Molecular Cancer Therapeutics

A Prodrug of Two Approved Drugs, Cisplatin and Chlorambucil, for Chemo War Against Cancer

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Abstract

Cancer cells maintain normal mitochondrial glutathione as one of the defense mechanisms to inhibit mitochondrial membrane polarization and hence apoptosis. A combinational therapeutic modality Platin-Cbl, a prodrug of FDA-approved chemotherapeutic agents, cisplatin and chlorambucil (Cbl), was synthesized and characterized to explore the potential of this compound to initiate chemo war on cancer cells using the active drugs, cisplatin and Cbl, when delivered to the cellular power house mitochondrion using a targeted nanoparticle designed to get associated with this organelle. Platin-Cbl demonstrated significantly high cytotoxic activity across a number of tumor cell lines as well as in a cisplatin-resistant cancer cell line compared with cisplatin or its mixture with Cbl suggesting its unique potency in cisplatin-resistant tumors. A mitochondria-targeted nanoparticle formulation of Platin-Cbl allowed for its efficacious mitochondrial delivery. *In vitro* studies documented high potency of Platin-Cbl nanoparticle formulations. Cisplatin-resistant cells upon treatment with Platin-Cbl were still able to manage energy production to a certain extent via fatty acid pathway; the advantage of using T-Platin-Cbl-NP is that this nanoparticle treatment causes impairment of all metabolic pathways in cisplatin-resistant cells forcing the cells to undergo efficient apoptosis. This study highlights a combination of several beneficial effects for a cascade of events to overcome resistance associated with single drug therapy. *Mol Cancer Ther; 16(4); 625–36. ©2017 AACR.*

Introduction

Metabolic transformations in cancer cells caused by mutations in oncogenes and tumor suppressor genes result in enhanced cellular stress (1–3). Long-term exposure of chemotherapeutic treatment to cancer triggers multidrug resistance (MDR) characterized by resistance to various kinds of chemotherapeutic agents (4). Significant roles of subcellular mitochondrion in energy production, metabolic process, apoptosis, necroptosis, and redox balance (5–8) identify this organelle as an alternative target to combat MDR by evading DNA repair and attacking metabolic pathways for cancer therapy. We previously reported several Pt(IV) prodrugs with ability to release Food and Drug Administration approved drug cisplatin and an additional therapeutic modality such as aspirin or GSH modulator (9, 10). Our recent work also demonstrated advantages of delivering Pt(IV) prodrugs to the mitochondria for local release of cisplatin with improved *in vitro* and *in vivo* properties compared with cisplatin or non-targeted counterparts (11, 12). Although the extent of glutathione (GSH) compartmentalization in the mitochondria is relatively less compared with its cytosolic concentration of 10 to 15 mmol/L; considering mitochondrial volume, mitochondrial GSH (mGSH) concentration attains approximately 10 to 14 mmol/L (13, 14). Cancer cells maintain normal mGSH (15). In the reactive oxygen species (ROS) rich mitochondrial environment, mGSH provides the buffering mechanism and normal mGSH levels inhibit mitochondrial membrane polarization and hence apoptosis (13–15). Thus, mitochondrial delivery of chemotherapeutics with ability to modulate mGSH can further control the fate of cancer cell death by apoptosis under cellular resistance (16). Chlorambucil (Cbl) is such a compound that has the ability to alter cellular GSH through glutathione S-transferase (GST) catalyzation (17). In addition, creation of different kinds of DNA damage such as a combination of alkylation and cross-linking can potentially combat resistance toward apoptosis, so that it becomes challenging for cells to execute defense mechanisms against different types of lesions and modulation of mGSH to induce apoptosis. A number of multithreat platinum (Pt)-based compounds were elegantly listed in the recent reviews (18–20).

We envisioned attacking multiple cellular pathways by constructing a prodrug of well-known DNA alkylating and GSH interacting agent nitrogen mustard or Cbl (21) with DNA crosslinking agent cisplatin (22–24) in the form of a prodrug by constructing Platin-Cbl and delivering in the mitochondrial network using a mitochondria-targeted delivery system (Fig. 1A). Fonseca and colleagues developed a mitochondria-targeted version of Cbl and demonstrated that by redirecting Cbl to the mitochondria, this new compound induced apoptosis in resistant cells (25). We recently demonstrated that targeting

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Note: Supplementary data for this article are available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

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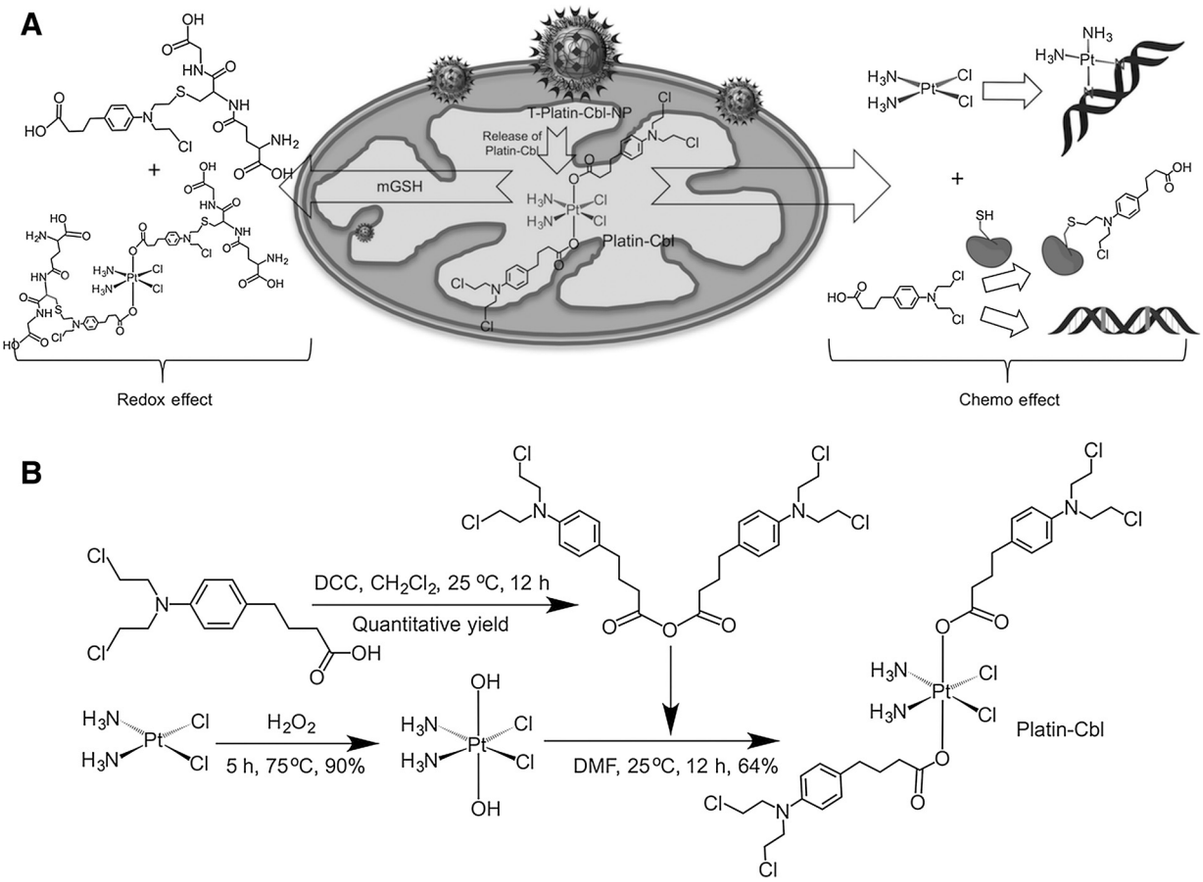


Figure 1.
A, Hypothesized mechanism of action of Platin-Cbl. **B,** Synthesis of Platin-Cbl.

mitochondrial DNA (mtDNA) by delivering a Pt(IV) prodrug which releases cisplatin in the mitochondrial lumen helps overcome resistance (11, 12). Thus, targeting mGSH with alkylating agents such as Cbl and use of a prodrug, which releases cisplatin as a DNA cross-linking agent and Cbl for DNA alkylation, and deactivation of mGSH can be a unique chemo-redox therapeutic approach of resistant cancers. Furthermore, formation of repair resistant crosslinks and alkylated adducts with mtDNA by delivering Platin-Cbl in the mitochondria can play additional role in better protection against resistance caused by DNA repair machinery.

Here, we describe a Pt(IV) prodrug of DNA crosslinking agent cisplatin and an alkylating agent Cbl with the intention to modulate cellular GSH level, cause a combination of mtDNA damage by alkylation and cross-linking, prevail against cellular DNA repair activities, thus forcing complete shutdown of the mitochondria and trigger apoptosis of cancer cells.

Materials and Methods

Materials and instrumentations

Details of materials and instrumentations used to conduct the studies mentioned in this work can be found in the Supplementary Information.

Cell lines and cell culture

Human prostate cancer PC3 cell line, human breast cancer MCF-7 cell line, and mouse breast cancer 4T1 were procured from ATCC. Professor Robert Brown, Imperial College London, kindly provided ovarian cancer A2780 cells. Cisplatin-resistant human ovarian carcinoma cell line A2780/CP70 was kindly provided by Dr. Thomas Hamilton (Fox Chase Cancer Center, Philadelphia, PA). PC3, mouse breast cancer 4T1, A2780, and A2780/CP70 cells were grown at 37°C in 5% CO_2 in RPMI1640 medium supplemented with 10% FBS, 1% penicillin/streptomycin, sodium pyruvate (100 mmol/L), HEPES buffer solution (1 mol/L), and L-glutamine (200 mmol/L). Human breast cancer MCF-7 cells were grown in DMEM medium containing 10% FBS and 1% penicillin/streptomycin at 37°C in an incubator maintained at 5% CO_2. Cells were passed every 3 to 4 days and restarted from frozen stocks upon reaching pass number 20.

Synthesis of anhydride of Cbl

A solution of Cbl (0.656 g, 2.16 mmol) in 20 mL of CH_2Cl_2 was prepared and a solution of N,N′-dicyclohexylcarbodiimide (DCC; 0.222 g, 1.08 mmol) in 5 mL of CH_2Cl_2 was added. The reaction mixture was stirred at room temperature for overnight. The byproduct, dicyclohexylurea (DCU), was filtered off using a glass filter and the resulting product was washed with a small amount

of CH_2Cl_2. The solvent was evaporated and the residue obtained was taken up in ethyl acetate. Residual DCU was removed by filtering the resulting suspension through a glass filter. The filtrate was evaporated to give Cbl-anhydride as a transparent thick oil. Yield: 0.690 g, 94%.

Synthesis of *c,c,t*-[$Pt(NH_3)_2Cl_2(OH)_2$]

This compound was prepared by following our previously reported methods (9, 26, 27). Yield: 90%. IR (KBr) υ_{max} (cm^{-1}): 3,803 (w), 3,515 (w), 3,458 (br, OH), 3,269 (w), 1,582 (s), 1,442 (s), 1,378(s), 1,074 (m, Pt-OH), 860 (br), 542 [br, Pt-N(O)]. HRMS *m/z* calculated for $Cl_2H_9N_2O_2Pt$: $(M+H)^+$ 333.9689; found 333.9683. Melting point: 295–300°C.

Synthesis of Platin-Cbl

A mixture of *c,c,t*-[$Pt(NH_3)_2Cl_2(OH)_2$] (0.129 g, 0.385 mmol) and Cbl-anhydride (0.683 g, 1.156 mmol) in 10 mL dry dimethylformamide (DMF) was stirred for 12 hours at room temperature to get a clear yellow solution. DMF was removed by rotary evaporation. The crude product was suspended in acetonitrile/dichloromethane and precipitated with diethyl ether. This process was repeated for five times. Finally, the product was isolated as a yellowish white solid by precipitating from the acetonitrile solution using diethyl ether. Yield 0.2 g, ~64%. ^{1}H NMR (DMSO-d_6, 400 MHz; Supplementary Fig. S1): δ ppm, 7.01 (d, 4H, $J = 8.3$ Hz), 6.65 (d, 4H, $J = 8.82$ Hz), 6.51 (m, 6H), 3.68 (s, 16H), 2.45 (t, 4H, $J = 7.5$ Hz), 2.18 (t, 4H, $J = 7.5$ Hz), 1.67 (t, 4H). ^{13}C NMR (DMSO-d_6, 100 MHz; Supplementary Fig. S2): δ ppm, 181.15, 144.80, 130.59, 129.89, 112.29, 52.68, 41.62, 40.62, 35.55, 33.87, 28.22. ^{195}Pt (DMSO-d_6, 107.6 MHz; Supplementary Fig. S3): δ ppm 1228.69. HRMS *m/z* calculated for $C_{28}H_{43}Cl_6N_4O_4Pt$: $(M+H^+)$ 907.4600. Found 907.1024 (Supplementary Fig. S4).

Details of nanoparticle (NP) synthesis, Pt and Cbl adduct formation, Platin-Cbl release from NPs, cytotoxicity, apoptosis, NCI-60 cell line assay, mitochondrial membrane potential by JC1, mitochondrial metabolism by Seahorse Analyzer, citrate synthase activity, and ATP synthase activity can be found in the Supplementary Data.

Results

Platin-Cbl and its controlled release nanoparticle formulations

A platinum(IV) prodrug, Platin-Cbl was designed to release cisplatin and an alkylating agent together in one molecular system to take the advantage of combined effects of cross-linking and alkylation properties of cisplatin and Cbl, respectively (Fig. 1A). An anhydride of Cbl was synthesized and reacted with *c,c,t*-[$Pt(NH_3)_2Cl_2(OH)_2$] (9, 26) to get combinational prodrug, Platin-Cbl (Fig. 1B). Extensive characterization of Platin-Cbl was carried out using spectroscopic and analytical techniques (Supplementary Fig. S1-^{1}H; Supplementary Fig. S2-^{13}C; Supplementary Fig. S3-^{195}Pt NMR; Supplementary Fig. S4-HRMS). Electrochemical reduction of the Pt(IV) center in Platin-Cbl to the corresponding Pt(II) center was observed at pH 7.4 and 6.0 (Supplementary Fig. S5). Reduction of Platin-Cbl with sodium ascorbate and subsequent reaction of the reduced species with 2′-deoxyguanosine 5′-monophosphate (5′-dGMP) demonstrated the ability of Platin-Cbl to form Pt-GG adducts using the cisplatin portion and alkylated products using Cbl fragment (Fig. 2A). Comparison of lipophilic character of Platin-Cbl with cisplatin, butyroplatin, a Pt(IV) compound devoid of Cbl, and Platin-*B*, a Pt(IV) compound recently reported by us (10) to interact with cellular GSH indicated that Platin-Cbl is more lipophilic than any of these Pt(IV) or Pt(II) compounds (Fig. 2B).

First, we evaluated the possibility of Platin-Cbl encapsulation inside the hydrophobic core of NPs by self-assembling a well-studied biodegradable polymer poly(lactic-*co*-glycolic acid) (PLGA)-polyethylene glycol (PEG) (PLGA-*b*-PEG-OH). Nontargeted (NT)-Platin-Cbl-NPs constructed using this polymer via a nanoprecipitation method were found to be monodisperse and spherical with a diameter of below <60 nm. These nanoparticles were found to be negatively charged (Fig. 3A, top, for diameter and zeta potential; Fig. 3B, top, for morphology). We observed efficient encapsulation of Platin-Cbl in the hydrophobic core of nanoparticles constructed using PLGA-*b*-PEG-OH block copolymer (Fig. 3C). We developed Platin-Cbl with the aim to target mGSH and form repair resistant cross-linked and alkylated mtDNA adducts for chemo-redox war on cancer cell mitochondria. Thus, next we assessed Platin-Cbl encapsulation inside PLGA-*b*-PEG-triphenylphosphonium (TPP) cation which utilizes the mitochondrial membrane potential ($\Delta\psi_m$) for mitochondrial association (28). Over the last few years, we demonstrated enormous ability of T-NPs formed using this PLGA-*b*-PEG-TPP polymer to deliver/associate several hydrophobic therapeutics to the mitochondria (11, 12, 28–31). Hence, we encapsulated Platin-Cbl inside PLGA-*b*-PEG-TPP to generate T-Platin-Cbl-NPs for mitochondrial delivery of Platin-Cbl (Fig. 3A, bottom, for diameter and zeta potential; Fig. 3B, bottom, for morphology; Fig. 3D for encapsulation efficiency). Small diameter, a positively charged surface, and high Platin-Cbl loading indicated that T-Platin-Cbl-NP will be able to deliver sufficient quantity of Platin-Cbl to the mitochondrial lumen. T/NT nanoparticles demonstrated release of Platin-Cbl under physiological conditions (pH 7.4 at 37°C), which followed controlled release kinetics (Fig. 3E). The release of Platin-Cbl from NT-NPs, in the initial period, was found to follow a slower kinetic compared with that observed with T-NPs (Fig. 3E).

The cytotoxic activity of Platin-Cbl and its T/NT-NPs were tested in a series of well-established cancer cell lines that are widely used under *in vitro* testing of chemotherapeutics. We used prostate cancer PC3, human breast cancer MCF-7, mouse breast cancer 4T1, and cisplatin-resistant ovarian cancer A2780/CP70 cells for analyzing IC_{50} of cisplatin, Cbl, a mixture of cisplatin and two equivalents of Cbl (cisplatin + 2Cbl), Platin-Cbl, NT-Platin-Cbl-NP, and T-Platin-Cbl-NP using the colorimetric MTT metabolic assay. A series of representative data and a table summarizing the IC_{50} values are shown in Fig. 4 and Supplementary Fig. S6.

Platin-Cbl in NCI-60 cell line panel

A comparison of effectiveness of Platin-Cbl in the National Cancer Institute (NCI)-60 human tumor cell lines of 9 nine different tissue origins with cisplatin and Cbl, the active drugs that the prodrug Platin-Cbl releases, is presented in Fig. 5. This figure describes the negative logarithm of GI_{50} values in molar (M) concentrations of Platin-Cbl, cisplatin, or Cbl that causes 50% of growth inhibition relative to the no drug-treated control. Summaries of single and five dose NCI-60 cell line data with Platin-Cbl are presented in Supplementary Figs. S7 and S8. For comparison, the negative logarithm of GI_{50} values in molar (M) for our previously reported Pt(IV) compound, Platin-*B*, in these 60 cell lines is also included in Fig. 5. Single and multidose NCI-60 cell line data for Platin-*B* are represented in Supplementary Figs. S9 and S10.

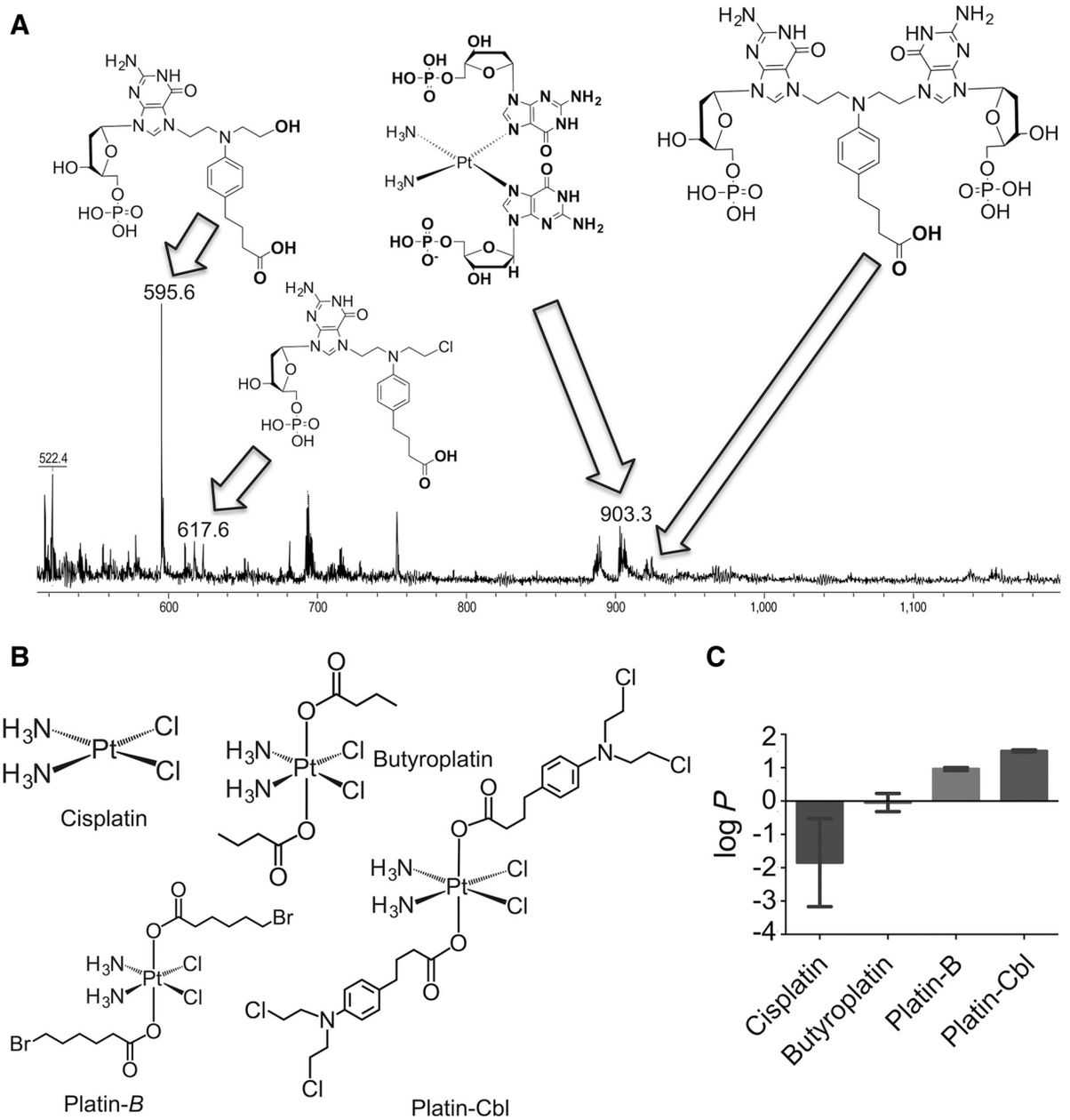


Figure 2.

A, Different adducts formed by Platin-Cbl with 5′-dGMP. **B,** Structures of different compounds used in the comparison of lipophilicity or log*P* values. **C,** Comparison of Platin-Cbl log*P* value with those of cisplatin, butyroplatin, and Platin-*B*.

Platin-Cbl induced apoptosis in cisplatin-resistant A2780/CP70 cells

Distinct populations of viable, early apoptotic, and late apoptotic A2780/CP70 cells upon treatment with cisplatin, Cbl, cisplatin+2Cbl, Platin-Cbl, NT-Platin-Cbl-NP, or T-Platin-Cbl-NP were identified by Annexin V-propidium iodide (PI) assay (Supplementary Fig. S11). The mitochondria targeted version of Platin-Cbl, T-Platin-Cbl-NP–treated cells were found to be in significantly greater early apoptotic state compared with those treated with NT-Platin-Cbl-NP or Platin-Cbl. This early apoptotic cell population in T-Platin-Cbl-NP–treated sample was also higher compared with the treatment of cisplatin, Cbl, or co-treatment with cisplatin and Cbl.

Cellular uptake of Platin-Cbl and its nanoparticles

Pt quantification in mitochondrial, nuclear, and cytosolic fractions of A2780/CP70 cells treated with NT/T-Platin-Cbl-NPs (25 μmol/L with respect to Platin-Cbl), Platin-Cbl (25 μmol/L), or cisplatin for 24 hours indicated that overall uptake of Platin-Cbl or its nanoparticles was relatively higher than cisplatin

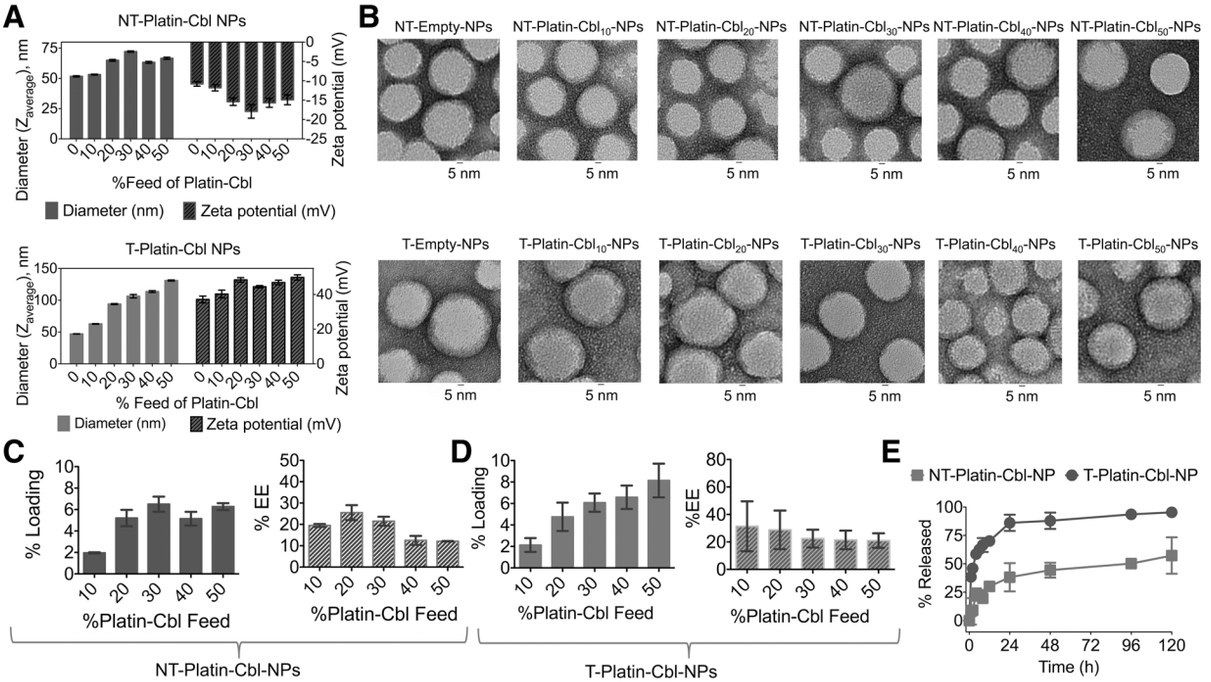


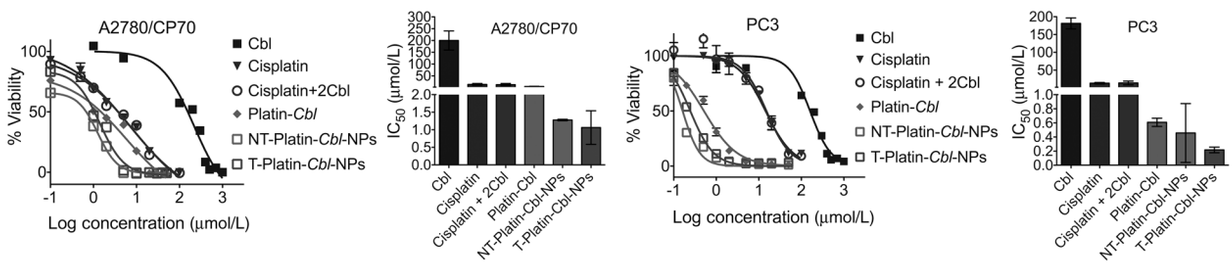
Figure 3.

A, Diameter and zeta potential of NT-Platin-Cbl-NPs and T-Platin-Cbl-NPs constructed by varying percent Platin-Cbl feed. **B,** Transmission electron micrographs of nontargeted (top) and targeted nanoparticles (bottom). Percent Platin-Cbl loading and encapsulation efficiency (EE) of Platin-Cbl–loaded (**C**) nontargeted and targeted nanoparticle (**D**). **E,** Platin-Cbl release from targeted and non-targeted nanoparticles at 37°C.

(Supplementary Fig. S12, top). Further, Pt-DNA adduct formation with genomic DNA in these treated cells was much higher for Platin-Cbl or its nanoparticles compared with cisplatin. The most interesting observation was T-Platin-Cbl-NP delivered cisplatin formed significantly higher adducts with mtDNA compared with cisplatin, Platin-Cbl, or NT-Platin-Cbl-NPs (Supplementary Fig. S12, bottom). These results indicated the advantage of using T-NPs to deliver Platin-Cbl in the mitochondrial network for formation of repair-resistant mtDNA-Pt adducts.

Effects on cellular GSH levels

We investigated the cellular GSH modulation by Platin-Cbl or its nanoparticles in A2780/CP70 cells. Analyses of GSH levels in these cells upon treatment with NT/T-Platin-Cbl-NPs (25 μmol/L



IC_{50} (μmol/L) of Platin-Cbl and its NPs in A2780/CP70 and PC3 cells lines						
Cell line	Cbl	Cisplatin	Cisplatin+2Cbl	Platin-Cbl	NT-Platin-Cbl-NP	T-Platin-Cbl-NPs
A2780/CP70	200±40	13.5±4	12±5	5.2±0.8	1.30±0.02	1.0±0.5
PC3	181±15	13.5±2	14±5	0.60±0.06	0.46±0.42	0.22±0.04

Figure 4.

IC_{50} values of chlorambucil (Cbl), cisplatin, cisplatin+2Cbl, Platin-Cbl, NT-Platin-Cbl-NP, T-Platin-Cbl-NP in prostate cancer PC3 and cisplatin resistant ovarian cancer A2780/CP70 cells as determined by the MTT assay.

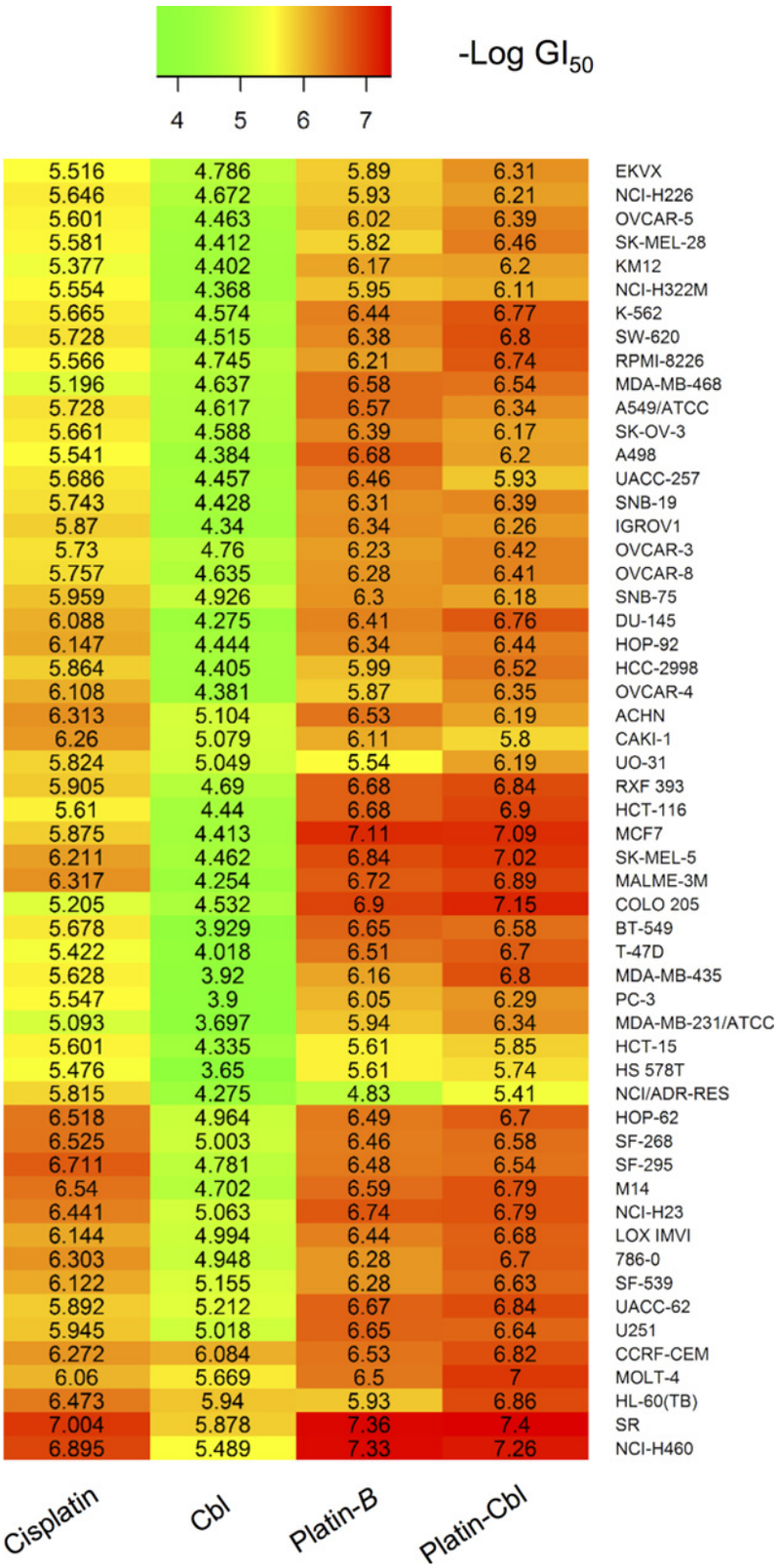


Figure 5.
Heatmaps showing the –logGI_{50} values of cisplatin, Cbl, Platin-*B*, and Platin-Cbl in NCI-60 human tumor cells of different origins. GI_{50} values are in molar.

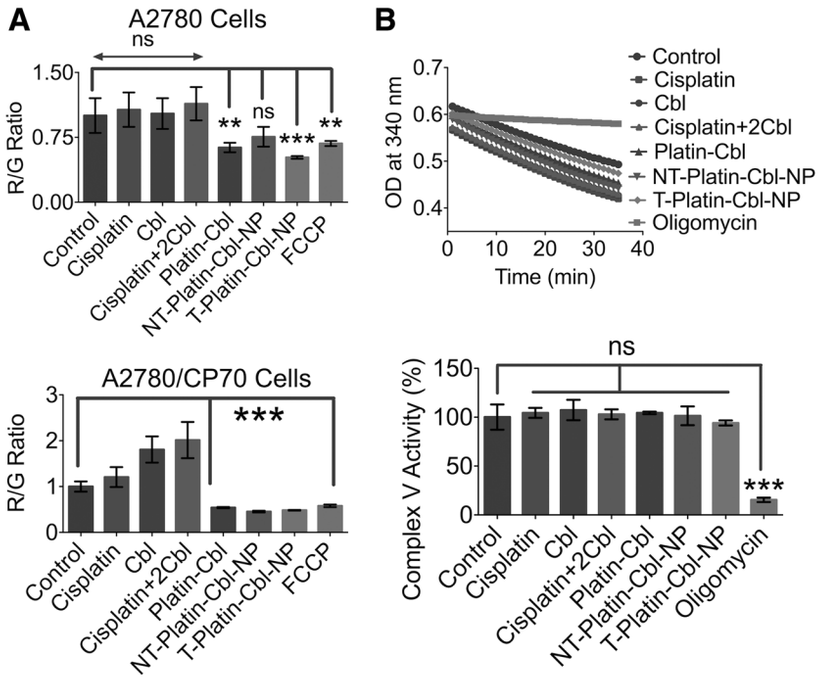


Figure 6.

A, Mitochondrial membrane potential analyses in A2780 cells (top) and A2780/CP70 cells (bottom) after treatment with cisplatin, Cbl, cisplatin+2Cbl, Platin-Cbl, NT-Platin-Cbl-NP, T-Platin-Cbl-NP, and FCCP by JC1 assay. R, red fluorescence from J aggregates in the mitochondria; G, green fluorescence from J monomers in the cytosols. Cells, were then treated with NT/T-Platin-Cbl-NPs, Platin-Cbl, cisplatin, cisplatin + 2Cbl, or Cbl. The medium was removed and JC1 was added into the cells and incubated for 30 minutes at 37°C in an incubator with 5% CO_2. As a control, FCCP was used simultaneously with JC1. **B,** Complex V or ATP synthase activity of bovine heart mitochondria in presence of cisplatin, Cbl, cisplatin+2Cbl, Platin-Cbl, NT-Platin-Cbl-NP, T-Platin-Cbl-NP. Oligomycin was used as a positive control to inhibit ATP synthase.

with respect to Platin-Cbl), Platin-Cbl (25 μmol/L), cisplatin (25 μmol/L), cisplatin (25 μmol/L) + Cbl (50 μmol/L), or Cbl (50 μmol/L) for 24 hours by a fluorometric GSH analysis kit indicated that the levels were reduced by Cbl and NT-Platin-Cbl-NPs to some extent, however the extent of GSH reduction was more prominent for T-Platin-Cbl-NP. Despite several attempts, we were unable to detect any GSH levels in T-Platin-Cbl-NP–treated cells (Supplementary Fig. S13).

Effects on $\Delta\psi_m$ of cancer cells

Alteration of $\Delta\psi_m$ is a hallmark for mitochondrial impairment, and has been reported in cells with mitochondrial dysfunctions. $\Delta\psi_m$ was evaluated by 5,6-dichloro-2-[(*E*)-3-(5,6-dichloro-1,3-diethyl-1,3-dihydro-2*H*-benzimidazol-2-ylidene)-1-prop-1-enyl]-1, 3-diethyl-1*H*-benzimidazolium iodide or JC1 assay (Fig. 6A). Accumulation of JC1 in mitochondria is $\Delta\psi_m$-sensitive with fluorescence shifts from red in mitochondria to green in cytosol when $\Delta\psi_m$ is collapsed. Thus, red/green (R/G) fluorescence intensity ratio can be used as an indicator of mitochondrial depolarization. Platin-Cbl and its nanoparticles showed significant reduction in R/G ratio to the levels as that of carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP) in A2780/CP70 cells used as a positive control for uncoupling mitochondrial membrane, suggesting depolarization of $\Delta\psi_m$ by Platin-Cbl and its nanoparticles (Fig. 6A). The effect of Platin-Cbl or its nanoparticles on the mitochondrial membrane potential was relatively less in A2780 cells. Noticeably, the $\Delta\psi_m$ was increased when cisplatin-resistant A2780/CP70 cells were treated with cisplatin, Cbl or their co-administration, indicating hyperpolarization by cisplatin treatment. However, this behavior did not appear in cisplatin-sensitive A2870 cells.

The complex V activity in presence of cisplatin, Cbl, and their combination was similar to untreated control, indicating that $\Delta\psi_m$ hyperpolarization in presence of cisplatin, Cbl, or cisplatin+2Cbl is not associated with complex V (Fig. 6B). In addition, Platin-Cbl and its nanoparticles demonstrated no alteration of complex V activity (Fig. 6B), indicating that Platin Cbl nanoparticles have no effect on complex V.

Effects of Platin-Cbl and its nanoparticles on mitochondrial metabolism

The effects of T-Platin-Cbl-NPs on the mitochondrial electron transport, mitochondrial respiration, extent of glycolysis, contributions of fatty acids and glutamine in A2780/CP70 cells were investigated to understand the changes Platin-Cbl can cause when delivered with a mitochondria-targeted nanoparticle. First, we studied mitochondrial bioenergetics in cisplatin-resistant A2780/CP70 cells in presence of Platin-Cbl and its nanoparticles by using mitochondrial respiration modulators oligomycin which is a mitochondrial complex V inhibitor, an ionophore FCCP as an uncoupler, and a mixture of antimycin A and rotenone as suppressors for mitochondrial complex III and I, respectively using Seahorse MitoStress assay (Fig. 7). Analyses of oxygen consumption rates (OCRs) in these treated cells indicated that T-Platin-Cbl-NP treatment results in disruption of mitochondrial respiration. Similar effect on mitochondrial bioenergetics was also observed with Platin-Cbl or NT-Platin-Cbl-NP; however, the extent of such effect was less than the cells which were treated with T-Platin-Cbl-NPs. Cisplatin, Cbl, or cisplatin + 2Cbl–treated cells demonstrated similar OCR levels as observed with control untreated cells. MitoStress studies were also performed with different concentrations of T-Platin-Cbl-NPs ranging from 1 to 4 μmol/L and it was observed that with increased concentrations of Platin-Cbl in these T-NPs, the effects on the mitochondrial respiration was more significant (Supplementary Fig. S14). From these studies, phenotypic analyses were performed which indicated that upon treatment with Platin-Cbl or its nanoparticles, the A2780/CP70 cells reach to a less energetic quiescent state compared with the control (Fig. 8A). The baseline OCR was significantly less when cells were treated with Platin-Cbl or its nanoparticles compared with

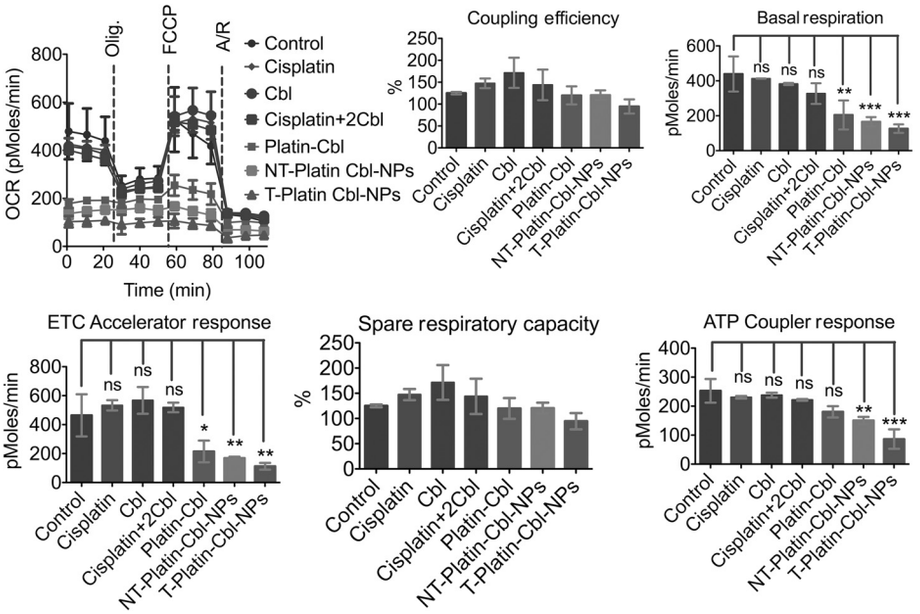


Figure 7.
Analysis of mitochondrial bioenergetics in A2780/CP70 cells upon treatment with Cbl, cisplatin, cisplatin + 2Cbl, Platin-Cbl, NT-Platin-Cbl-NP, T-Platin-Cbl-NP using Seahorse analyzer.

cisplatin, Cbl, or their combination, which was similar to untreated control. When stressed by FCCP, the OCR level was increased, Platin-Cbl or its NPs still maintains less OCR than its precursor compound treatment and untreated control. Furthermore, higher concentration of T-Platin-Cbl-NP promotes A2780/CP70 cells to a less metabolically active state further demonstrating the ability of Platin-Cbl to modulate mitochondrial metabolism when delivered with a mitochondria-targeted nanoparticle (Fig. 8B). We also studied the mitostress behavior of prostate cancer PC3 cells (Supplementary Fig. S15A) and cisplatin sensitive A2780 ovarian cancer cells (Supplementary Fig. S16A) in presence of Platin-Cbl and its nanoparticles. These studies were conducted at a lower concentration (0.5 μmol/L for A2780 cells, and 2 μmol/L for PC3 cells) with an incubation time of 12 hours. A2780 cells were more sensitive to cisplatin, Platin-Cbl and Cbl compared with A2780/CP70 and PC3 cells. Similar decreased OCR level was observed in cisplatin, Cbl and their combinations, Platin-Cbl and its nanoparticles in A2780 cells. At a concentration of 2 μmol/L, there was not much mitochondrial toxicity of Cbl treatment for PC3 cells. All other cisplatin-based therapeutics

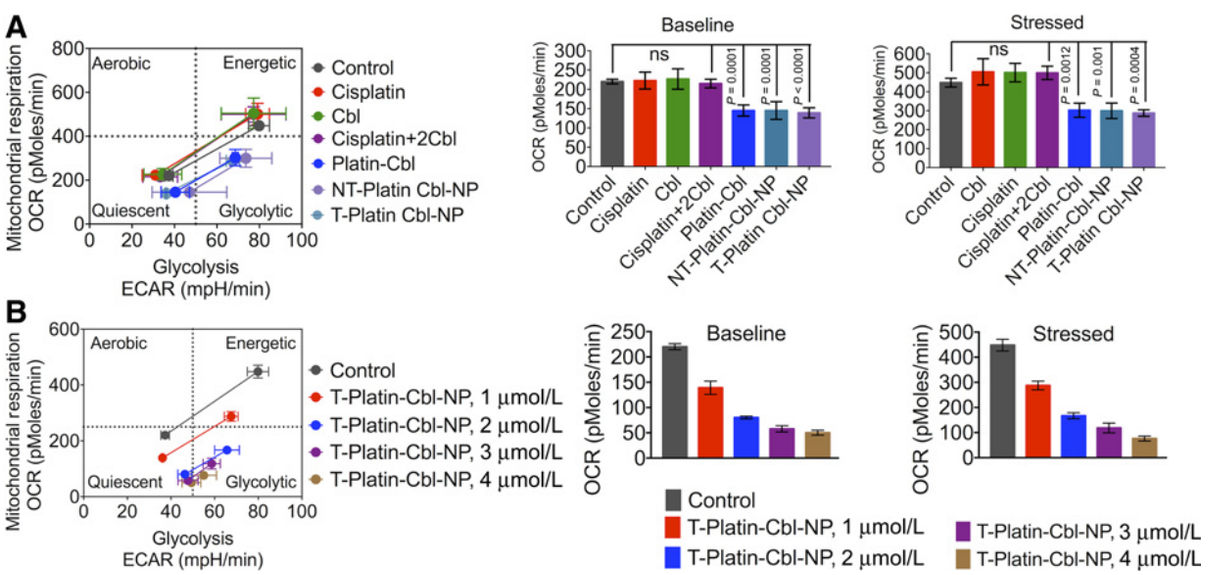


Figure 8.
A, Phenotypic analyses of A2780/CP70 cells upon treatment with Cbl, cisplatin, cisplatin + 2Cbl, Platin-Cbl, NT-Platin-Cbl-NP, T-Platin-Cbl-NP using Seahorse analyzer. **B,** Phenotypic analyses of A2780/CP70 cells upon treatment with different concentrations of T-Platin-Cbl-NP for 24 hours.

show comparable mitochondrial damage in PC3 cell line. Phenotypic analyses of PC3 (Supplementary Fig. S15B) and A2780 (Supplementary Fig. S16B) cells indicated that cisplatin-sensitive A2780 cells reach to a more energetic state when stressed by FCCP. The treatments with cisplatin, Cbl, or their combinations, Platin-Cbl, and its nanoparticles show a similar less metabolically active state compared with the control. Under cellular stress, PC3 became more glycolytic instead of energetic. The treatment with Platin Cbl and its nanoparticles, cisplatin, or cisplatin+2Cbl had similar level of stressed OCR and extra cellular acidification rate (ECAR) and promoted these cells to a less energetic state compared with the control. Treatment with Cbl had no effect on cellular metabolic state. These results indicated that PC3 cells are more quiescent in nature.

We next assessed the metabolic dependence of A2780/CP70 cells on glucose, glutamine, and fatty acids in presence of Platin-Cbl and its T/NT nanoparticles. Most cancer cells use both glucose and glutamine to support the energy requirement of their extraordinary growth. In this metabolic scenario, glucose is metabolized mostly via aerobic glycolysis (32) at an accelerated rate generating lactate thereby resulting in decreased feed of pyruvate to the tricarboxylic acid cycle (TCA) for mitochondrial respiration and conversion of glutamine to glutamate by the mitochondrial enzyme glutaminase (GLS) for supply of glutamate-derived TCA cycle intermediate (33). A very recent study demonstrated that cisplatin-resistant ovarian cancers are glutamine dependent to support the energy need for their growth (34). Thus, we studied whether Platin-Cbl can modulate the glutamine pathway in cisplatin-resistant ovarian cancer A2780/CP70 cells. In tumor, the biosynthesis of fatty acids from acetyl-CoA and malonyl-CoA can be more operative (35, 36). Fatty acid synthase (FASN) complex participates in fatty acid synthesis. The expression of this FASN enzymatic system is low in normal tissues, but many types of tumors show overexpression of FASN (35). To understand these different metabolic pathways, we used a pharmacologic inhibitor of mitochondrial pyruvate carrier (MPC), α-cyano-β-(1-phenylindol-3-yl)-acrylate (UK5099) to understand the effects of glucose homeostasis; etomoxir, an inhibitor of fatty acid beta oxidation, for fatty acid metabolic pathway; and an allosteric GLS inhibitor, bis-2-(5-phenylacetamido-1,2,4-thiadiazol-2-yl)ethyl sulfide (BPTES) to understand the effects of glutamine metabolism.

The Fuel Flex assay was conducted using Seahorse XFe96 analyzer on A2780/CP70 cells treated with different test articles followed by addition of different metabolic inhibitors and measuring the OCR levels. The adhered cells were treated with NT/T-Platin-Cbl-NPs (2 μmol/L with respect to Platin-Cbl), Platin-Cbl (2 μmol/L), cisplatin (2 μmol/L), or cisplatin (2 μmol/L) + 2Cbl (4 μmol/L) for 24 hours. Addition sequence of UK5099, etomoxir, and BPTES was varied to understand the contributions from glucose, fatty acid, and glutamine, respectively. Glucose dependency of Platin-Cbl or its nanoparticles was measured by using XF fuel flex optimization assay medium and injection of UK5099 and then simultaneous addition of etomoxir and BPTES (3.0 μmol/L); the injection order was also reversed to study the glucose capacity. The glutamine and fatty acid dependency and capacity were studied in a similar way as glucose pathway except the injection chemicals were varied. For glutamine metabolism, BPTES, etomoxir plus UK5099 were injected to analyze the glutamine dependency. Reverse injection order was used to study the glutamine capacity. For fatty acid dependency, etomoxir, BPTES plus UK5099 were injected. The reversed order was conducted to evaluate fatty acid capacity. The flexibility was calculated by Flexibility (%) = Capacity (%) − Dependency (%). These studies together indicated that upon treatment with Platin-Cbl or its T/NT nanoparticles, there is no dependency on glucose or glutamine metabolism pathway. After adding inhibitors BPTES, etomoxir, or UK5099, T-Platin Cbl-NP–treated cells did not show any decrease in OCR level, indicating no dependence on glucose, glutamine and fatty acid for energy production. Platin-Cbl treatment significantly decreased the dependency of fatty acid as source for energy production. Cbl and cisplatin did not alter glucose, glutamine, or fatty acid as energy source uptake as they had comparable OCR level when compared with control. Cisplatin+2Cbl treatment decreased the glucose dependency but had no effect on fatty acid and glutamine dependency (Fig. 9A and B). These preliminary studies indicated that upon treatment with Platin-Cbl, A2780/CP70 cells are able to rely on fatty acid metabolism pathway to some extent for their survival; T-Platin-Cbl-NP–treated cells, however, was not able to utilize this alternate fatty acid mediated metabolism for their survival (Fig. 9C). Thus, these preliminary studies indicated that T-Platin-Cbl-NP is able to effectively destroy the cisplatin-resistant cells attacking all possible metabolic pathways (Fig. 9).

To further understand the effect of these test articles on glycolysis, a GlycoStress test was performed on A2780/CP70 cells which indicated although Platin-Cbl or its nanoparticles alter the glycolytic capacity or reserve, but these articles have minimal effects on the extent of glycolysis (Supplementary Fig. S17).

Effects on mitochondrial mass

Mitochondrial activity of Platin-Cbl and its nanoparticles was further confirmed by analyzing the enzyme, citrate synthase (CS) (37) which acts on the initial step of TCA cycle (Fig. 9D). This enzyme activity serves as an excellent marker for mitochondrial respiration and overall mitochondrial mass. CS activity of A2780/CP70 cells showed that Platin-Cbl or its nanoparticles inhibited CS activity to a greater extent compared with cisplatin, Cbl, or their mixture (Fig. 9D). The activity was significantly reduced by Platin-Cbl-NP formulations compared with Platin-Cbl and cisplatin.

Discussions

Design strategy of Platin-Cbl allowed us to deliver a potent anticancer drug cisplatin and a potent alkylating agent Cbl in the relatively acidic cancerous cell milieu by detachment of the axial bonds during the reduction of Pt(IV) to active Pt(II) species. In addition, Cbl may protect the early sequestration/detoxification of Pt drug from biological thiols. In addition to alkylation, Cbl can contribute in increasing the lipophilicity of Pt(IV) compounds which may help in internalization of Platin-Cbl. Extensive characterizations and *in vitro* studies indicated that two FDA approved drugs can be combined to result in Platin-Cbl with ability to release both the drugs in their active forms. We previously reported a similar Pt(IV) prodrug Platin-*B* with ability to release cisplatin and an alkylating agent/GSH modulator 6-bromohexanoic acid with high cytotoxic property which takes advantage of protein interaction using pendent -Br moieties on 6-bromohexanoic acid to localize in the mitochondria (10). However, lipophilicity of Platin-*B* was not enough for its incorporation inside a hydrophobic core of polymeric nanoparticle for better delivery.

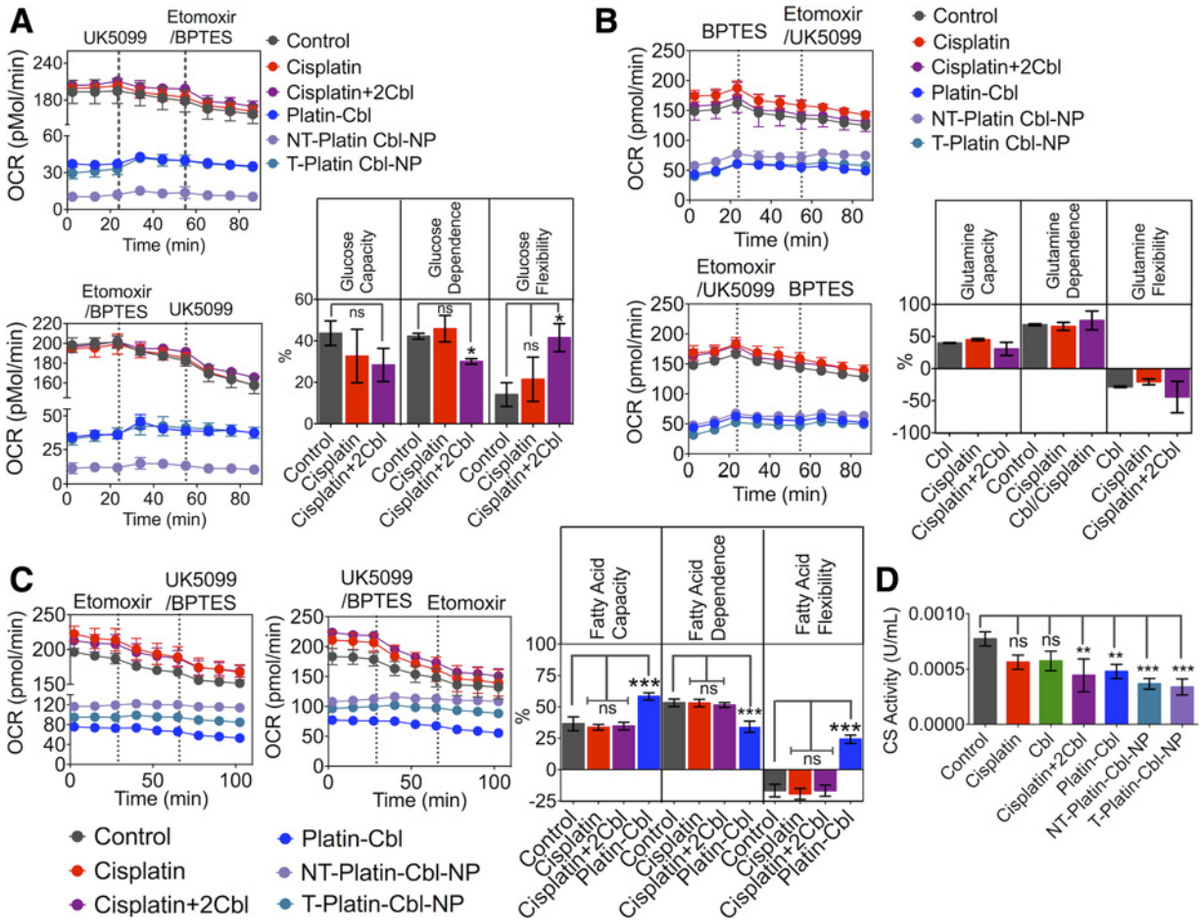


Figure 9.

Glucose (**A**), glutamine (**B**), and fatty acid (**C**) metabolisms of A2780/CP70 cells in presence of NT/T-Platin-Cbl-NPs, Platin-Cbl, cisplatin, or cisplatin + 2Cbl by using Fuel Flex assay on a Seahorse Analyzer. Addition sequence of UK5099, etomoxir, and BPTES was varied to understand the contributions from glucose, fatty acid, and glutamine, respectively. **D,** Citrate synthase activity in A2780/CP70 cells in the presence of cisplatin, Cbl, cisplatin+2Cbl, Platin-Cbl, NT-Platin-Cbl-NP, or T-Platin-Cbl-NP.

Significantly higher logP value of Platin-Cbl compared with Platin-*B* indicated that this compound will be a suitable candidate for its encapsulation in the hydrophobic core of controlled release polymeric nanoparticles. Targeted and nontargeted Platin-Cbl–loaded nanoparticles were synthesized and their small diameter, a positively charged surface, and high Platin-Cbl loading indicated that T-Platin-Cbl-NPs will be able to deliver Platin-Cbl to the mitochondrial network. Release studies indicated that the nanoparticles can release Platin-Cbl under physiologic conditions in a controlled manner.

In PC3 and MCF-7 cells, Platin-Cbl and its nanoparticles were significantly more active than the free drugs cisplatin or Cbl as well as their combination. However, in cisplatin-resistant A2780/CP70 cells, the activity of Platin-Cbl was much higher than cisplatin, Cbl, or their mixture. This activity of Platin-Cbl was further enhanced upon its encapsulation in NT or T-NPs. From NCI-60 cell line assay, it was seen from a comparison of negative logarithmic values of GI_{50} in different cell lines, Platin-Cbl is more active in most of the cell lines compared with cisplatin and Cbl. Platin-*B* and Platin-Cbl showed similar activities across these 60 cell lines. Thus, when Platin-Cbl is used *in vivo* in a nano delivery vehicle which can improve its stability, pharmacokinetics, and biodistribution properties, it might show much better therapeutic window compared with cisplatin or Cbl. NCI-60 cell line assay and apoptosis study together indicated that Platin-Cbl is significantly more active in human cancer cells and its activity can be further enhanced upon encapsulation in a mitochondria-targeted nanoparticle. Uptake of Platin-Cbl or its nanoparticles in cisplatin-resistant A2780/CP70 ovarian cancer cells demonstrated higher cellular Pt concentrations. Pt-adduct analyses with both genomic and mtDNA evidenced significantly greater Pt-mtDNA adducts formation when Platin-Cbl is delivered with a mitochondria-targeted nanoparticle system. This observation highlights the advantage of using a mitochondria-targeted delivery system because the ability to form repair-resistant Pt-mtDNA adducts can potentially play key roles in overcoming chemo-resistance often show by cisplatin-treated tumors due to enhanced DNA repair by the NER system. Cellular GSH quantification further supported the advantage of delivering Platin-Cbl with T-NPs because the GSH level in A2780/CP70 cells treated

with T-Platin-Cbl-NPs was significantly lower than Platin-Cbl, its NT-NP, or other relevant controls.

Mitochondrial membrane depolarization studies indicated that for cisplatin, Cbl, and their combination, R/G ratios were enhanced compared with untreated control, indicating hyperpolarization of mitochondrial membrane in A2780/CP70 cells. The result is consistent with report that cisplatin causes hyperpolarization of $\Delta\psi_m$ by inducing cellular resistance, inhibiting protein kinase C (PKC)-α and activating extracellular signal-regulated kinases 1 and 2 (38, 39). Ca^{2+} is reported to regulate $\Delta\psi_m$ by either hyperpolarization caused by inhibition of complex V or depolarization inducing mitochondrial permeability transition pore (PTP; ref. 40). There is an inverse relationship between $\Delta\psi_m$ and Ca^{2+}, whereas hyperpolarization is related to low Ca^{2+} accumulation (41). The alteration of $\Delta\psi_m$ attributes to the disturbance of the electron transport chain (ETC). It is reported that malfunction of complex V leads to proton leaks into the cytoplasm and changes the state of $\Delta\psi_m$ (38, 42, 43). Complex V activity studies indicated that Platin Cbl or its nanoparticles have no effect on complex V. Mitochondrial bioenergetics studies and CS activity indicated an overall loss of mitochondrial functions and mass in Platin-Cbl or its nanoparticle-treated cells. Further in depth studies are required to enlighten the activity of Platin-Cbl or its nanoparticles on the mitochondria.

Reduced glucose uptake is reported in ovarian cancer cells upon treatment with cisplatin (44). A study of renal function in rabbit model indicated that cisplatin causes glycosuria by inhibiting Na^+-pump activity resulting in decreased glucose reabsorption (45). Glucose transporter 1 (GLUT1) is overexpressed as key modulators for promoting glucose uptake, cell proliferation, and aerobic glycolysis in many cancers (46, 47). Cisplatin is known to inhibit the activity and expression of GLUT1 in head and neck cancer cells regardless of normoxic or hypoxic conditions (48). Thus, the activity of Platin-Cbl or its nanoparticles to interrupt the glucose consumption for energy production might be due to inhibition of GLUT1 activity and expression, or Na^+-pump activity, or reduced binding area for active glucose absorption. Glutamine is an important fuel for many cells and is also a precursor for GSH synthesis (49). Besides, glutamine possesses antioxidant properties to prevent cellular systems from apoptosis caused by external stimuli (50). Glutamine is reported to protect cells from cisplatin-induced nephrotoxicity, genotoxicity, and intestinal mucosal injury (51–53). A2780/CP70 cells were found not be dependent on glutamine metabolism pathway. Platin-Cbl and its nanoparticles demonstrated reduced glucose dependency. Fatty acid metabolism in cancers by cisplatin-based treatment is seldom investigated. Fatty acid is also required for energy storage, cellular proliferation, and signaling (36). Platin-Cbl and its nanoparticles demonstrated enhanced fatty acid flexibility. Thus, T-Platin-Cbl is sensitive to interrupt and alter mitochondrial metabolism.

In this article, we describe the design, synthesis, and *in vitro* antitumor activity of Platin-Cbl, a prodrug, constructed on the basis of two FDA-approved drugs, cisplatin and chlorambucil. Furthermore, Platin-Cbl was successfully encapsulated in the hydrophobic core of polymeric nanoparticles for future *in vivo* applications. These Platin-Cbl–loaded nanoparticles were modified with mitochondria-targeted TPP cation to access the mtDNA and mGSH for effective chemotherapy. Platin-Cbl showed enhanced cytotoxicity compared with the individual drugs, cisplatin and chlorambucil and their equimolar mixture. This cytotoxic potency was further increased with mitochondria targeted Platin-Cbl nanoparticles. Cancer cell growth inhibitory property of Platin-Cbl was accessed in the panel of NCI-60 cells and compared with the individual drugs, cisplatin and chlorambucil. In most of the NCI-60 cell lines, Platin-Cbl showed better activity as compared with the parental drugs. Mitochondrial metabolism studies in resistant cancer cells indicated activity of T-Platin-Cbl-NPs to interrupt energy production via glucose, glutamine, and fatty acid pathways compared with NT-Platin-Cbl or Platin-Cbl alone. Citrate synthase activity confirmed that the mitochondrial action of Plaitn-Cbl and its nanoparticle formulation is associated with diminished CS activity. Furthermore, extensive *in vivo* studies are required to establish the exact therapeutic role of this unique prodrug of two FDA-approved drugs, Platin-Cbl and its nanoparticle formulation towards the development of effective therapeutic modality.

Disclosure of Potential Conflicts of Interest

N. Kolishetti is a Research Director at Partikula LLC. S. Dhar is a Chair of Scientific Advisory Board member of Partikula LLC. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

Conception and design: R.K. Pathak, S. Dhar
Development of methodology: R. Wen, R.K. Pathak, S. Dhar
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): R. Wen, R.K. Pathak, N. Kolishetti
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): R. Wen, R.K. Pathak, N. Kolishetti, S. Dhar
Writing, review, and/or revision of the manuscript: R. Wen, R.K. Pathak, N. Kolishetti, S. Dhar
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): S. Dhar
Study supervision: S. Dhar

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