Biomaterials Science

Polymer Nanoparticle Delivery of Dichloroacetate and DACH-Pt to enhance antitumor efficacy and lower systemic toxicity

Journal:	*Biomaterials Science*
Manuscript ID	BM-ART-10-2015-000439.R1
Article Type:	Paper
Date Submitted by the Author:	19-Dec-2015

Complete List of Authors:	Yan, Lesan; State Key Laboratory of Polymer Physics and Chemistry, Changchun Institute of Applied Chemistry, Chinese Academy of Sciences, Yang, Qiang; Huazhong University of Science and Technology, Department of Obstetrics and Gynecology, Union Hospital, Tongji Medical College Cai, Jing; Huazhong University of Science and Technology, Department of Obstetrics and Gynecology, Union Hospital, Tongji Medical College Sun, Si; Huazhong University of Science and Technology, Department of Obstetrics and Gynecology, Union Hospital, Tongji Medical College Kang, Xiang; Huazhong University of Science and Technology, Department of Obstetrics and Gynecology, Union Hospital, Tongji Medical College Guo, Jing; Huazhong University of Science and Technology, Department of Obstetrics and Gynecology, Union Hospital, Tongji Medical College Zhu, Yapei; Huazhong University of Science and Technology, Department of Obstetrics and Gynecology, Union Hospital, Tongji Medical College Jing, Xiabin; Changchun Institute of Applied Chemistry, State Key Laboratory of Polymer Physics and Chemistry Wang, Zehua; Huazhong University of Science and Technology, Department of Obstetrics and Gynecology, Union Hospital, Tongji Medical College

Journal Name

ARTICLE

Polymer Nanoparticle Delivery of Dichloroacetate and DACH-Pt to enhance antitumor efficacy and lower systemic toxicity

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

www.rsc.org/

Qiang Yang,[†a] Jing Cai,[†a] Si Sun,[a] Xiang Kang,[a] Jing Guo,[a] Yapei Zhu,[a] Lesan Yan[*,b] Xiabin Jing,[b] Zehua Wang[*a]

Platinum agents can cause DNA damage and thus induce apoptosis to cancer cells, which has made them the backbone of cancer chemotherapy regimens. However, most cancers will develop drug resistance over the course of the treatment. Meanwhile, most tumors meet energy needs largely by aerobic glycolysis (glycolysis in the presence of oxygen, called the Warburg effect), which is related to their resistance to apoptosis. Therefore, we have used a biodegradable polymer carrier to conjugate with DACH-Pt and dichloroacetate, a PDK inhibitor that can reverse the Warburg effect and derepress the resistance to apoptosis, thus sensitizing cancer cells to platinum. The as-prepared polymer-drug conjugates can be assembled into nanoparticles for effective delivery and better synergism. *In vitro* and *in vivo* studies revealed that the combination of polymer-DCA and polymer-DACH-Pt are much better than the free drugs administered simultaneously, in terms of both safety and antitumor efficacy.

Introduction

It has been almost 40 years since cisplatin, the very first platinum anticancer drug, was approved to cure testicular and ovarian cancer by the FDA in 1978[1; 2]. Since then, a variety of platinum compounds have been successfully developed and become the backbone of cancer chemotherapy[3; 4]. Cisplatin, carboplatin, and oxaliplatin, representative of the first, second, and third generation of platinum drugs respectively, are still being widely used in the clinic to treat patients with numerous kinds of tumors[3; 5; 6]. However, multiple cycles of chemotherapy can cause serious side effects and drug resistance, leading to recurrence of some advanced diseases and ultimately the failure of chemotherapy[2; 7-9].

Most solid tumors feature a hyperpolarized mitochondrial phenotype, and thus a unique cellular energy metabolic profile; unlike normal cells, which meet energy needs by means of mitochondrial glucose oxidation, cancer cells use glycolysis for energy production even in the presence of oxygen. This aerobic glycolysis phenomenon is called the Warburg effect[10-12]. It is usually thought to be a result of cancer progression; however, recently researchers proposed that it may be an early adaption to the hypoxic microenvironment in carcinogenesis, and that it persists because it is associated with mitochondrial dysfunction and resistance to apoptosis[10; 13; 14] and subsequently enables cancer cells to survive. Since the antineoplastic potential of platinum compounds relies on inducing apoptosis in cancer cells by forming crosslinks with DNA that causes DNA damage[1; 2], it is assumed that the Warburg effect may affect the response of cancer cells to platinum drugs through the suppression of apoptosis. Therefore, researchers proposed and verified that the reversal of the Warburg effect can sensitize cancers to platinum agents[15; 16].

Dichloroacetate (DCA) is a small molecule used for decades as a treatment for various acquired and congenital metabolic disorders intermediated by mitochondria[17; 18]. It activates pyruvate dehydrogenases (PDH) by inhibiting pyruvate dehydrogenase kinase (PDK), which promotes pyruvate entry into the mitochondrion, increases glucose oxidation, and depolarizes the mitochondrion; this induces apoptosis in cancer cells but not in non-cancerous cells[19; 20]. In this way, DCA was given new consideration as an anticancer drug and/or an "apoptosis-sensitizer" agent that could be combined with traditional cytotoxic drugs like platinum and bortezomib, etc.[15; 21-23].

In view of the possible connections among the platinum drugs, DCA and cancer, we assume that the combination of platinum and DCA can obtain a promising result. To further improve therapeutic efficacy, we conjugated DCA and 1R,2R-

[a] *Department of Obstetrics and Gynecology, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430022, China. Tel.: +86-2785351649. E-mail: zehuawang@163.net;*
[b] *State Key Laboratory of Polymer Physics and Chemistry, Changchun Institute of Applied Chemistry, Chinese Academy of Sciences, Changchun 130022, People's Republic of China. Email: lesanyan@gmail.com*
Graduate University of the Chinese Academy of Sciences, Beijing 100039, P. R. China
† *These authors contributed equally to this work.*

diaminocyclohexane platinum(II) (DACHPt, a precursor of oxaliplatin) to separate biodegradable polymer carriers, and then administered the two assembled nanoparticles to achieve a better synergism than simple administration of free platinum plus DCA.

Experimental

Synthesis of polymer conjugates P(Pt) and P(DCA)

Regents

Dichloroacetate (DCA) was purchased from Shanghai Jieshi Chemical Co., Ltd. (Shanghai, China). Oxaliplatin was obtained from Yangtze River Pharmaceutical Group (Jiangsu, China). 1R,2R-diamine-cyclohexane-platinum(II) dichloride (DAHPt(II)) was prepared as previously described[24]. The block polymer $MPEG_{5000}$-*b*-P(LA_{1000}-*co*-MCC_{960}) and $MPEG_{5000}$-*b*-P(LA_{1000}-*co*-MCC_{960}-OH) were synthesized as previously described[24; 25]. N,N-dicyclohexylcarbodiimide (DCC) and 4-dimethylaminopyridine (DMAP) were purchased from Sigma-Aldrich (USA). Mammalian genomic DNA extraction kit was obtained from Beyotime Biotechnology (Shanghai, China). Other chemicals and solvents were obtained commercially and used directly as needed.

General Methods

Inductively coupled plasma mass spectrometry (ICP-MS, Xseries II, Thermoscientific, USA) was used for quantitative determination of the total platinum content in the MPEG-*b*-P(LA-*co*-DHC/Pt(IV)) conjugate and trace levels of platinum in DNA-Pt adducts. The morphology of the polymer micelles was measured on a JEOL JEM-1011 electron microscope. Particle size and zeta potential measurements were conducted on a Malvern Zetasizer Nano ZS90.

Cell lines and cell culture

Human breast cancer cell line MCF-7 was purchased from the China Center for Type Culture Collection (Wuhan, China), and cultured in DMEM (Gibco, USA) supplemented with 10% Fetal Bovine Serum (Hyclone, USA) and 1% penicillin/streptomycin. Murine liver cancer cell line H22 was a generous gift from Tongji Hospital, Wuhan, China, and was cultured in RMPI-1640 (Gibco, USA) containing 10% FBS and 1% penicillin/streptomycin. The cells were incubated at 37°C in a humidified atmosphere of 5% CO_2.

Synthesis of conjugates MPEG-b-P(LA-co-MCC/Pt) (P(Pt))

P(Pt) was prepared as previously described[25].

Synthesis of MPEG-b-P(LA-co-MCC-OH/DCA) (P(DCA))

MPEG-*b*-P(LA-*co*-MCC-OH/DCA) was synthesized by DCC coupling reaction. Briefly, 100 mg MPEG-*b*-P(LA-*co*-MCC-OH) was dissolved in 10 mL dry dichloromethane in a flask, to which 30 mg DCC and 10 mg DAMP were added, followed by 10 mg DCA. This reaction mixture was left stirring for 1 h, after which it was filtered. The filtrate was then poured into 200 ml cold ether to precipitates the product, which was further purified by dialysis against water (MW=3,000 Da) overnight and then lyophilized to powder.

Preparation of drug loaded nanomicelles M(Pt) and M(DCA)

Nanomicelles were prepared by nano-precipitation method. M(Pt) micelles were prepared as previously described[24]. M(DCA) were made from P(DCA). Briefly, P(DCA) was dissolved in acetone in a conical flask at a total polymer concentration of 10% w/v. Water (two times the volume) was added drop-wise under stirring to form a micellar solution. After one hour stirring, the solution was rotary evaporated to remove the acetone and then freeze-dried to obtain the DCA-loaded nanomicelles M(DCA).

***In vitro* cytotoxicity assessment**

The *in vitro* cytotoxicity of all drugs was assessed by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay as described previously [26; 27].

MCF-7 cells were plated in 96-well plates at a density of 5000 cells/well. On the second morning, the medium was replaced with fresh medium (200 μl/well) that dosed with various drugs at predetermined concentrations. After 48 hours incubation, 20 μl of MTT solution (5 mg/ml) was added to each well and cells were incubated for another 4 hours, followed by removal of the MTT contained culture medium. 150 μl DMSO was then added to each well to dissolve the formazan crystals formed within live cells. Finally, the plates were incubated for 10 minutes at 37 °C, and the absorbance of the formazan product was measured at 495 nm on a microplate reader.

***In vitro* cellular uptake of RhB-labeled nanomicelles**

Rhodamine B was chemically conjugated to the polymer MPEG-*b*-P(LA-*co*-MCC-OH) as previously described[28]. The fluorescently-labeled copolymer MPEG-b-P(LA-co-MCC-OH/RhB) can further self-assemble into micelles.

For cellular uptake examination, MCF-7 cells were seeded at a density of 2×10^4 cells per well in 24-well plates and incubated for 24 h. When cells reached approximately 80% confluency, the medium was replaced with 500 μl of culture medium containing RhB-labeled nanomicelles (0.2 μg/ml RhB concentration), and either 0.1% NaN_3 (an endocytic inhibitor) or no NaN_3 (control). After incubation for 1 h or 4 h at 37 °C, the cells were washed three times with cold PBS, mounted in a Dako fluorescent mounting medium, and then observed under a confocal fluorescent microscope (FluoView™ FV1000, OLYMPUS, Japan). The relative intensity of RhB signal from each digital image was processed by the software Ver.4.2 supplied by the manufacturer. A region of interest (ROI) was drawn around each individual cell, and the average fluorescence intensity was measured in each image. Similarly, the average fluorescence intensity from an ROI of equal size drawn around a background region was also measured for each image. Numerical values of the background subtracted fluorescence intensity were then compared with each other.

Establishment of subcutaneous tumor xenografts

All animal experiments were approved by the Animal Care and Use Center of Tongji medical college, Huazhong University of Science and Technology. Female Kunming (KM) mice (6-8 weeks old, 20-25 g) were purchased from Tongji Medical College, Huazhong University of Science and Technology (Wuhan, China). All mice were maintained under Specific Pathogen Free conditions and had free access to food and water throughout the experiments. To develop tumor xenografts, H22 cells (5×10^5 cells in 0.1 mL of PBS) were injected into the lateral aspect of the anterior limb of the mouse. Tumor xenografts were allowed to grow to a desirable volume before subjected to further use. Tumor length (major axis), width (minor axis) and mouse body weight were measured every two days. The tumor volume was calculated as: Tumor volume (V)= length *width^2/2, as previously described[8; 25].

Assessment of *In vivo* antitumor efficacy

The tumor model was established as described in the previous paragraph. When tumor nodules grew to about 200-250 mm^3, tumor-bearing KM mice were randomly assigned to 7 groups with 6 mice per group, and were administrated assigned drugs through the tail vein on Day 1, 3, and 5. The drug formulations and doses are listed as follows: DCA (40 mg/kg); M(DCA) (40 mg DCA/kg); oxaliplatin (5 mg Pt/kg); M(Pt) (5mg Pt/kg); oxaliplatin +DCA (40 mg DCA/kg+5 mg Pt/kg); M(DCA) +M(Pt) (40 mg DCA/kg+5 mg Pt/kg); and saline (200 μl, the same volume of the other groups). After administration, the mice were monitored for 2 weeks. There were no mouse deaths over the course of the experiment.

***Ex vivo* tumor DNA-Pt adduct formation**

When tumor size reached to approximately 2000 mm^3, tumor-bearing mice were randomly assigned to 4 groups with 3 mice per group, and each group was intravenously injected with platinum-contained drug formulations. The drug formulations and doses are listed as follows: oxaliplatin (5 mg Pt/kg); M(Pt) (5mg Pt/kg); oxaliplatin +DCA (40 mg DCA/kg+5 mg Pt/kg) and M(DCA) +M(Pt) (40 mg DCA/kg+5 mg Pt/kg). 24 hours after injection, mice were sacrificed to harvest tumor xenografts. DNA was extracted using a Mammalian genomic DNA extraction kit (Beyotime Biotech, Shanghai, China), according to the manufacturer's instructions, and was quantified by UV-Vis spectrophotometry. The platinum content in DNA was determined by ICP-MS. The amount of DNA-Pt adduct was expressed as "pg Pt per μg DNA".

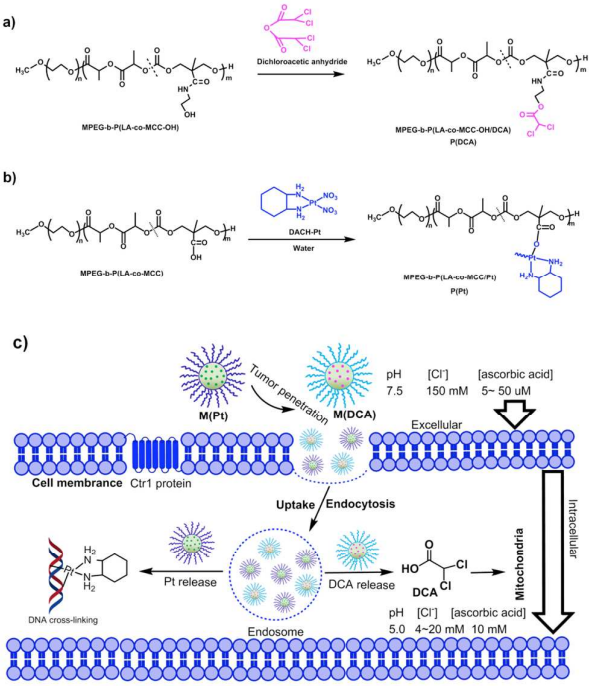
Statistical analysis

Data were expressed as mean ± standard deviation (SD). Student's t-test was used to determine the statistical difference between the combination indexes. The differences of DNA-Pt adduct formation among the four groups and the statistical significant of the antitumor efficacy were determined by one-way ANOVA and followed by *post hoc test* for subsequent multiple comparisons. All statistical analyses were carried out with SPSS 15.0 computer software (SPSS, Inc., Chicago, USA). $P < 0.05$ was considered statistically significant.

Results and Discussion

Synthesis and Characterization of polymer conjugates P(Pt) and P(DCA)

Similarly to our previous work[24; 25], we chelated DACHPt(II) onto the pendant carboxyl groups of methoxyl-poly(ethylene glycol)-block-poly(L-lactide-co-2-methyl-2-carboxyl-propylene carbonate) (MPEG-b-P(LA-co-MCC)), a commonly used biodegradable amphiphilic polymer, to make the polymer-DACHPt conjugate P(Pt) (the initial "P" represents "polymer") (Scheme 1). P(Pt) conjugates can self-assemble into micelles in aqueous solution. Due to the hydrophobicity of the P(LA-co-MCC/Pt) segment, the platinum species will be located in the inner core of the micelles and thus be protected against the environment. At the same time, the PEG shell decreases opsonin adsorption and immune recognition and thus assists P(Pt) in enhancing its circulatory time, functioning as "immune escape"[29; 30]. In the P(Pt) conjugate, the platinum content was 10 wt% as measured by inductively coupled plasma mass spectrometry (ICP-MS).



Scheme 1. Design of a biodegradable polymer platform for delivering DCA to sensitize platinum therapy. (a, b) Synthesis of the polymer-DCA conjugate, P(DCA) and polymer-DACHPt conjugate, P(Pt). (c) Possible mechanisms of action of M(DCA) and M(Pt).

A simple chemical reaction was used to conjugate DCA with MPEG-b-P(LA-co-MCC-OH), forming the biodegradable polymer-DCA conjugate (Scheme 1). This conjugate was characterized by ^{1}H NMR (Fig. 1). According to the supplier's data, the methinic proton peak from dichloroacetatic anhydride is at 6.12 ppm. Comparing the ^{1}H NMR of P(DCA) (Fig. 1) with that of the parent polymer MPEG-*b*-P(LA-*co*-MCC-

OH)[24], the newly emerged major NMR peak at 6.02 ppm clearly indicated the presence of DCA. By comparing the integration at 6.02 ppm (h in DCA) with protons in PEG at 3.65 ppm (-CH_2CH_2O- in polymer), we deduced the DCA content in P(DCA) of 9 wt% and the conjugation efficiency was ~100%.

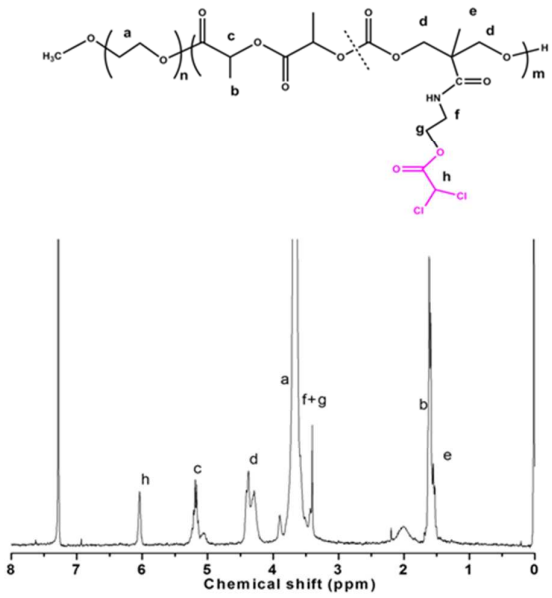


Figure 1. ^{1}HNMR of the conjugate P(DCA) in $CDCl_3$.

Preparation and Characterization of nanomicelles M(Pt) and M(DCA)

The preparation of nanomicelles was described in our previous work[24; 25]. Briefly, conjugates of P(Pt) and P(DCA) were first dissolved in acetone. By adding water in a drop-wise manner under stirring, the hydrophilic MPEG block forms the shell of the nanomicelles and the hydrophobic P(LA-co-MCC/Pt) or P(LA-co-MCC-OH/DCA) segment develops the inner core, which helps protect DACHPt and DCA from blood clearance. Nanomicelles containing DACHPt and DCA are referred to as M(DACHPt) and M(DCA), respectively. The nanomicelles are expected to be taken up by cancer cells via endocytosis, which is more efficient than passive diffusion or uptake by ion channel[31; 32]. Once internalized by cancer cells, the two nanomicelles would escape from the endosomes and then release the platinum species and DCA respectively, targeting the cell nucleus and mitochondria respectively. Moreover, the polymer carrier itself could be degraded into small inorganic molecules and ultimately excreted (Scheme 1).

The micelles were characterized by TEM and DLS. As shown in Fig. 2a, the M(DCA) is 59 nm in diameter by DLS with a PDI at 0.14. Fig. 2b is a representative TEM image of M(DCA), showing that M(DCA) are in spherical structures. As previously described[25], the M(Pt) is ~40 nm in diameter.

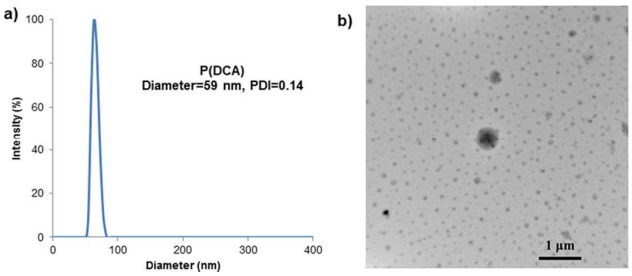


Figure 2. Representative DLS curve (a) and TEM images (b) of M(DCA) in aqueous solution at 0.5 mg/ml. The nanomicelles are spherical with 59 nm in diameter.

In vitro cytotoxicity of single drugs

In vitro cytotoxicity of the micelles was evaluated by MTT assay using human breast cancer cell line MCF-7.

First, we tested the cytotoxicity of the free drug oxaliplatin and the platinum loaded micelles M(Pt). As shown in Fig. 3a and Table 1, both free oxaliplatin and M(Pt) were moderately toxic towards cancer cells. IC_{50} values of oxaliplatin and M(Pt) were 23.6 μM and 21.3 μM respectively, revealing that the M(Pt) is comparable to oxaliplatin. This was somewhat surprising, considering our later finding that the nanomicelles have increased uptake into cells via endocytosis, thus resulting in enhanced intracellular accumulation of platinum[31]. Here, the enhanced intracellular accumulation did not cause a corresponding increase in cytotoxicity. This can be explained by the conjugation of the DACHPt to the polymer carrier; while more platinum was present within the cell, the platinum species may have been less available due to prolonged association with the polymer, so the cytotoxicity of M(Pt) did not increased with the intracellular accumulation.

Table 1. 48 h IC_{50} of the drugs against MCF-7 cell line

Drug	IC_{50}
Oxaplatin	23.6 μM
M(Pt)	21.3 μM
DCA	33.5 mM
M(DCA)	1.1 mM
OxaPt+DCA	12.3 μM
M(DCA)+M(Pt)	6.7 μM

Next, we evaluated the cytotoxicity of DCA and DCA-loaded nanomicelles M(DCA). Fig. 3b and Fig. 3c show the dose dependent response of DCA and M(DCA) on MCF-7 cancer cells at 48 h respectively. Note that the X-axial unit of Fig. 3b is mM, while for Fig. 3c it is μM. Results clearly showed that DCA had very limited toxicity towars MCF-7 cells by itself, even at concentrations as high as 20 mM (less than 40% inhibition rate). On the other hand, M(DCA) was much more toxic; the IC_{50} of M(DCA) was 1.1 mM, while that of free DCA was 33.5 mM. Considering that there were ~10 DCA molecules in one

polymer chain, the DCA IC_{50} at 1.1 mM corresponded to the a polymer concentration at 1 mg/ml, which lies within the "safety zone"[25]. The enhanced efficacy of DCA after polymer conjugation may be due to increased DCA accumulation within the cancer cells in the nanoparticle formulation. It is important to note that the effective concentration of micellar DCA is significantly higher than was seen with the platinum agents -- in the millimolar range, while platinum was micromolar, and we can say that even the M(DCA) is relatively "nontoxic" compared to the platinum agents. Though neither DCA formulation is ideal as an anticancer drug by itself, we can still use it to sensitize cancer cells to platinum agents, achieving a better antitumor efficacy through the synergistic effect of the two substances.

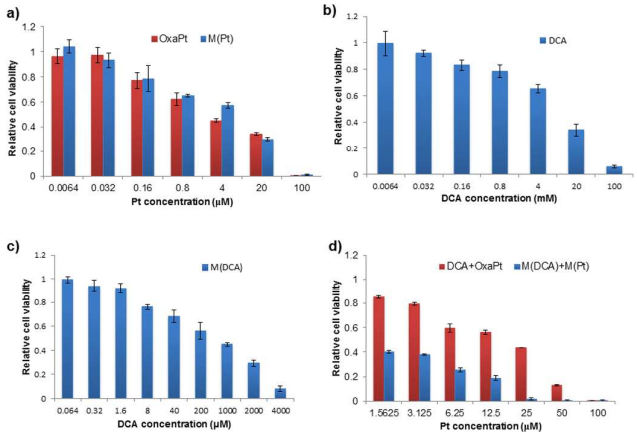


Figure 3. *In vitro* cytotoxic evaluation of OxaPt and M(Pt) (a), DCA and M(DCA) (b, c), and the combination therapies DCA/Pt and M(DCA/Pt) (d) in MCF7 cells at 48 h. Data were shown as mean ± S.D(n=3).

***In vitro* cytotoxicity of drug combination and synergism analysis**

To verify that the DCA can sensitize cancer cells to oxaliplatin, and to evaluate the effectiveness of administrating two nanomicelles simultaneously, we tested both the free drug combination "DCA+oxaliplatin" and the micellar combination "M(DCA)+M(Pt)" regimens. The MTT results were plotted in Fig. 3d, and the IC_{50} values were shown in Table 1. In the presence of 2 mM DCA, the IC_{50} of oxaliplatin is 12.3 μM, which is half of that of the oxaliplatin alone, i.e., a two-fold increase of cytotoxicity of the oxaliplatin was observed. For the nanomicelles, even with the same amount of DCA, the "M(DCA) +M(Pt)" regimen was much more efficient in cancer cell-killing than the free combination; the IC_{50} of the combination micelle treatment was 6.7 μM, much lower than that of nonmicellar DCA+oxaliplatin (12.3 μM) or oxaliplatin (23.6 μM). This justified our strategy of using nanomicelles to co-deliver DCA and DACHPt: more than simply wrapping up the two substances, this method constitutes a new and promising approach.

To quantitatively analyze the interaction between DCA and oxaliplatin/DACHPt, we calculated the combination index (CI) to determine the presence and extent of any synergism. For the combination of two drugs, CI was defined as a certain inhibitory effect of x% as shown in the following equation:

$$CI_x = \frac{IC_{x,Ac}}{IC_{x,As}} + \frac{IC_{x,Bc}}{IC_{x,Bs}}$$

Where CI_x represents the CI values at an inhibitory effect of x%, $IC_{x,Ac}$ and $IC_{x,Bc}$ represent the concentration of drug A/B at x% inhibitory effect in combination use, and $IC_{x,As}$, and $IC_{x,Bs}$, represent the concentration of drug A/B at x% inhibitory effect in single use.

According to Chou[33], the results of combination can be classified as additive effect (CI = 1), synergism (CI < 1), and antagonism (CI > 1), with lower CI values representing better synergism.

To discuss the synergy effect, we have chosen an inhibitory effect at 80%, whereas the cell proliferation is maximally suppressed under this circumstance, and thus represents the best combination index [34]. Therefore, the CI_{80} were calculated based on the IC_{80} of DCA, P(DCA), OxaPt, M(Pt), OxaPt+DCA and M(Pt)+M(DCA). The data were listed in the following table 2:

Table 2. IC_{80} of DCA and IC_{80} of Pt in various drug formulations, and the CI of the two combinations.

Drug	IC_{80} of DCA (mM)	IC_{80} of Pt (uM)	CI_{80}
DCA	6.21	0	N.A.
OxaPt	0	51.4	N.A.
M(DCA)	3.61	0	N.A.
M(Pt)	0	47.5	N.A.
OxaPt+DCA	2.00	45.7	0.92
M(Pt)+M(DCA)	2.00	10.3	0.78

As we can see, the CI values were smaller than 1, indicating that both drug combination showed a synergistic effect. M(Pt)+M(DCA) showed a slightly lower CI_{80} value, which means greater synergy.

Cellular uptake of nanomicelles

To visually observe the nanoparticles within the cancer cells and preliminarily study the cellular uptake mechanism, the effect of endocytic inhibition on RhB-labeled nanoparticles uptake was evaluated in MCF-7 cells. As shown in Fig. 4, the RhB-labeled nanoparticles were distributed throughout the cytoplasm, providing visually proof that this nanoparticle can effectively deliver drugs into the cells. From Fig.4, we can also see that the RhB fluorescence within the cells treated with NaN_3 was weaker than that of the control cells, i.e., the uptake of nanomicelles was suppressed by NaN_3. These results indicated that the nanoparticle uptake was endocytosis-dependent, as we discussed above.

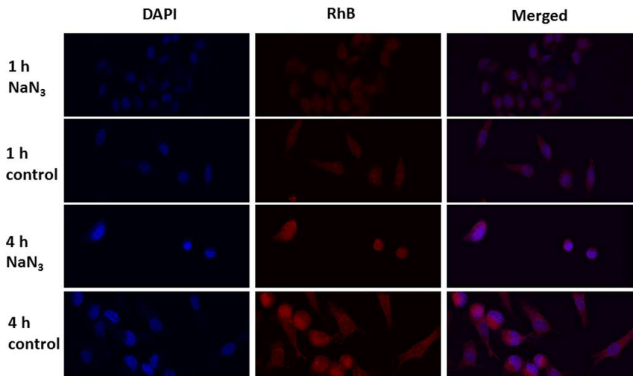


Figure 4. *In vitro* cellular uptake of RhB-labeled nanomicelles. In the presence of NaN_3 (0.1%), the uptake was suppressed compared to control.

In vivo antitumor study

To confirm the antitumor efficacy *in vivo*, H22 subcutaneous xenograft models were established in female KM mice. The mice were randomly divided into 7 groups as the tumors reached around 200 mm^3, about 7 days after inoculation of cancer cells. Drugs were then administered intravenously on day 1, 3 and 5, taking the day of first injection as day 1. The dosage of DCA in any group (if it contained DCA) was equivalent to 40 mg DCA compound per kg body weight; and that the dosage for platinum was equivalent to 5 mg platinum species per kg body weight. The tumor size and body weight were monitored every 2 days for a total of 2 weeks and were plotted in Fig. 5.

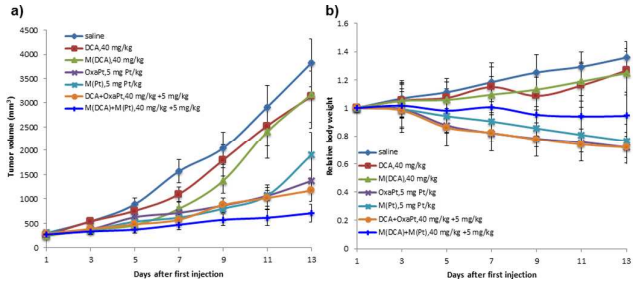


Figure 5. *In vivo* antitumor evaluation. Mice were injected with DCA (40 mg/kg), OxaPt (5 mg Pt/kg), DCA (40 mg/kg) + OxaPt (5 mg Pt/kg), M(Pt) (5 mg Pt/kg), M(DCA) (40 mg/kg), and M(DCA/Pt) (DCA: 40 mg/kg; Pt: 5 mg/kg) and saline. Six mice were in each group. (a) Tumor volume versus days post first injection; (b) Relative body weight versus days post first injection.

As shown in Fig. 5a, we observe the following phenomena: 1) The micellar drugs M(DCA) and M(Pt) displayed better antitumor effects than the same dosage of free drug DCA or oxaliplatin, in accordance with the *in vitro* results; this can be attributed to the nanotechnology approach, which enhanced the intracellular accumulation of the substances and prolonged circulation time[31]. 2) Both oxaliplatin and M(Pt) were superior to the DCA and M(DCA), in terms of inhibiting tumor growth. This may be attributed to the weak cytotoxicity of DCA, as described above. The *in vivo* study conforms that, although DCA may reverse the Warburg effect in cancer cells, the DCA by itself might not qualify as a sufficient anticancer agent[13]. 3) The combination of free DCA and oxaliplatin showed a considerable antitumor effect, which was even better than the micellar platinum drug M(Pt), i.e., the DCA sensitized cancer cells to oxaliplatin *in vivo*, just as it did *in vitro*. 4) What's more, the M(DCA)+M(Pt) displayed the best antineoplastic effect, better than single use of DCA or oxaliplatin or even the combined free drugs.

Fig. 5b displayed the relative body weight of the mice during the experiment. The three groups that did not receive platinum treatment did not suffer weight loss at all, while the other four groups lost weight to some extent. However, the weight loss of the M(DCA) +M(Pt) group was the least among all four groups subjected to platinum treatment; the mice lost about 6% of initial body weight by day 13, while the oxaliplatin group and the DCA+oxaliplatin group almost lost 30% of their initial body weights. Taking the tumor weight into account, this difference could be even more significant, because the tumors of the M(DCA)+M(Pt) group were the smallest ones. The *in vivo* xenograft model experiment confirmed the efficacy and safety of the nanoparticle mediated simultaneous delivery of DCA and DACHPt.

Ex vivo tumor DNA-Pt adduct formation

It is well known for all the platinum drugs kill cancer cells mainly by binding with DNA to induce DNA damage. Because of this, we measured the quantity of DNA-platinum adducts in the *ex vivo* tumor xenografts. The tumor-bearing mice were subjected to drug administration when tumor size reached ~2000 mm^3; 24 hours after that, the mice were sacrificed to harvest tumor xenografts. DNA was extracted and quantified by UV-Vis spectrophotometry, and the platinum content in DNA was determined by ICP-MS.

As shown in Fig. 6, the mice that received M(Pt) contained 17.66 pg Pt/ug DNA within their tumors, this was 5 times higher than those that received oxaliplatin, which had just 3.5 pg Pt/ug DNA ($p<0.001$). A similar outcome was seen with M(DCA)+M(Pt) vs DCA+oxaliplatin, which showed 15.90 pg Pt/ug DNA vs 3.32 pg Pt/ug DNA, respectively ($p<0.001$). This strongly validated that the nanomicelles can greatly increase the amount of DNA-Pt adducts. This increase can be attributed to the increased internalization of nanomicelles by cancer cells via endocytosis, and potentially by the EPR effect of solid tumors.

Another observation from Fig.6 is that the DCA did not enhance the DNA-Pt adducts formation (OxaPt *vs* DCA+OxaPt: 3.50 *vs* 3.32, $p>0.05$; M(Pt) *vs* M(DCA)+M(Pt): 17.66 *vs* 15.90, $p>0.05$). Therefore, the enhancement of cytotoxicity *in vitro* and the improved antitumor efficacy *in vivo* by the drug combination was not caused by increased cellular uptake or DNA-Pt adduct formation by DCA. This lack of DNA-Pt adduct increase is commonly seen in other forms of combination therapy, such as DCA and oxaliplatin[25] or cisplatin and paclitaxel[28].

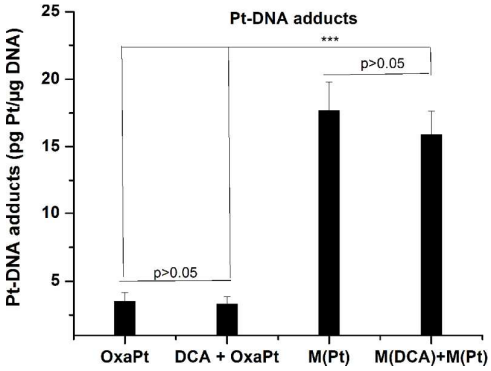


Figure 6. DNA-Pt adducts formed after 24 h treatment with various drugs. Data were shown as mean ± S.D (n=3). ***p<0.001

Conclusions

In summary, we have conjugated biodegradable polymers with DCA and DACH-Pt, to obtain a polymer-DCA conjugate P(DCA) and a polymer-DACH-Pt conjugate P(Pt), which were each assembled into nanomicelles. DCA was selected due to its ability to reverse the aerobic glycolysis found in cancer cells and consequentially derepress apoptosis, which could ultimately sensitize cancer cells to platinum agents. The nanomicelles loaded with DCA, M(DCA), and with DACH-Pt, M(Pt), were spherical and had mean diameters of about 60 nm and 40 nm respectively. *In vitro* cytotoxicity data showed the combination of micellar DCA and DACH-Pt was much efficacious than the free drug combination; the IC_{50} of the former was 6.7 μM while that of the latter was 12.3 μM, meaning that a two-fold increase of the sensitization was observed in cancer cells in the presence of DCA. The *in vivo* antitumor evaluation showed that the M(DCA) +M(Pt) was most effective in inhibiting tumor growth, and was also the least toxic (represented by body weight loss) than equivalent dosages of platinum. In conclusion, we showed a promising approach to sensitize cancers to platinum based chemotherapy.

Acknowledgements

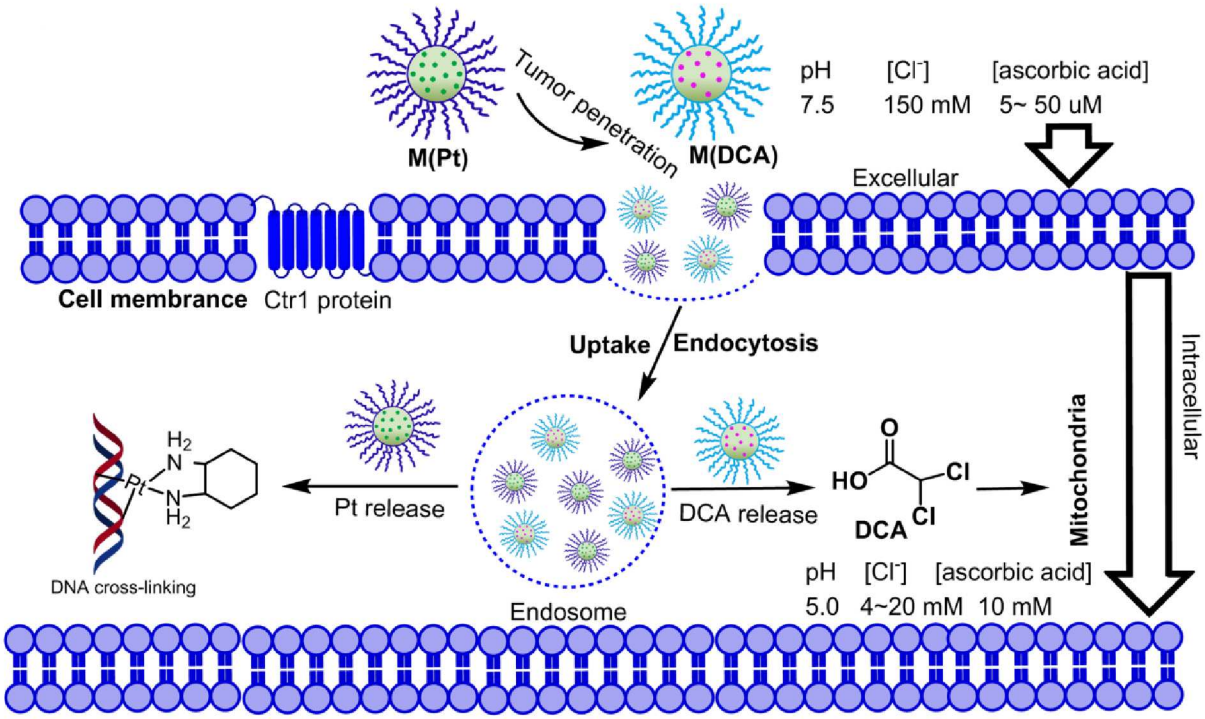
We give our sincere thanks to Dr. Elizabeth M Higbee-Dempsey from the School of Medicine at University of Pennsylvania for reviewing and editing the manuscript.

This work was supported by National Natural Science Foundation of China (81272860), and Wuhan Science and Technology Bureau (2014060101010042).

References

1 D. Wang and S. J. Lippard, *Nature reviews. Drug discovery*, 2005, **4**, 307-320.
2 A. Davis, A. V. Tinker and M. Friedlander, *Gynecologic oncology*, 2014, **133**, 624-631.
3 J. T. Reardon, A. Vaisman, S. G. Chaney and A. Sancar, *Cancer research*, 1999, **59**, 3968-3971.
4 G. Doshi, G. Sonpavde and C. N. Sternberg, *Expert opinion on drug metabolism & toxicology*, 2012, **8**, 103-111.
5 D. Baratti, S. Kusamura and M. Deraco, *The Lancet. Oncology*, 2014, **15**, e249.
6 G. Zhang, X. P. Li, B. J. Liu, J. L. Wang, S. J. Wang, H. Cui and L. H. Wei, *Chinese medical journal*, 2013, **126**, 4477-4482.
7 N. Eckstein, *Journal of experimental & clinical cancer research : CR*, 2011, **30**, 91.
8 J. Cai, C. Yang, Q. Yang, H. Ding, J. Jia, J. Guo, J. Wang and Z. Wang, *Oncogenesis*, 2013, **2**, e75.
9 Y. Min, C. Q. Mao, S. Chen, G. Ma, J. Wang and Y. Liu, *Angewandte Chemie*, 2012, **51**, 6742-6747.
10 R. A. Gatenby and R. J. Gillies, *Nature reviews. Cancer*, 2004, **4**, 891-899.
11 D. J. Granville and R. A. Gottlieb, *Current medicinal chemistry*, 2003, **10**, 1527-1533.
12 G. Bianchi, R. Martella, S. Ravera, C. Marini, S. Capitanio, A. Orengo, L. Emionite, C. Lavarello, A. Amaro, A. Petretto, U. Pfeffer, G. Sambuceti, V. Pistoia, L. Raffaghello and V. D. Longo, *Oncotarget*, 2015.
13 E. D. Michelakis, L. Webster and J. R. Mackey, *British journal of cancer*, 2008, **99**, 989-994.
14 D. R. Plas and C. B. Thompson, *Trends in endocrinology and metabolism: TEM*, 2002, **13**, 75-78.
15 R. A. Cairns, I. Papandreou, P. D. Sutphin and N. C. Denko, *Proceedings of the National Academy of Sciences of the United States of America*, 2007, **104**, 9445-9450.
16 J. W. Kim and C. V. Dang, *Cancer research*, 2006, **66**, 8927-8930.
17 P. W. Stacpoole, T. L. Kurtz, Z. Han and T. Langaee, *Advanced drug delivery reviews*, 2008, **60**, 1478-1487.
18 W. Li, M. O. James, S. C. McKenzie, N. A. Calcutt, C. Liu and P. W. Stacpoole, *The Journal of pharmacology and experimental therapeutics*, 2011, **336**, 87-94.
19 J. G. Pastorino, J. B. Hoek and N. Shulga, *Cancer research*, 2005, **65**, 10545-10554.
20 M. C. Sugden and M. J. Holness, *American journal of physiology. Endocrinology and metabolism*, 2003, **284**, E855-862.
21 S. Bonnet, S. L. Archer, J. Allalunis-Turner, A. Haromy, C. Beaulieu, R. Thompson, C. T. Lee, G. D. Lopaschuk, L. Puttagunta, S. Bonnet, G. Harry, K. Hashimoto, C. J. Porter, M. A. Andrade, B. Thebaud and E. D. Michelakis, *Cancer cell*, 2007, **11**, 37-51.
22 W. Y. Sanchez, S. L. McGee, T. Connor, B. Mottram, A. Wilkinson, J. P. Whitehead, S. Vuckovic and L. Catley, *British journal of cancer*, 2013, **108**, 1624-1633.
23 S. Fujiwara, Y. Kawano, H. Yuki, Y. Okuno, K. Nosaka, H. Mitsuya and H. Hata, *British journal of cancer*, 2013, **108**, 170-178.
24 H. Xiao, D. Zhou, S. Liu, Y. Zheng, Y. Huang and X. Jing, *Acta biomaterialia*, 2012, **8**, 1859-1868.
25 Q. Yang, H. H. Xiao, J. Cai, Z. G. Xie, Z. H. Wang and X. B. Jing, *Rsc Adv*, 2014, **4**, 61124-61132.
26 L. Yan, W. Wu, W. Zhao, R. Qi, D. Cui, Z. Xie, Y. Huang, T. Tong and X. Jing, *Polym. Chem.*, 2012, **3**, 2403–2412.
27 H. Xiao, W. Li, R. Qi, L. Yan, R. Wang, S. Liu, Y. Zheng, Z. Xie, Y. Huang and X. Jing, *J. Controlled Release*, 2012, **163**, 304–314.
28 H. Xiao, H. Song, Q. Yang, H. Cai, R. Qi, L. Yan, S. Liu, Y. Zheng, Y. Huang, T. Liu and X. Jing, *Biomaterials*, 2012, **33**, 6507-6519.
29 F. Ni, L. Jiang, R. Yang, Z. Chen, X. Qi and J. Wang, *Journal of nanoscience and nanotechnology*, 2012, **12**, 2094-2100.
30 M. Cavadas, A. Gonzalez-Fernandez and R. Franco, *Nanomedicine*, 2011, **7**, 730-743.

31 C. M. Beddoes, C. P. Case and W. H. Briscoe, *Advances in colloid and interface science*, 2015, **218**, 48-68.
32 M. Bezanilla, A. S. Gladfelter, D. R. Kovar and W. L. Lee, *The Journal of cell biology*, 2015, **209**, 329-337.
33 T. C. Chou, *Cancer research*, 2010, **70**, 440-446.
34 M. Hu, P. Huang, Y. Wang, Y. Su, L. Zhou, X. Zhu, and D. Yan, *Bioconjugate Chem*, *2015*, **26**, 2497−2506



Nanoparticles loaded with Dichloroacetate and DACHPt showed potential to sensitize cancer cells to chemotherapy *in vitro* and *in vivo*.