 4. For all groups, there was no obvious damage in the H&E staining images of heart, lung, liver, spleen and kidney, indicating the safety of this strategy for $Cu(DDC)_2$ administration. The mesentery image of saline displayed that the mesothelioma cells formed papillary nodules which attached on the surface of mesentery. And the mesentery images of blank NPs and DS/NP + Cu^{2+} formulations showed the normal tissue architecture of mesentery while the mesentery in DS/NP + Cu^{2+}/NP group showed papillary lesion. To the nodules images of saline, blank NPs and DS/NP + Cu^{2+} formulations, papillomatous growth of the mesothelioma cells were clearly observed, and partial cells were destroyed in the image of DS/NP + Cu^{2+} formulation. As the nodules in DS/NP + Cu^{2+}/NP group were too rare and little to be separated, thus, no H&E staining image was received. The above H&E staining results revealed the anticancer efficacy of DS/NP + Cu^{2+}/NP formulation was the best.

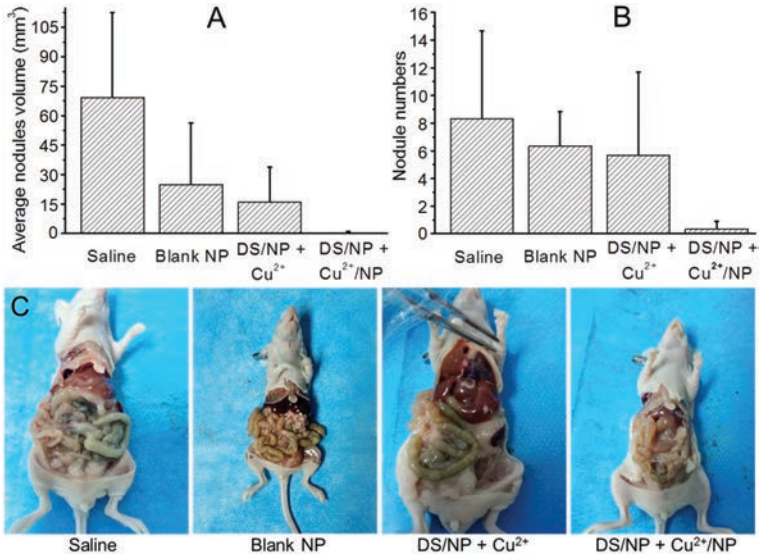


Fig. 3. The *in vivo* anticancer activity in nodules volumes (A), nodule numbers (B) and image of mesothelioma-bearing mice with four formulations.

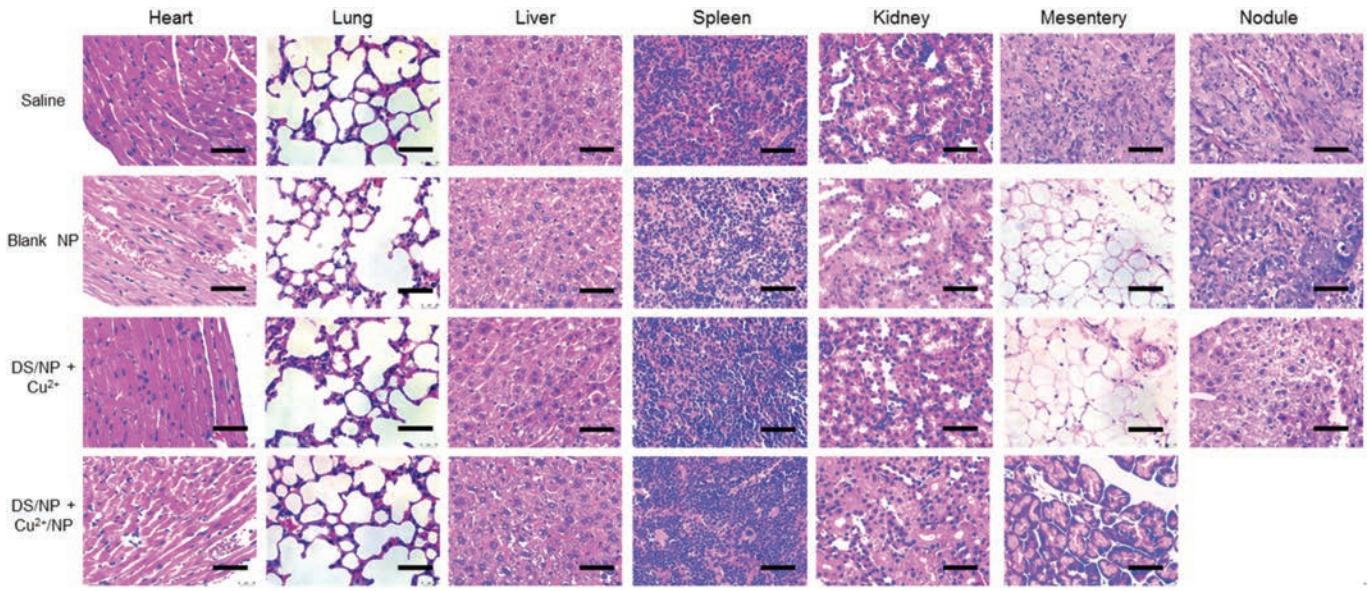


Fig. 4. H&E staining images of organs and tumor nodules. The scale bar is 50 μm.

In other subcutaneous tumor models, the excellent therapeutic effect of nanomedicine would be consistent with good results in both body weight increase and none or weak toxicity observed in H&E staining images. To our research, it was very different. The relationship between body weight variation and therapeutic effects on nodule volumes and numbers as well as H&E staining images seemed contradictory. In fact, these results were not conflict. The mesothelioma tumor models were implanted in the peritoneum of mice, abundant abdominal dropsy was produced. We measured the abdominal dropsy in the four groups and the volumes were 3, 1.98, 6.38 and 0.2 mL to saline, blank NP, DS/NP + Cu^{2+} and DS/NP + Cu^{2+}/NP groups. The abdominal dropsy gave great contribution to the body weight increase, thus, resulted in the contradictory appearance. The smallest volume of 0.2 mL abdominal dropsy to DS/NP + Cu^{2+}/NP formulation was perfectly consistent with the results of nodules and H&E staining images.

In summary, a new strategy of nanomedicine application with *in situ* formation of $Cu(DDC)_2$ complex as the chemotherapeutic was introduced. Two kinds of PLGA75/25 nanoparticles trapped with disulfiram and copper gluconate were administrated *via* intraperitoneal injection to the mesothelioma-bearing nude mice. The released disulfiram and Cu^{2+} were complexed as $Cu(DDC)_2$ in peritoneum to resolve the encapsulation problem of $Cu(DDC)_2$ precipitation and exert excellent chemotherapeutic function. The side effect of $Cu(DDC)_2$ was greatly reduced and efficient *in vivo* anticancer activity was received. Therefore, this strategy could be potential for the application of disulfiram in chemotherapy.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (No. 51773130), Natural Science Foundation of Shandong Province (No. ZR2017MC072). The authors thank Prof. Weiguang Wang for providing mesothelioma cells.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.cclet.2020.04.051.

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