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A Spermine-Conjugated Lipophilic Pt(IV) Prodrug Designed to Eliminate Cancer Stem Cells in Ovarian Cancer

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We developed a spermine-conjugated lipophilic Pt(IV) prodrug that are able to reduce the cancer stem cell population in ovarian cancer. The therapeutic effect is attributed to the hydrophobic tail and cationic spermine head group, the combination of which allows the Pt(IV) prodrugs to localize in mitochondria and induce corresponding damages.

The long-standing challenge of treating ovarian cancer is cancer recurrence accompanied with drug resistance to the conventional platinum-based chemotherapy.[1] Cancer stem cells (CSCs) are believed to be the root of this problem.[2, 3] CSCs, also known as tumor-initiating cells, are a small population of cancer cells that have tumorigenic ability and are responsible for tumor metastasis, cancer relapse, and development of drug resistance.[3, 4] CSCs are highly resistant to chemotherapy, partially due to their intrinsic features, including enhanced DNA damage repair ability and overexpression of anti-apoptotic proteins, drug efflux transporters and detoxifying enzymes.[2, 5] Currently, there is no effective clinical treatment for eradicating CSCs in tumors.

Targeting mitochondria is one of the most promising therapeutic routes to eradicate CSCs.[6, 7] Recent studies indicate that the CSC population found in various cancer types favor oxidative phosphorylation in mitochondria for their energy production, and contain higher mitochondria mass compared to non-CSCs.[7, 8] Increasing attention has been drawn to develop new metallodrugs that target mitochondrial functions.[9, 10] Notably, Suntharalingam and coworkers have developed the first metallopeptide that can eradicate breast CSCs via triggering mitochondrial damage.[11] Song reported a Ru(II) complex that can effectively eliminate CSCs in the MCF7 breast cancer cell line by targeting mitochondria and endoplasmic reticuli.[12] In spite of the recent developments of metallodrugs for breast CSCs, metallodrugs that can treat CSCs in ovarian cancer have not been reported.

In this communication, we present the first platinum-based metallodrug that is able to reduce the CSC population in ovarian cancer via triggering mitochondrial damage. It was previously reported that the lipophilic fatty acid-like Pt(IV) prodrugs exhibit high *in vitro* and *in vivo* potency and promising pharmacokinetics, by virtue of their lipophilic tails.[13, 14] In this work (Fig 1A), we have modified such Pt(IV) prodrugs with the attachment of a cationic spermine moiety. This modification endorses the Pt(IV) prodrugs with both cationic and lipophilic features, therefore facilitating their accumulation in mitochondria for elimination of CSCs.

We first explored a new design of developing mitochondrial-damaging Pt(IV) prodrugs. Attachment of cationic moieties to the lipophilic fatty acid-like Pt(IV) prodrugs is expected to promote their accumulation in mitochondria, due to the negative mitochondrial membrane potential ($\Delta\psi_m$ ~ 150–180 mV). However, the conventional mitochondria-targeted cationic moiety, triphenylphosphonium, is hydrophobic, likely leading to much lower solubility of such lipophilic prodrugs. Alternatively, we sought to use a hydrophilic moiety, spermine, as shown in Fig 1A. Under physiological conditions, the conjugated spermine moiety carries a charge of +3. The synthetic route of the spermine-conjugated lipophilic Pt(IV) prodrug (**1**) was described in Scheme S1 of the ESI. Briefly, tri-Boc-spermine was conjugated to the fatty acid-like Pt prodrug with a C16 tail via a HATU-catalyzed amide-bond formation reaction. Boc deprotection was carried out by stirring the compound in 10% TFA dichloromethane solution at r.t. for 3.5 h. This newly synthesized Pt(IV) prodrug has been characterized using multinuclear (^{1}H and ^{13}C) NMR spectroscopy, high-resolution mass spectrometry, and analytical HPLC (Fig S1-3 in the ESI).

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Electronic Supplementary Information (ESI) available: Experimental details regarding the synthesis and characterization of **1**, cell culture, the MTT assay, cellular uptake, and flow cytometric analysis are presented in the ESI. See DOI: 10.1039/x0xx00000x

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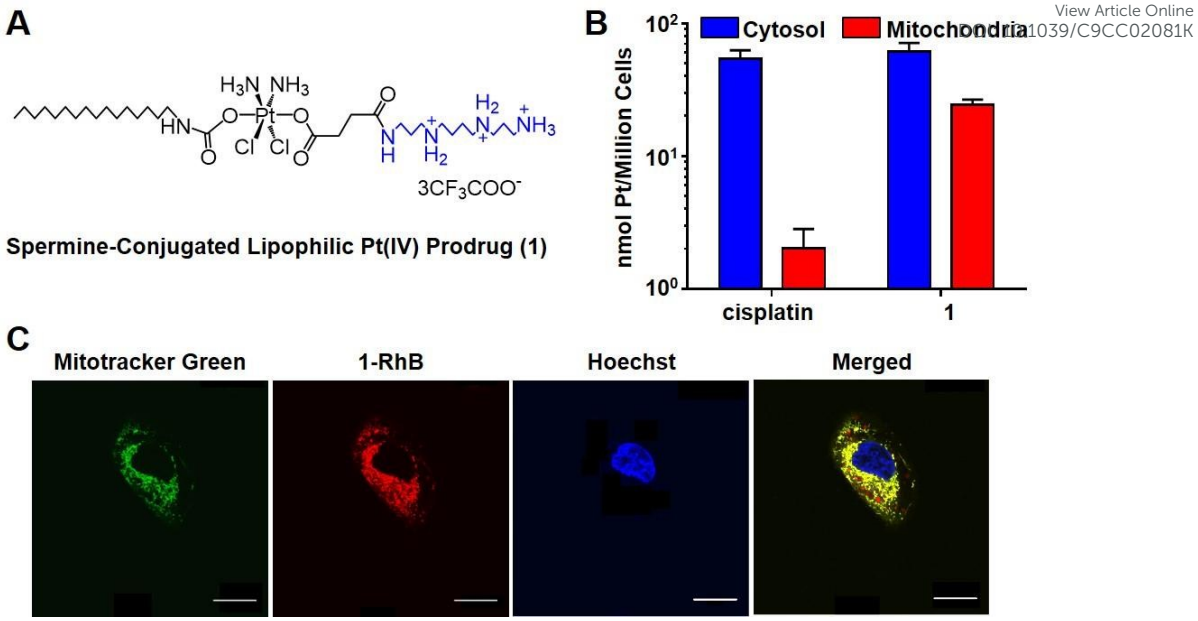

Figure 1. Spermine-conjugated lipophilic Pt(IV) prodrugs accumulate in mitochondria: (A) chemical structure of the Pt(IV) prodrug (**1**); (B) cytosolic and mitochondrial Pt contents of SKOV3 cells treated with cisplatin (150 μM), **1** (5 μM); (C) images of MDA-MB-231 cells treated with Mitotracker Green, **1** labeled with Rhodamine B (10 μM), and Hoechst for 1 h at 37 °C 5% CO_2. (Scale bar = 20 μm).

Next, accumulation of the Pt(IV) prodrug in mitochondria was confirmed. Graphite furnace atomic absorption spectroscopy (GFAAS) was used to measure the Pt contents in the isolated mitochondria and cytosol of SKOV3 ovarian cancer cells treated with the different platinum compounds (cisplatin, **1**). Cisplatin was engaged as a control, since it does not prefer to accumulate in mitochondria. For the SKOV3 cells treated with cisplatin (Fig 1B), a minimal amount of Pt (2.03±0.79 nmol Pt/10^6 cells) was found in the mitochondria, while the majority (54.1±8.6 nmol Pt/10^6 cells) was detected in cytosol. In contrast, **1** delivered a large amount of Pt content (24.5±2.1 nmol Pt/10^6 cells) to the mitochondria, which is comparable to the amount (61.3±10.0 nmol Pt/10^6 cells) delivered to the cytosol, as shown in Fig 1B. Furthermore, we carried out fluorescence imaging studies to pinpoint the mitochondrial accumulation of the Pt compound in live cells. For visualizing its distribution, the Pt(IV) prodrug (**1**) was labeled with Rhodamine B via the reaction between **1** and Rhodamine B isothiocyanate (see ESI). MitoTracker Green FM was used to label mitochondria. We already confirmed mitochondrial accumulation of **1-RhB** using GFAAS (Fig S5 in the ESI), which is similar to that of **1**. As expected, co-localization of the different fluorescence signals of **1-RhB** and MitoTracker Green FM was observed (Fig 1C). In sum, the combined evidence from both fluorescence imaging and GFAAS studies support accumulation of the spermine-conjugated lipophilic Pt(IV) prodrugs in the mitochondria.

Subsequently, we determined the mitochondrial damage triggered by the Pt(IV) prodrug. Mitochondrial ROS levels, a biomarker for evaluating mitochondrial damage, was determined using the MitoSOX™ Red reagent. We used flow cytometry to measure the mitochondrial ROS levels of the SKOV3 cells treated with the Pt(IV) prodrug (**1**) and cisplatin, respectively. As shown in Fig 2A (left panel), the treatment of **1** (10 μM, 24 h) increased the mitochondrial ROS level as compared to the control. Again, the treatment of cisplatin (60 μM, 24 h) resulted in insignificant change with respect to mitochondrial ROS levels, in line with its mechanism of action. In addition, mitochondrial membrane potential ($\Delta\psi_m$), a key parameter for assessing mitochondrial functions, was determined using Mitostatus™ reagent. As shown in Fig 2A (right panel), the treatment of **1** (10 μM, 24 h) induced the loss of $\Delta\psi_m$ of the mitochondria of SKOV3 cells. Finally, we used ATP luminescence assays to measure cellular ATP levels, as ATP is mainly produced in mitochondria. As shown in Fig 2B, cisplatin (2.5–20 μM, 24 h) does not deplete cellular ATP levels in SKOV3 cells. On the other hand, the addition of **1** (2.5–20 μM, 24 h) significantly suppressed the ATP production (8–79% reduction), which is likely attributed to the mitochondrial damage. To summarize, the spermine-conjugated lipophilic Pt(IV) prodrug can damage mitochondria of ovarian cancer cells. Given that nuclear DNA is a well-known intracellular target for platinum-based anticancer agents, we further analyzed nuclear DNA damage by probing phosphorylation of H2AX (γH2AX), a DNA damage biomarker. Notably, as shown in Fig S6, the Pt(IV) prodrug (**1**) is able to induce a marked increase in γH2AX as determined by flow cytometry.

Further experimentation indicated that the spermine-conjugated lipophilic Pt(IV) prodrug was able to reduce the CSC population in ovarian cancer. Since mitochondria play a central role in supporting cellular metabolism and survival of ovarian CSCs, the abovementioned mitochondrial damage triggered by the Pt(IV) prodrug is expected to damage and reduce the CSCs population in ovarian cancer. SKOV3 tumor spheroids were formed in CSC media in ultra-low attachment flasks or 6-well

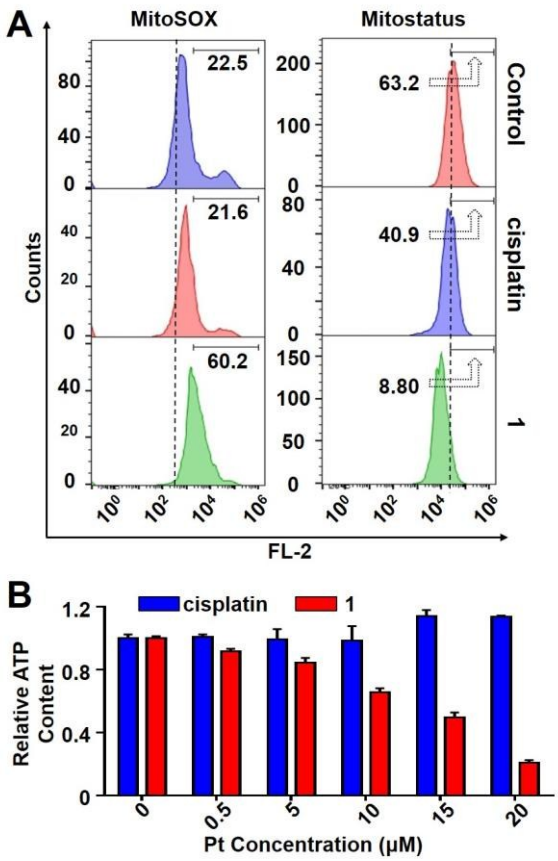

Figure 2. The spermine-conjugated lipophilic Pt(IV) prodrug induced mitochondrial damage and depleted ATP in ovarian cancer cells: (A) flow cytometric analysis of mitochondrial ROS level (left, MitoSOX assays) and membrane potentials (right, MitoStatus assays) of SKOV3 cells treated with cisplatin (60 μM) and **1** (10 μM) for 24 h at 37 °C 5% CO_2; (B) luminescence ATP detection assay of SKOV3 cells treated with cisplatin and **1** for 24 h at at 37 °C 5% CO_2.

plates by following the reported procedure.[15] As expected, the treatment of cisplatin (10 μM, 72 h) enriched the CSCs population in SKOV3 spheroids, and the enrichment was confirmed by flow cytometric analysis of CD44 (FL-1), CD117 (FL-2), and CD133 (FL-4) of the treated cells (Fig 3A, middle panel).[15, 16] In contrast, the treatment of **1** (1 μM, 72 h) significantly decreased the CSC population in the SKOV3 spheroids as shown in Fig 3A (bottom panel). In addition, spheroid-formation assays were used to further support the reduction of the CSC population in SKOV3 by **1**. In the experiment, SKOV3 spheroids were treated with cisplatin (5 μM) or **1** (2.5 μM). After 72 h, the SKOV3 cells were harvested, trypsinized, and re-seeded in ultra-low attachment 96-well microplates (5000 cells per well). After 72 h, the numbers of tumor spheroids formed in each well were counted under an optical microscope. As shown in Fig 3B, the treatment of **1** dramatically diminished the capability of SKOV3 cells from forming spheroids (10±2 spheroids/10^6 cells), but in the control and cisplatin-treated samples, large tumor spheroids were observed (55±4 spheroids/10^6 cells for control group and 27±3 spheroids/10^6 cells for cisplatin-treated group). In summary, the experimental results indicate that SKOV3 ovarian cancer cells treated with the spermine-conjugated lipophilic Pt(IV) prodrug exhibit a lower CSC population as compared to the control or cisplatin-treated group.

Finally, we found that the spermine-conjugated lipophilic Pt(IV) prodrug exhibits promising *in vitro* efficacy against ovarian cancer cells. MTT assays were performed to test *in vitro* efficacy of the new Pt agent (**1**). A panel of human cancer cell lines, including A2780, A2780cis, SKOV3, MDA-MB-231, and A549, were employed in the MTT assays. Cancer cells were treated with cisplatin or **1** for 72 h and cell viability was evaluated. IC_{50} values, which represent the concentration required to inhibit growth by 50%, are shown in Fig 4A. Across all the tested cell lines, **1** exhibits much higher *in vitro* potency as compared to cisplatin. For example, in the A2780cis cell line, the IC_{50} value of **1** (1.12±0.18 μM) is 12 times lower than that of cisplatin (13.19±1.84 μM), and these data have been further validated using cell counting assays (Fig S7). Resistance factor,

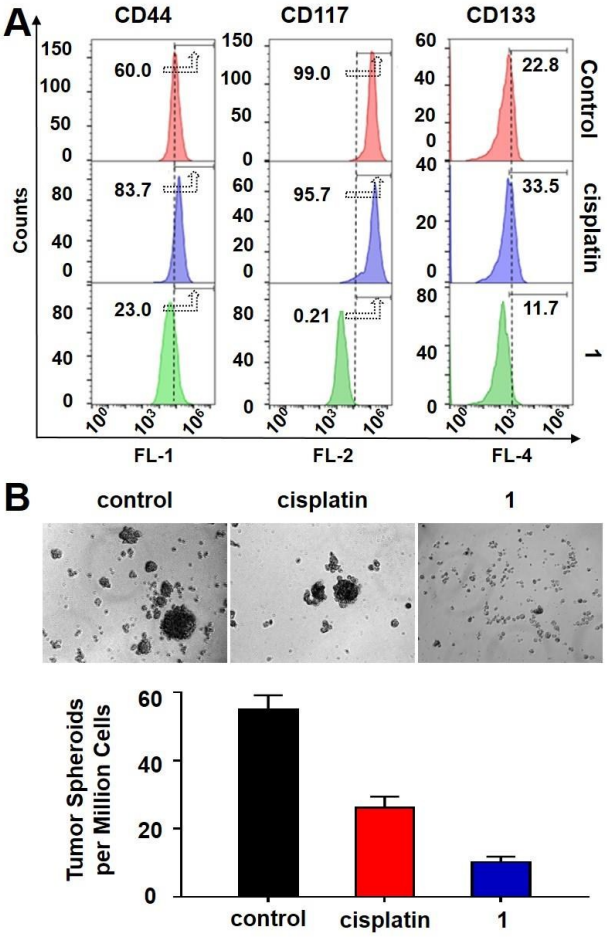

Figure 3. The spermine-conjugated lipophilic Pt(IV) prodrug is able to reduce the CSC-like population in ovarian cancer cells: (A) flow cytometric analysis of CSC biomarkers of SKOV3 cells treated with cisplatin (10 μM) and **1** (1 μM) for 72 h at 37 °C 5% CO_2; (B) spheroid-formation assays of SKOV3 cells treated with cisplatin (5 μM) and **1** (2.5 μM) for 72 h at 37 °C 5% CO_2.

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A

IC_{50} (μM)	A2780 Ovarian Cancer Sensitive to cisplatin	A2780cis Ovarian Cancer Resistant to cisplatin	SKOV-3 Ovarian Cancer	MDA-MB-231 Breast Cancer	A549 Lung Cancer	Resistance Factor $\frac{IC_{50}(A2780cis)}{IC_{50}(A2780)}$
cisplatin	1.60 ± 0.45	13.19 ± 1.84	16.31 ± 3.92	23.68 ± 1.74	12.03 ± 1.44	8.2
1	0.75 ± 0.11	1.12 ± 0.18	0.94 ± 0.38	1.79 ± 0.13	1.66 ± 0.14	1.5

B

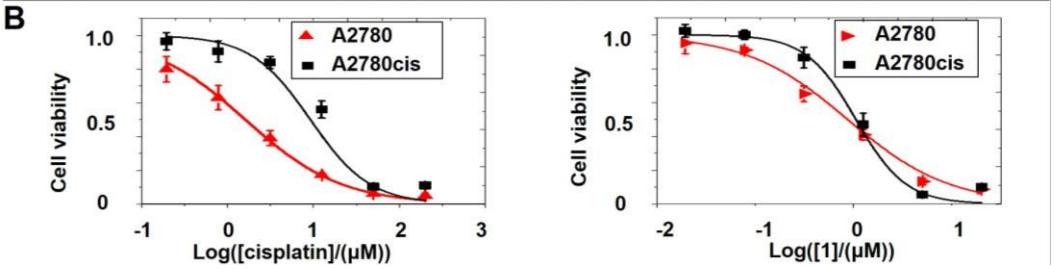

Figure 4. The spermine-conjugated lipophilic Pt(IV) prodrug overcomes drug resistance and exhibits high potency *in vitro*: (A) cytotoxicity profiles of cisplatin and spermine-conjugated lipophilic Pt(IV) prodrug (**1**) against a panel of human cancer cell lines, resistance factor = (IC_{50}(A2780cis)/IC_{50}(A2780); (B) representative killing curves of cisplatin (left) and **1** (right) against A2780 (cisplatin sensitive) and A2780cis (cisplatin resistant) ovarian cancer cell lines.

the fold change in IC_{50} values [(IC_{50}(A2780cis)/IC_{50}(A2780)], reflects drug resistance of the Pt compounds tested. As shown in Fig 4A and 4B, the Pt(IV) prodrug (**1**) displayed low cross-resistance as compared to cisplatin, and the resistance factors are 1.5 for **1** and 8.2 for cisplatin. In summary, the data from the MTT assays indicate that the newly developed Pt(IV) prodrug shows superior therapeutic indexes as compared to cisplatin.

In conclusion, we have demonstrated a new chemical design of a mitochondrial-damaging platinum agent that can reduce the CSC population and overcome drug resistance in ovarian cancer. Unlike classic platinum drugs, which mainly target nuclear DNA, the spermine-conjugated lipophilic Pt(IV) prodrug tends to accumulate in mitochondria, due to its cationic and lipophilic structural features. Subsequently, the mitochondrial damage triggered by the Pt(IV) prodrug reduced the CSC population in ovarian cancer. In addition to its interesting mechanism of action, this new platinum agent displays superior therapeutic indexes as compared to cisplatin. Due to the different attacking mode, this Pt(IV) prodrug shows low cross-resistance as compared to cisplatin. In addition, this compound exhibits much higher *in vitro* potency than cisplatin. This work offers a new approach to design Pt anticancer agents to target the root of cancer relapse and drug resistance in ovarian cancer.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

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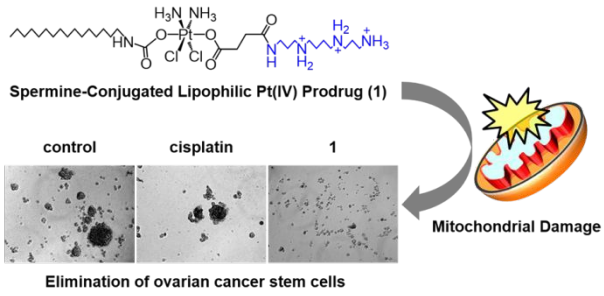

The spermine-conjugated lipophilic Pt(IV) prodrug is designed to induce mitochondrial damage and eliminate ovarian cancer stem cells.