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COMMUNICATION

A nanodrug to combat cisplatin-resistance by protecting cisplatin with *p*-sulfonatocalix[4]arene and regulating glutathione S-transferases with loaded 5-fluorouracil

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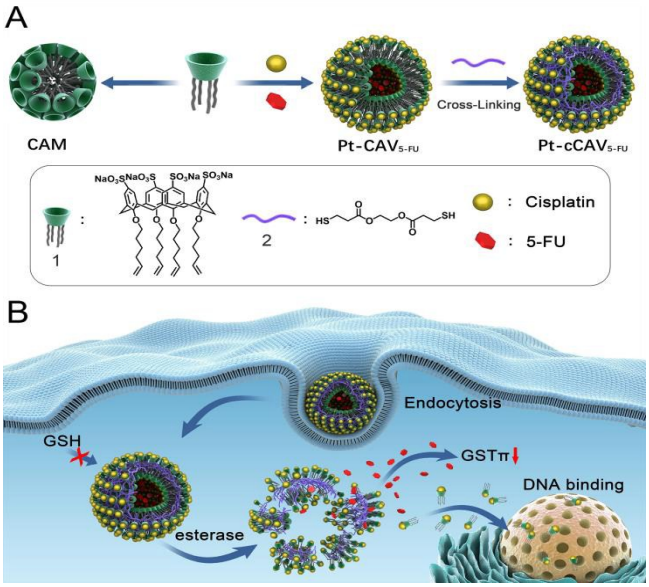
A nanodrug that can effectively combat cisplatin-resistant A549/CDDP cells was developed by protecting cisplatin from glutathione (GSH) detoxification trough a host-guest interaction between cisplatin and *p*-sulfonatocalix[4]arene. The enzymatic activity of glutathione S-transferases (GSTs) was also regutated by loaded 5-fluorouracil (5-FU).

Cisplatin was approved by the U.S. Food and Drug Administration (FDA) for clinical application early in 1978. However, patients receiving this chemotherapy usually suffer from severe drug resistance.[1] Studies on the mechanism of cisplatin resistance have revealed that while the highly reactive Pt (II) can facilitate intercalation of DNA to exert cytotoxicity, it can also be easily inactivated by various sulfhydryl-containing molecules *in vivo*, such as human serum albumin (HSA), metallothionein (MT), and especially glutathione (GSH) in tumor cells.[2] GSH complexes with cisplatin in the form of dimers by -SH occupying the drug's active site of the leaving group Cl^-, which causes drug efflux and the inability to form cisplatin-DNA adducts, thus blocking the apoptosis induced by failure of the DNA mismatch repair pathway.[3] Notably, clinical studies have indicated that detoxification of GSH is associated with glutathione S-transferases (GSTs), which are overexpressed in resistant cells and can catalyse the detoxification.[4] To overcome GSH/GSTs pathway-induced resistance to cisplatin, there are two strategies that should both be pursued: making cisplatin itself more resistant to sulfhydryl detoxification without impairing its cytotoxicity, and modulating intracellular GSH or GST levels by various means.

Thanks to advances in nanomedicine, it is possible to simultaneously combine these two strategies into one nanodrug carrier to achieve a synergistic effect.[5] Nanodrug carriers are now widely used in the delivery of platinum drugs



Scheme 1. Schematic illustrations of the preparation of Pt-cCAV5-FU (A) and the mechanism to combat cisplatin-resistant A549/CDDP cells (B).

in vivo, for they can improve the solubility of hydrophobic drugs and enrich them in tumor tissues through enhanced permeability and retention (EPR) effects, thereby optimizing the biological distribution and reducing systemic toxicity. There have been several reports about nanoparticle platforms used to overcome GSH/GSTs pathway-induced cisplatin resistance by combining the two strategies simultaneously. As a typical example, Ling et al. co-delivered Pt (IV) prodrugs and poly (disulfide amide) polymers within nanoparticles constructed by lipid-PEG to combat cisplatin-resistant A2780cis ovarian cancer.[6] Li et al. reported an ethacrynic acid-modified Pt (IV) prodrug in lipid-PEG micelles, which significantly enhanced antitumor outcomes against cisplatin-resistant BEL7404-CP20 liver cancer cells both *in vitro* and *in vivo*.[7] As a key part of these combination strategies, the cisplatin was covalently modified into Pt (IV) prodrugs, which compared to Pt (II) are much more resistant to deactivation by

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reductants.[8] However, this prodrug strategy of covalently modifying cisplatin is time-consuming and costly. Besides, due to the need to add adjuvants such as PEG, the effective drug loading of these models is relatively low, and the loose nano-assemblies may also be unstable in a physiological environment.

Recent studies have shown that supramolecular chemotherapies utilizing macrocyclic molecules to non-covalently protect drugs can effectively prolong their half-lives.[9] For example, protected by *p*-sulfonatocalix[4]arene, the half-life of the new platinum drug ME5SS can be prolonged by as much as 3.2 times in a sulfhydryl-rich environment compared to the unprotected drug while maintaining cytotoxicity.[10] Inspired by these promising results, we herein report a supramolecular nanodrug that can protect cisplatin and simultaneously deliver a GST regulator to combat GSH/GSTs pathway-induced cisplatin resistance. As shown in **Scheme** 1, cisplatin can co-assemble with **1** by a host–guest interaction, then **1** evolves into a cisplatin-induced *p*-sulfonatocalix[4]arene vesicle (Pt-CAV) in water, while it forms a *p*-sulfonatocalix[4]arene micelle (CAM) structure when **1** self-assembles alone. Crosslinked Pt-CAV (Pt-cCAV) can be easily obtained by crosslinking Pt-CAV with a linker containing an ester bond. Through the successful protection to cisplatin in a sulfhydryl environment by the calixarene cavity of **1**, nanoparticles showed good cytotoxicity both to human non-small cell lung cancer (A549) cells and their cisplatin-resistant derivative, A549/CDDP. Furthermore, when the soluble GST regulator 5-FU was loaded in the inner hydrophilic region of Pt-cCAV, the resulting Pt-cCAV$_{5\text{-FU}}$ further regulated GSTs and effectively reversed the drug resistance of A549/CDDP. This novel nanodrug possesses the advantages of good biocompatibility, high loading of cisplatin, and high stability in a physiological environment, thus giving it great potential to combat GSH/GSTs pathway-induced cisplatin resistance.

Calixarenes are third-generation macrocyclic molecules, among which calix[4]arenes have been studied most extensively for their applications in medicine.[11] The calix[4]arene can be endowed with excellent water solubility and has good biocompatibility when modified with sulfonic groups on the upper rim (*p*-sulfonatocalix[4]arenes).[12] To make them self-assemble into nanocarriers in water, we modified the lower rim of *p*-sulfonatocalix[4]arenes with 6-bromo-1-hexene to construct amphiphilic molecule **1** (see **Supporting Information** for the synthetic route).

By dissolving **1** in water and adding cisplatin at a ratio of 1:1, a very clear and transparent solution of Pt-CAV was obtained. Dynamic light scattering (DLS) showed that the hydration kinetic radii of the Pt-CAV were about 58.8 nm (**Fig**. 1A). The vesicle structure was confirmed by fluorescence leakage assays, where the surfactant Triton X-100 destroyed the assembly of Pt-CAV, and the maxima of excitation and emission fluorescence intensity of the indicator Phloxine B increased more than 6 times (**Fig**. 1B).[13] MALDI-TOF-MS spectrum showed that the host–guest complex assembled only

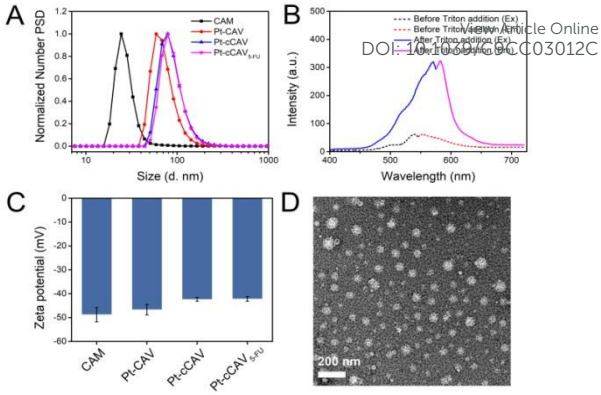

Fig. 1 Characteristics of Pt-cCAV$_{5\text{-FU}}$. Distribution of the hydrodynamic diameter sizes of CAM, Pt-CAV, Pt-cCAV, and Pt-cCAV$_{5\text{-FU}}$ (A). Vesicle structure verification with fluorescence leakage tests by encapsulating Phloxine B (B). Zeta potentials of CAM, Pt-CAV, Pt-cCAV, and Pt-cCAV$_{5\text{-FU}}$; [**1**] = 2 mM (C). Representative TEM of Pt-cCAV$_{5\text{-FU}}$ (D).

by neutral cisplatin and **1** in a ratio of 1:1, suggesting the hydrophobic cavity of **1** is the binding site (**Fig**. S1). A series of ratios of cisplatin to **1** were tested, and when the ratio of cisplatin to **1** was < 1.0, they could not co-assemble well and had inhomogeneous particle sizes and a high polydispersity index (PDI). When the ratio was ≥ 1.0, the final loading amount of cisplatin could not be further increased (**Table** S1), confirming the formation of 1:1 host–guest complexes between cisplatin and **1**. When no cisplatin was added, **1** self-assembled into micelle structures (CAM, **Scheme** 1), and the hydration kinetic radii were reduced to 29.0 nm. In order to further clarify the binding mode of cisplatin to molecule **1**, we set a control group with cisplatin and 4 eq. of sodium 4-(hex-5-en-1-yloxy) benzenesulfonate **5**, which is the monomer of **1**.[14] It was found that the vesicle structure was not assembled by [cisplatin + **5**] as confirmed by the DLS and fluorescence leakage test (**Fig**. S2). This result indicated that the "guest-induced assembly" could only be achieved through the hydrophobic binding of cisplatin to the preorganized hydrophobic cavity of **1**.[15]

Pt-cCAV was obtained by triggering a "thiol-ene" click reaction at the hydrophobic region of Pt-CAV in the presence of compound **2** (see **Supporting Information** for details). The successful crosslinking was confirmed by ^{1}H NMR, where 81% of double bonds were consumed, and all peaks became broad (**Fig**. S4), and gel permeation chromatography (GPC), where a great increase in molecular weight was determined [M_n = 56916 g mol^{-1}, and PDI = 2.061 (**Fig**. S5)]. Dilution experiments showed that Pt-cCAV maintained a stable particle size upon dilution, as low as 10 μg mL^{-1} in water, while Pt-CAV disintegrated when below 60 μg mL^{-1} (**Fig**. S6A). When incubated in 10% fetal bovine serum, Pt-cCAV kept well after 1 h, but no reasonable particle size could be detected under the same conditions for Pt-CAV (**Fig**. S6B). These results suggested high tolerance of crosslinked nanoparticles for extremely dilute concentrations or a complex bloodstream environment.[16]

The final Pt-cCAV$_{5\text{-FU}}$ was prepared with the same procedure as for Pt-cCAV, except for employing a solution containing 5-FU instead of pure water. DLS showed that 5-FU entrapment did not disturb the particle size of Pt-cCAV (**Fig**. 1A). 5-FU entrapment also did not change the zeta potential of Pt-cCAV (**Fig**. 1C). Representative transmission electron microscopy (TEM) images showed that nanoparticles of Pt-cCAV$_{5\text{-FU}}$ were spherical in shape without adhesion or aggregation (**Fig**. 1D), with a diameter of 70 nm, which was consistent with the DLS results. Inductively coupled plasma (ICP) tests showed that the loading content of cisplatin was 13 wt%. Encapsulation percentage (EN%) assays showed that the maximum encapsulation percentage of 5-FU was 19% (**Table**. S2), and the final molar ratio of cisplatin to 5-FU in Pt-cCAV$_{5\text{-FU}}$ was calculated to be 4.04:1.

The human non-small cell lung cancer cell line A549 and its cisplatin-resistant derivative A549/CDDP were chosen to test the effectiveness of Pt-cCAV$_{5\text{-FU}}$ by comparison with cisplatin, Pt-cCAV, and [cisplatin + 5-FU]. Results of 48-h dose-dependent cell inhibition assays are shown in **Fig**. S7, and the corresponding half inhibitory concentrations (IC$_{50}$) and resistance fold are shown in **Table** 1. Compared with free cisplatin, the Pt-cCAV group decreased the drug resistance fold of A549/CDDP from 4.98 to 1.76, suggesting that the protective effect provided by *p*-sulfonatocalix[4]arene made cisplatin more resistant to detoxification by various intracellular sulfhydryl-containing molecules, including GSH. When treated with Pt-cCAV$_{5\text{-FU}}$, the drug resistance fold of A549/CDDP decreased to 0.75, demonstrating that when loaded with 5-FU, the nanodrug further reduced cell resistance to cisplatin. Notably, the crosslinked micelles (cCAM, see **Supporting Information** for details), equivalent to the pure carriers in Pt-cCAV,[17] showed negligible toxicity to cancer or normal cells over the whole experimental concentration range (**Fig**. S8), suggesting that the toxicity of Pt-cCAV originated entirely from the guest cargo cisplatin instead of the carriers. By contrast, the free [cisplatin + 5-FU] combination showed only a slight reduction in drug resistance (**Table** 1).

Table 1 Comparison of IC$_{50}$ and derived drug resistance fold of cisplatin, Pt-cCAV, Pt-cCAV$_{5\text{-FU}}$, and [cisplatin + 5-FU] to A549 and A549/CDDP cells. Data are presented as means ± SD (n = 3).

Test Group	IC$_{50}$ (μM)		Resistance Fold
	A549	A549/CDDP	
Cisplatin	7.5 ± 0.5	37.6 ±1.3	4.98
Pt-cCAV	25.7 ± 2.0	45.1 ± 0.5	1.76
Pt-cCAV$_{5\text{-FU}}$	18.6 ± 1.2	14.0 ± 2.2	0.75
Cisplatin + 5-FU	6.2 ± 0.7	21.0 ± 1.0	3.38

Since free 5-FU showed little toxicity over the whole tested concentration (**Fig**. S7), the effect of 5-FU on GST-π expression and GST activity in A549/CDDP cells was checked to clarify whether 5-FU reduced cell resistance by inhibiting the GST pathway. GST-π (a member of the GST family) has been confirmed in a large number of studies to be the most directly related marker of cisplatin resistance and is the main target of

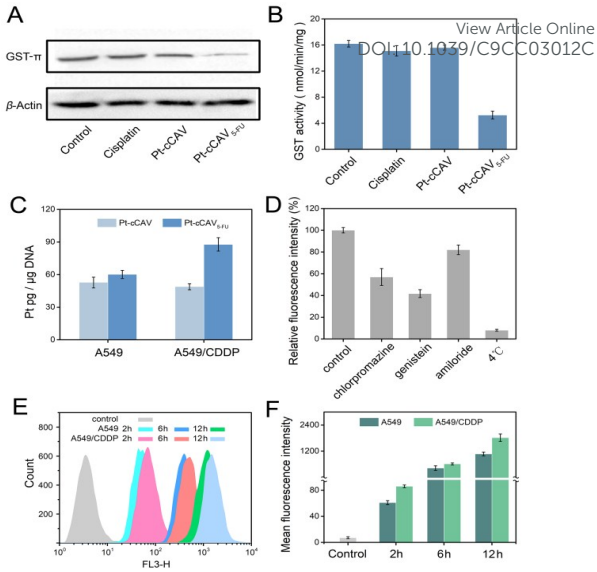

Fig. 2 Regulatory effect of Pt-cCAV$_{5\text{-FU}}$ on GSTs and cell uptake of Pt-cCAV$_{5\text{-FU}}$ by A549 and A549/CDDP. Western blots of GST-π expression (A) and GST activity of of A549/CDDP cells (B) after treatment with cisplatin, Pt-cCAV, and Pt-cCAV$_{5\text{-FU}}$ for 12 h at a Pt concentration of 50 μM; no treatment was the control. Platinum content in the genomic DNA of A549 and A549/CDDP cells after incubation with Pt-cCAV and Pt-cCAV$_{5\text{-FU}}$ at a Pt concentration of 50 μM for 12 h (C). The relative cell uptake efficiency of DiI-labeled Pt-cCAV$_{5\text{-FU}}$ under different inhibitors; chlorpromazine inhibits the endocytosis pathway mediated by clathrin, genistein inhibits the endocytosis pathway mediated by caveolin, and amiloride inhibits the endocytosis pathway mediated by micropinocytosis (D). The flow cytometric profiles (E) and mean fluorescence intensity (F) of DiI-labeled Pt-cCAV$_{5\text{-FU}}$ after 2, 6, and 12 h incubations with A549 and A549/CDDP cells, respectively; untreated cells were used as a control.

5-FU when used in combination with cisplatin.[18] Tominaga et al. found that a combination of 5-FU and cisplatin effectively treated recurrent oesophageal cancer through immunohistologic assays because of the significantly reduced expression of GST-π.[19] Nishiyama et al. found that GST-π gene expression is significantly reduced when 5-FU and cisplatin are combined, which induces synergistic toxicity against cisplatin-resistant hepatocellular carcinoma HCC-48 cells.[20] As shown in **Fig**. S9, western blotting showed that A549/CDDP cells indeed had higher GST-π expression compared to A549 cells. When A549/CDDP cells were treated with Pt-cCAV, GST-π expression did not change compared with the control and free cisplatin. However, GST-π expression was obviously reduced when cells were treated with Pt-cCAV$_{5\text{-FU}}$ (**Fig**. 2A), which indicated that 5-FU loaded in nanoparticles downregulated the expression of GST-π. GST activity experiments further showed that only the Pt-cCAV$_{5\text{-FU}}$ group had the distinct effect of reducing GST activity in A549/CDDP cells (**Fig**. 2B), confirming that the enzymatic activity of GSTs decreased with the significant inhibition of the GST-π expression level.

Intuitively, by inhibition of the pathways leading to cisplatin resistance, the resistance fold of A549/CDDP cells can be reduced and ideally approach 1.0. Surprisingly, when treated with Pt-cCAV$_{5\text{-FU}}$, the resistance fold of A549/CDDP cells decreased to as low as 0.75, suggesting that Pt-cCAV$_{5\text{-FU}}$ was even more toxic to A549/CDDP than A549 cells. The genomic DNA Pt content test also showed that Pt-cCAV$_{5\text{-FU}}$ resulted in 1.45 times more nuclear platinum in A549/CDDP than A549 cells (**Fig**. 2C), which confirmed that Pt-cCAV$_{5\text{-FU}}$ exerted greater cytotoxicity to A549/CDDP cells.[21] In order to determine how the reversal of drug resistance in A549/CDDP cells occurs, we further studied the specific cellular internalization of the nanoparticles by co-incubating A549 cells with 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perch-lorate (DiI)-labeled Pt-cCAV$_{5\text{-FU}}$ and checking the inhibition of internalization mechanisms with various inhibitors (See **Supporting Information** for details). As shown in **Fig**. 2D, compared with chlorpromazine and amiloride, cells treated with genistein showed the least uptake according to relative intracellular fluorescence, indicating that the internalization pathway of Pt-cCAV$_{5\text{-FU}}$ was through caveolin-mediated endocytosis, a pathway commonly adopted for nanoparticles with sulfonic groups on the surface, which have special affinity to caveolin.[22] Since drug-resistant strains usually overexpress caveolin, they often have higher uptake of caveolin-mediated nanoparticles than non-resistant strains.[23] Flow cytometry was used to examine the different uptake efficiencies between A549 and A549/CDDP cells. We co-incubated DiI-labeled Pt-cCAV$_{5\text{-FU}}$ with the two cell lines and detected cytoplasmic fluorescence intensities after 2, 6, and 12 h, and A549/CDDP cells consistently took in more nanoparticles (**Fig**. 2E, F). Confocal laser scanning microscopy was used to visualize the time-dependent accumulation of nanoparticles, showing them mainly distributed in the cytoplasm (**Fig**. S10). The above results illustrate that Pt-cCAV$_{5\text{-FU}}$ not only protected cisplatin from detoxification by GSH and regulated the enzymatic activity of GSTs, but was also uptaken faster by A549/CDDP cells, which facilitated the intracellular accumulation of more drugs. All of these factors contributed to Pt-cCAV$_{5\text{-FU}}$ showing more toxicity to the resistant cell lines than to non-resistant cell lines.

In summary, a supramolecular-based strategy was successfully used to create the nanodrug Pt-cCAV$_{5\text{-FU}}$. This drug combats GSH/GSTs pathway-induced cisplatin resistance by protecting cisplatin from glutathione (GSH) detoxification through host–guest interactions between cisplatin and *p*-sulfonatocalix[4]arene, and by regulating GSTs with loaded 5-FU. Compared with the free drug, Pt-cCAV reduced the drug resistance fold of A549/CDDP cells from 4.9 to 1.7, and Pt-cCAV$_{5\text{-FU}}$ further reduced it to 0.75 via 5-FU regulation of GST-π expression and reduction of GST activity. In addition to the pathway inhibition, Pt-cCAV$_{5\text{-FU}}$ was uptaken faster by A549/CDDP cells through caveolin-mediated endocytosis, resulting in higher cytotoxicity to A549/CDDP than A549 cells. The novel nanodrug Pt-cCAV$_{5\text{-FU}}$ thus effectively reversed A549/CDDP resistance and holds great potential in cancer therapy.

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Conflicts of interest

There are no conflicts to declare.

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