Prodrug Design

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Enzyme-Instructed Intracellular Molecular Self-Assembly to Boost Activity of Cisplatin against Drug-Resistant Ovarian Cancer Cells

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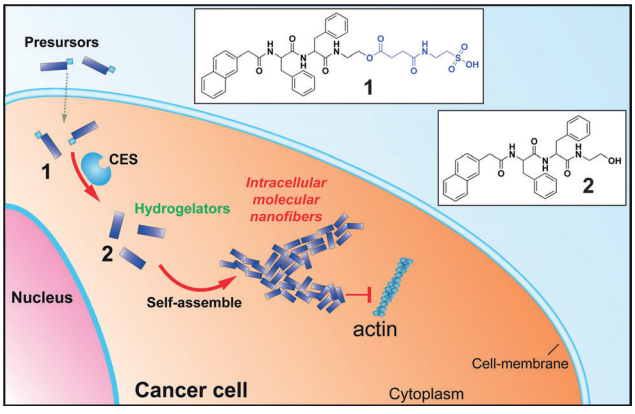
Abstract: *Anticancer drug resistance demands innovative approaches that boost the activity of drugs against drug-resistant cancers without increasing the systemic toxicity. Here we show the use of enzyme-instructed self-assembly (EISA) to generate intracellular supramolecular assemblies that drastically boost the activity of cisplatin against drug-resistant ovarian cancer cells. We design and synthesize small peptide precursors as the substrates of carboxylesterase (CES). CES cleaves the ester bond pre-installed on the precursors to form the peptides that self-assemble in water to form nanofibers. At the optimal concentrations, the precursors themselves are innocuous to cells, but they double or triple the activity of cisplatin against the drug-resistant ovarian cancer cells. This work illustrates a simple, yet fundamental, new way to introduce non-cytotoxic components into combination therapies with cisplatin without increasing the systemic burden or side effects.*

Since its serendipitous discovery five decades ago,[1] cisplatin has become the most successful therapeutic agent for anticancer chemotherapy.[2] Particularly, cisplatin has drastically extended the progression-free survival (PFS) of patients with ovarian cancers.[3] However, owing to the lack of early detection of ovarian cancer and the almost inevitable relapse in the patients with advanced ovarian cancer, drug resistance remains a major obstacle in treating ovarian cancers.[4] Many approaches have been investigated to address the urgent need of treating drug-resistant ovarian cancers. One of the most explored strategies is combination chemotherapy (such as the combination of cisplatin with other therapeutics) because the advantages of cisplatin promote the rapid translation from preclinical to clinical settings. Despite the remarkable clinical success of combination therapies,[3] the 5-year relative survival rate of ovarian cancer hardly improved over the past decade (45% (2004–2010) vs. 45% (1996–2003)).[4e] Thus, there is an urgent need for innovative approaches in cisplatin-based combination therapies.

We have been exploring enzyme-instructed molecular self-assembly[5] inside cells,[6] and we recently showed that intracellular molecular nanofibers promiscuously interact with cytoskeleton proteins[7] yet selectively inhibit cancer cells.[8] Recently, Maruyama and co-workers,[9] Pires and Ulijn,[10] Yang et al.,[11] and Wells[12a] also reported inhibition of cancer cells by nanofibers formed by the self-assembly of small molecules. The exceptional selectivity[8] and new mechanisms[7,13] of the molecular nanofibers against cancer cells encouraged us to explore the utilization of enzyme-instructed intracellular molecular self-assembly for combination therapy with cisplatin. Unlike the previous approaches, this work focuses on the use of D-peptides for intracellular self-assembly, and is the first demonstration of combining intracellular enzyme-instructed self-assembly with cisplatin. We design and synthesize two enantiomeric peptidic precursors (L-**1** and D-**1**) that turn into the self-assembling molecules (L-**2** and D-**2**) upon the catalysis of carboxylesterases (CES; Scheme 1).[14] Our study confirms that CES are able to convert both L-**1** and D-**1** into the corresponding molecules of



Scheme 1. Enzymatic transformation of the precursor (**1**) as a substrate of carboxylesterase (CES) to the corresponding hydrogelator (**2**) for intracellular self-assembly.

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L-**2** and D-**2**, respectively. L-**2** or D-**2** self-assembles in water to form molecular nanofibers. At the optimal concentration, L-**1** or D-**1** is innocuous to cells. The co-incubation of L-**1** or D-**1** at the optimal concentration with cisplatin significantly boosts the activity of cisplatin against SKOV3 and A2780cis, two lines of drug resistant ovarian cancer cells. The efficacy of this simple approach (inhibiting over 80% of SKOV3 by 20 μM of cisplatin and 15 μg mL^{-1} of D-**1**), in fact, is comparable to that of the innovative approach based on the co-delivery of siRNA and cisplatin nanoparticles (80% inhibition of SKOV3 by 75 μM of cisplatin).[15] We chose to work on the enantiomeric precursors L-**1** and D-**1** to assess the influences of the cell uptake of the precursors and the proteolytic stability of the intracellular nanofibers to the efficacy of combination therapy. These results confirm that enzyme-instructed self-assembly promises a new approach to boost the activity of cisplatin against drug-resistant ovarian cancers without increasing the systemic toxicity.

The synthesis of **1** and **2** is simple and straightforward. The facile synthetic route (Supporting Information, Scheme S1) combines liquid-phase synthesis and solid-phase peptide synthesis (SPPS) for making the precursors. For example, by loading *N*-Fmoc-protected phenylalanine (Fmoc-Phe-OH) onto 2-chlorotrityl resin and carrying out SPPS, we obtain Nap-FF[16] for coupling with ethanolamine to produce L-**2**. After L-**2** reacts with succinic anhydride, another step of amide bond formation allows the attachment of taurine to form L-**1**. After the purification by HPLC, the overall yield of L-**1** is about 60%. The same synthetic approach also produces D-**1**.

Because enzyme-instructed self-assembly usually leads to the formation of supramolecular hydrogels,[17] we evaluated the hydrogelation resulting from the esterase catalyzed conversion of L-**1** and D-**1** as a facile method to assay the self-assembly. After obtaining the precursors, we tested the use of CES to convert the precursors into the hydrogelators that self-assembly in water to form molecular nanofibers. The addition of L-**1** (or D-**1**) in PBS buffer at pH 7.4 at a concentration of 0.4 wt% (5.5 mM) afforded a transparent solution. After the addition of CES (2 U mL^{-1}) into the solution of L-**1** (or D-**1**), a translucent hydrogel formed after 24 h. We also found the minimum gelation concentration (mgc) of L-**2** or D-**2** is about 0.1 wt% (1.4 mM). While CES efficiently converts both L-**1** and D-**1** into L-**2** and D-**2**, respectively, the hydrogel of L-**2** is apparently weaker than the hydrogel of D-**2** (Supporting Information, Figure S5). We speculate that this subtle difference might originate from weaker interactions between D-**2** and CES than between L-**2** and CES. The transmission electron microscopy (TEM) images of the resulting hydrogels reveal the formation of uniform nanofibers after the addition of CES (Figure 1A,B). The diameters of the nanofibers of the hydrogel formed by L-**2** or D-**2** after the addition of CES in the solution of L-**1** or D-**1** are 10 ± 2 nm or 8 ± 2 nm, respectively.

Our preliminary test of the cytotoxicity of L-**1** and D-**1** indicates that L-**1** and D-**1** show significant cytotoxicity to SKOV3 cells at concentrations below the mgc (Supporting Information, Figure S4). Thus, we used static light scattering (SLS) to help verify the existence of nanoscale assemblies (for example, nanofibers or nanoparticles) in the solution of L-**1** (or D-**1**) at concentrations lower than the mgc and after the addition of CES (2 U mL^{-1}). We chose the concentrations from 10 μM to 100 μM to analyze whether there are differences in self-assembly of the molecules before and after the addition of CES. Before being treated with CES, the signal intensity ratios of the solution of L-**1** (or D-**1**) at concentrations from 10 μM to 50 μM were close to zero (Figure 1C,D), indicating that there are hardly any assemblies of L-**1** (or D-**1**) in the solution. When the concentration of the solution of L-**1** (or D-**1**) increases to 100 μM, there was a slight increase of intensity ratio, suggesting that small amounts assemblies of L-**1** (or D-**1**) exist in the solution. In contrast, the addition of CES to the solution of L-**1** (or D-**1**) at concentrations from 10 μM to 100 μM results in a significant increase of the signal intensity ratios, especially when the concentration of L-**1** (or D-**1**) is at or above 50 μM. For example, the signal intensity ratio of the solution of L-**1** (or D-**1**) at 50 μM drastically increased from about zero (before the addition of CES) to about 17 (after the addition of CES), which revealed the formation of assemblies of L-**2** (or D-**2**). Moreover, the solution of 100 μM L-**1** showed a 9-fold increase of the signal intensity ratio after the addition of CES, indicating the formation of a larger amount of assemblies after enzymatically converting the precursors to the hydrogelators (Supporting Information, Figure S6). Similarly, the signal intensity ratio of the solution of 100 μM D-**1** increased significantly after the addition of CES, which agrees with the observation that CES converts D-**1** into D-**2** to form self-assembling nanoscale assemblies of D-**2** in water (Figure 1B).

After confirming that CES converts the precursor L-**1** (or D-**1**) into the hydrogelator L-**2** (or D-**2**), we determined the stability of the precursors (L-**1** or D-**1**) when incubated with the ovarian cancer cells. After culturing the precursors with SKOV3 or A2780cis cells at 37 °C for 4 h, we collected the cell

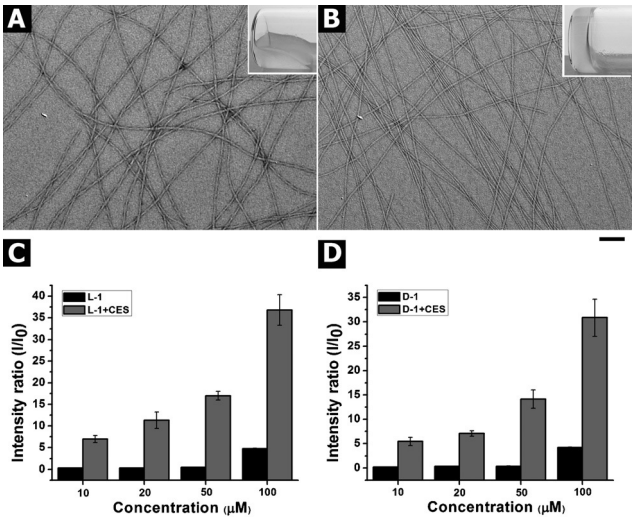

Figure 1. TEM images of the hydrogels (inset: optical images) formed by the addition of CES (2 U mL^{-1}) to the solution of A) L-**1** or B) D-**1** at the concentration of 0.4 wt% in PBS buffer (Scale bar: 100 nm). The signal intensity ratio of static light scattering (SLS) of the solution of C) L-**1** or D) D-**1** at concentrations from 10 to 100 μM before (black bar) and after (gray bar) being treated CES (2 U mL^{-1}) for three hours.

lysates and culture medium for liquid chromatography–mass spectrometry (LC-MS) analysis and determined the intracellular concentrations of the precursors, the hydrogelators, and the relevant proteolyzed products. After incubation with SKOV3 or A2780cis cells for 4 h, more than 85% of the precursors (L-**1** or D-**1**) turned into the corresponding hydrogelators (L-**2** or D-**2**; Table 1). Moreover, the intracellular

Table 1: The intracellular concentrations of the precursors and hydrogelators in SKOV3 and A2780cis cells.

Compd.	Precursor (**1**) [μM]	Hydrogelator (**2**) [μM]	Ratio[a]
L-**1**[b]	62	431	6.95
D-**1**[b]	16	108	6.75
D-**1**[c]	69	582	8.43

[a] ratio of hydrogelator to precursor after 4 h. [b] The cell lysates of SKOV3 cells were collected after 4 h incubation with 20 μM (15 μg mL^{-1}) of D-**1** or with 50 μM (37 μg mL^{-1}) of L-**1** at 37 °C. [c] The cell lysates of A2780cis cells were collected after 4 h incubation with 100 μM (73 μg mL^{-1}) of D-**1** at 37 °C.

concentrations of the hydrogelators were all above 100 μM, which indicates the intracellular self-assembly of the hydrogelators. The cumulative intracellular concentration of L-**1** and L-**2** was also about 10-fold higher than the incubation concentration of L-**1**, and the cumulative intracellular concentration of D-**1** and D-**2** was about 5-fold higher than the incubation concentration of D-**1**. These results not only indicate that the cellular uptake of L-**1** is more efficient than that of D-**1**, but also confirm that the selective retention of hydrogelators inside the cells originates from ester bond cleavage catalyzed by CES. A fluorescent esterase substrate, 6-CFDA,[18] also confirmed high esterase activity in SKOV3 cells (Supporting Information, Figure S7). We also analyzed the culture medium containing L-**1** (or D-**1**), which was incubated with SKOV3 cells or A2780cis cells. After 4 h incubation with SKOV3 cells, about 19% of L-**1** in the medium turned into L-**2** (Supporting Information, Table S2), and the concentration of L-**1** in the medium decreased from 50 μM to 39 μM; about 15% of D-**1** converted D-**2**, and the concentration of D-**1** in the medium decreased from 20 μM to 16 μM. A similar trend was also observed in A2780cis cells. These results further validate the hypothesis that intracellular enzymatic conversion of the precursors catalyzed by CES results in the intracellular self-assembly of the hydrogelators.

To evaluate the effect of intracellular self-assembly of L-**2** or D-**2** for cisplatin-based combination therapy, we test the cell viability of three ovarian cancer cell lines by incubating them with the mixture of precursors and cisplatin (CDDP). After 72 h, the mixture of CDDP (6 μg mL^{-1}) with D-**1** (15 μg mL^{-1}) or L-**1** (37 μg mL^{-1}) inhibits about 74% or 87%, respectively, of SKOV3 cells (Figure 2A). In contrast, D-**1** (15 μg mL^{-1}) or L-**1** (37 μg mL^{-1}) alone is almost innocuous to the cells, and CDDP (6 μg mL^{-1}) alone inhibits only 48% SKOV3 cells. We also used another method to treat the SKOV3 cells, in which the D-**1** (or L-**1**) were added 12 h after the addition of CDDP to SKOV3 cells. As shown in Figure 2A, 72 h after the addition of D-**1** (15 μg mL^{-1}) or L-**1** (37 μg mL^{-1}) following the addition of CDDP (6 μg mL^{-1}),

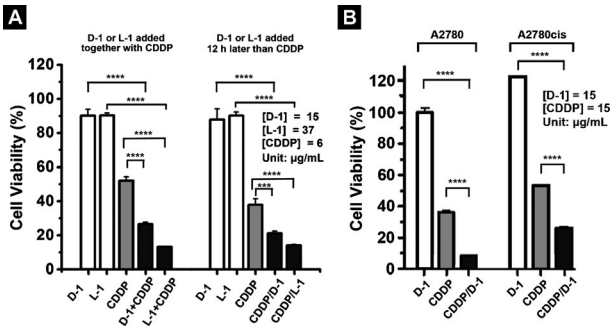

Figure 2. Cell viability of ovarian cancer cells incubated with the precursors with and without cisplatin (CDDP). A) The cell viability of SKOV3 cells incubated with the precursors D-**1** or L-**1** alone, or in combination with CDDP for 72 h. B) The cell viability of A2780 cells and A2780cis cells incubated with the precursors D-**1** alone, or in combination with CDDP for 72 h (*** = $p \le 0.001$, **** = $p \le 0.0001$).

the inhibition of SKOV3 is about 80% or 86%, respectively. The higher efficacy exhibited by L-**1** agrees with the higher uptake and incubation concentration of L-**1**.

We also tested the combination of CDDP and D-**1** for treating A2780cis (cisplatin-resistant) and A2780 (cisplatin-sensitive) cells. D-**1** (15 μg mL^{-1}) alone hardly exhibited any cytotoxicity to A2780cis cells (Figure 2B). The combination of D-**1** and CDDP inhibited 70% of A2780cis cells, which is double the activity of CDDP. The combination of D-**1** and CDDP significantly inhibits A2780 cell viability and decreases the viability of A2780 from about 38% (without adding D-**1**) to only 9%. Since SKOV3 and A2780cis are two drug-resistant ovarian cell lines, CDDP shows lower inhibition ability against these two cell lines compared with A2780 cells. These results confirm that the addition of the precursors of self-assembling small molecules in combination with cisplatin drastically boosts the activity of cisplatin against drug-resistant ovarian cancer cells. The IC_{50} values of L-**1** and D-**1** against the ovarian cancer cells are 62–94 μM and 48–69 μM, respectively (Supporting Information, Table S2), but their concentrations for the combination therapy can be lower than IC_{50} values because EISA accumulates the hydrogelators intracellularly. Furthermore, the intracellularly formed nanofibers (of D-**1**) are about seven times more effective against HeLa cells than the nanofibers of the dipeptides reported previously (Nap-FF[8]; Supporting Information, Table S3).

To verify the critical role of enzyme-instructed self-assembly, we synthesize a control compound (**3**), which replaces the ester bond in D-**1** by an amide bond (Supporting Information, Scheme S2). This change (−COO− to −CONH−) renders **3** resistant to CES. Control compound **3** (500 μM) alone hardly inhibited SKOV3 cells after 72 h incubation (Supporting Information, Figures S8A, S9A). After 72 h incubation with SKOV3 cells, while CDDP (6 μg mL^{-1}) alone causes about 40% cell death, the mixture of **3** (15 μg mL^{-1}) and CDDP (6 μg mL^{-1}) inhibited only about 32% of SKOV3 cells. The innocuous effects of **3** also exclude the possibility that L-**1** or D-**1** act as a surfactant to inhibit cell survival. A similar trend is observed in A2780cis cells (Supporting Information, Figures S8B, S9B). These results further confirm that enzyme-instructed self-assembly inside

cells is the main cause for boosting the efficacy of CDDP in the combination therapy of CDDP with the precursors (D-**1** and L-**1**). Some of the cell viabilities slightly exceed 100% (for example, Figure 2B) because MTT assay measures the activity of mitochondrial reductase and it is not unusual for treated groups to have higher enzyme activity than the control group.

To gain insight into the action of intracellular self-assembly within cells, we examined the change of the actin filaments inside cells. SKOV3 cells treated by D-**1** (20 μM (15 μg mL^{-1}), 20 h) exhibit far fewer well-defined, long actin filaments than that in the control SKOV3 cells (without the treatment of D-**1**; Figure 3). This trend becomes more

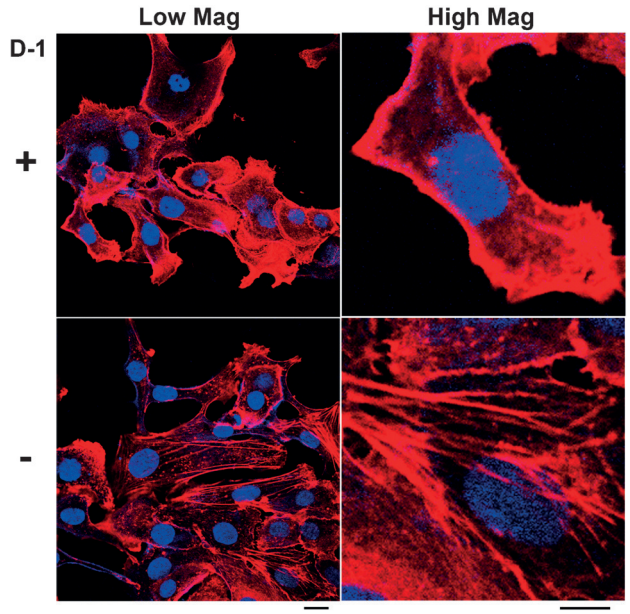

Figure 3. The fluorescence images of SKOV3 cells stained with Alexa Fluor 633 Phalloidin (F-actin) and Hoechst (nuclei) (upper) after treatment of D-**1** at concentration of 20 μM for 20 h or (bottom) without the treatment of D-**1**. Scale bars: left = 20 μm, right = 10 μm.

pronounced after increasing the concentration from 20 to 100 μM (Supporting Information, Figure S11), as evidenced by the number of the actin filaments in the cells (Supporting Information, Figure S19). This observation agrees with hypothesis that the intracellular nanofibers of small peptides interact with actin.[7] To verify the reversible assembly of D-**2** inside cells, we treated the SKOV3 cells with D-**1** at the concentrations of 20, 50, and 100 μM respectively, for 20 h, then replaced the media with fresh media and incubated the cells for an additional 20 h. Actin filaments recovered after being treated with fresh medium for 20 h when the concentrations of D-**1** were 20 and 50 μM (Supporting Information, Figure S12). The incomplete recovery of actin filaments, when [D-**1**] = 100 μM, supports the hypothesis that D-**1** starts to self-assemble at 100 μM. After being incubated with L-**1**, SKOV3 cells exhibited similar behavior (Supporting Information, Figures S13, S14): after 20 h, cells incubated with L-**1** (50 μM) exhibited fewer well-defined actin filaments compared with the cells without the treatment of L-**1**. However, 20 h after exchanging the media, the morphology of actin filaments was restored to normal. These results suggest that intracellular nanofibers formed by enzyme-instructed self-assembly exhibit transient cytotoxicity that should help minimize long-term systemic burden in combination therapy. Dissociation likely reduces the long-term cytotoxicity after the apoptosis of cells so that the precursors and nanofibers cause minimal systemic toxicity.

In conclusion, we demonstrated that enzyme-instructed intracellular self-assembly of small molecules is a new approach to boost the activity of CDDP against two drug-resistant ovarian cancer cell lines. Moreover, at the optimal concentrations, 20 μM (D-**1**) and 50 μM (L-**1**) used for boosting the activities of the cisplatin, L-**1** and D-**1** hardly inhibit HS-5 and PC-12 cells (Supporting Information, Figures S15, S17), despite cisplatin significantly inhibiting HS-5 and PC-12 cells (Supporting Information, Figure S16).[19] Intravenous injection of L-**1** or D-**1** hardly affected the weight and organ index of mice (Supporting Information, Figure S18), confirming the low systemic toxicity of the precursors. The genome analysis according to The Cancer Genome Atlas (TCGA) indicates the amplification of CES in certain tumors (for example, breast and ovarian cancer; Supporting Information, Figure S23), which not only supports the observations in this work, but also provides useful guidance for treating other cancers based on the self-assembly of intracellular nanofibers. This work, together with other emerging evidence,[6–10,12] indicates that enzyme-instructed self-assembly promises a new way for developing combination therapy for cancer treatment. Other than cisplatin, carboplatin has been used as the preferred platinum-based drug,[20] and we will use carboplatin for our future work.

Acknowledgements

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