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Platinum(IV)-Ferrocene Conjugates and Their Cyclodextrin Host–Guest Complexes

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Abstract

Reported here are new platinum(IV) (Pt(IV)) complexes bearing ferrocene (Fc) moieties. These systems differ from one another only by the nature of the functional group (ester vs amide) connecting the linker to the Fc subunits. This minor structural variation (one atom difference) leads to major differences in solubility, stability, and antiproliferative activity against lung (A549) cancer cells. The host–guest chemistry of these complexes was investigated in an aqueous medium in the presence of β -cyclodextrins (β -CD), either free or in the form of a covalently linked Fc-Pt- β -CD hybrid. An inclusion complex between Fc and β -CD is formed in aqueous media, presumably as a result of hydrophobic interactions involving the Fc and the inner β -CD cavity. Consequently, it proved possible to use a β -CD-based strategy to purify the Pt-Fc conjugates in this study under aqueous conditions (by means of C18 silica gel columns). The use of a β -CD adjuvant also allowed dimethyl sulfoxide (DMSO) to be avoided as an organic cosolvent in cell studies. The amide version reported here (**2**) proved to be more soluble, more stable, and more active than the ester analogue (**11**) in A549 cells. The use of a β -CD functionalized with a fluorescent probe allowed intracellular Pt-Fc localization to be visualized by confocal fluorescence microscopy.

Graphical Abstract

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Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.inorg-chem.9b00570.

General procedures; synthesis and characterization of compounds **1**, **2**, and **3** and complexes **2**@ β -CD, β -CD-SiR, and **2**@ β -CD-SiR; antiproliferative activity (MTT assays); cell uptake studies (ICP-MS); fluorescence microscopy analyses; and X-ray experimental data; (PDF)

Accession Codes

CCDC 1890547–1890548 contain the supplementary crystallographic data for this article. These data can be obtained free of charge via www.ccdc.cam.ac.uk/data_request/cif, or by emailing data_request@ccdc.cam.ac.uk, or by contacting The Cambridge Crystallographic Data Centre, 12 Union Road, Cambridge CB2 1EZ, UK; fax: +44 1223 336033.

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INTRODUCTION

Biochemical processes necessary for life require a complex network of intra- and extracellular pathways, many of which are interrelated. For instance, signal transduction is achieved through a series of simple stimuli that typically allow highly specific functions to be readily controlled. Within this latter paradigm, reactive oxygen species (ROS) play a critical role as small-molecule signal mediators in numerous physiological processes. Like other cellular mechanisms, the homeostasis of these reactive chemical species is critical to proper cell functioning. The chemical or environmental modulation of ROS can shift the so-called cellular redox potential outside of normal salutary levels. In cancer cells, the aberrant production of ROS imparts an inherent sensitivity that makes these diseased cells susceptible to ROS-mediated cell death.

A number of cancer drugs have functions that are tied directly or indirectly to changes in ROS levels. This includes platinum-based agents. However, the Pt drugs are not equal. For instance, in contrast to cisplatin,¹ oxaliplatin is able to stress the endoplasmic reticulum (ER)² and induce a complex cascade of events. The resultant immune response plays a crucial role in the efficacy of oxaliplatin (commonly used to treat colorectal cancer) as demonstrated by several *in vivo* studies.³ ER stress is also associated with an increase in ROS production, particularly H₂O₂.^{4,5} We thus envisaged that a marriage between a platinum(IV) oxaliplatin prodrug and an appropriate ROS inducer might enhance the efficacy of platinum-based cancer therapeutics.

The use of redox cycling functionalities holds broad utility in cancer therapy. Indeed, the medicinal chemistry of ferrocene (Fc) has been studied extensively *in vitro* and *in vivo*,⁶ with its anticancer activity having been demonstrated in several cancer cell lines. Mechanistic analyses have provided support for the suggestion that the cytotoxic activity of Fc originates from the generation of ROS through redox cycling between the Fe(III) (ferrocenium, Fc⁺) and Fe(II) (Fc) states.⁷ The resultant increase in ROS leads to the oxidative damage of important biomolecules, such as DNA. A very elegant example of a cisplatin Pt(IV) derivative with Fc groups in the axial positions that underwent activation in the presence of H₂O₂ was recently reported.⁸ Another study, published by Keppler and coworkers, details the synthesis and antiproliferative activity of new oxaliplatin Pt(IV)-ferrocene conjugates, including **11** and **12** (Scheme 1, numbering from the original report),

that are active in three different cell lines; these latter species were prepared by tethering Fc species to appropriately chosen Pt(IV) precursors.⁹ However, these recent advances notwithstanding, Pt-Fc constructs are still few in number. Further classes of conjugates combining these elements are needed if the potential synergistic benefits of Pt + Fc combination therapy are to be assessed in detail.

Here we report the synthesis of a series of new platinum-(IV)-ferrocene conjugates. Modification of the functional group that connects the Fc moieties to the linker, specifically amide vs ester, has allowed us to study the effect of this structural feature on the antiproliferative activity of the complexes. Also reported is the use of Fc- β -cyclodextrin (β -CD) host-guest chemistry to purify these new Pt-Fc constructs and improve their water solubility and cellular uptake, as determined by ¹H NMR spectroscopy, UV-visible spectroscopy, ITC analyses, fluorescence microscopy utilizing a silicon-rhodamine B-labeled β -CD (SiR β -CD), and induced coupled plasma mass spectrometry (ICP-MS). In addition, the use of a host-guest Fc- β -CD construct was found to suppress the need for organic cosolvents, such as dimethyl sulfoxide (DMSO), which can affect biological assays.¹⁰ A polymeric species composed of a mixed Fc and β -CD axially coordinated Pt(IV) (**3**) was also prepared and studied by a variety of analytical methods including scanning electron microscopy (SEM). The redox potentials of these compounds were measured by cyclic voltammetry, and their antiproliferative activities were tested against the A549 cancer cell line in the absence and presence of β -CD by means of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide dye reduction) assays.

RESULTS AND DISCUSSION

As detailed by Keppler and co-workers, the iterative construction of platinum(IV)-ferrocene compounds via ester or amide coupling allowed for the facile synthesis of **11** and **12** (Scheme 1A).⁸ The antiproliferative properties (IC₅₀), as evaluated by MTT assays, led to the inference that the linking unit plays a major role in mediating the potency of the corresponding conjugate. Modification of the ester subunit to an amide yielded higher activity in A549 lung cancer cells. We postulated that such differences might reflect a relative increase in the stability and solubility of the amide-based Pt-Fc complexes in aqueous environments¹¹ or differences in the redox potentials that would be expected to affect their ability to produce reactive oxygen species (ROS).¹² Efforts to explore such mechanistic hypotheses led us to prepare compound **2**, an analogue of **11** and **12** exclusively containing amide bonds (Scheme 1). Compound **2** was obtained by reacting precursor **1** with **L3** using PyBOP (benzotriazol-1-yloxytripyrrolidino-phosphonium hexafluorophosphate) as a coupling agent; this gave **2** in a 60% isolated yield after silica gel column chromatographic purification. Compounds **2** and **11** were observed to have markedly different retention times by RP-HPLC (Supporting Information, Figure S3), with **11** appearing to be more hydrophobic. Compound **11** was also found to be less soluble in aqueous media than its new analogue, conjugate **2**.

A comparative analysis of the proton NMR spectral features for **11** and **2** revealed differences in the chemical shifts for the -CH₂- signals located on the ethylene diamide linker as well as for the NH protons (Figure 1); these differences are rationalized in terms of

the formation of different types of intra- or intermolecular hydrogen bonds in the case of these two congeners.

Unfortunately, despite the increase in water solubility relative to **11**, complex **2** remains as a suspension in most aqueous media. An effort was made to address this deficiency. It is known that ferrocene is a suitable guest for cyclodextrin,¹³ a class of receptors that have been extensively exploited in a range of pharmaceutical applications.¹⁴ The host–guest properties of **2** were thus studied in the presence of β -CD. Toward this end, conjugate **2** was dissolved in deuterated dimethyl sulfoxide (DMSO-*d*₆). Deuterated water (D₂O) was then added progressively until a final mixture consisting of 9:1 DMSO/water (v/v) was obtained. The resulting mixture proved turbid, and only weak, broad signals were observed in the ¹H NMR spectrum. Such findings are consistent with **2** being only partially dissolved under these conditions (Figure S10 in the Supporting Information). However, upon addition of β -CD (2 equiv) a clear solution was obtained. Moreover, the peaks corresponding to the Fc moieties became sharper and more intense (cf. the signals for H_a, H_b, and H_c in Figure 2). It was thus concluded that **2** was solubilized.

An analysis of the NMR spectrum recorded in D₂O revealed that the signals corresponding to H_a, H_b, and H_c were shifted to lower field ($\delta = -0.04$ ppm) in the presence of β -CD. Such shifts were also observed with L3 (SI Figure S12) and are consistent with what is expected when an Fc moiety is encapsulated within the cavity of a β -CD.¹⁵ Moreover, the signal attributed to H_a, initially a singlet, was split upon addition of β -CD. This is ascribed to this proton sampling different chemical and magnetic environments as a result of complexation. Similar spectral changes were observed in the case of L3 (SI, Figure S12). The inferred inclusion events are likely driven by hydrophobic interactions and thus are expected to be favored in 100% water. In fact, when compound **2** was stirred into an aqueous solution of β -CD (D₂O; [2] = 3.3 mM; [β -CD] = 9 mM), the final mixture was free of any observable precipitate. Moreover, the ¹H NMR spectral features under these conditions proved comparable to those obtained when the spectrum was recorded in a D₂O/DMSO-*d*₆ mixture (Figure 2, spectrum d). Nevertheless, one of the signals corresponding to the –CH₂– from the ethylene diamide linker is affected by the addition of β -CD. Specifically, the triplet features expected for this signal are not well defined, and the peak itself is shifted to lower field upon the addition of β -CD. (See the resonance for H_d, Figure 2.) On the other hand, no change is observed for the CH₂ signal from the succinate moiety (Supporting Information, Figure S13) presumably because these latter protons are relatively far from the Fc core.

Recent studies have revealed that oxaliplatin interacts with CDs and cucurbit[7]uril through the encapsulation of the hydrophobic diaminocyclohexane (DACH) moiety.¹⁶ In the case of **2**, however, the peaks corresponding to the DACH CH₂ protons are not affected by the addition of β -CD (up to 4 equiv). Such a finding is rationalized in terms of the Fc outcompeting the DACH moiety in the formation of the inclusion complex. On the other hand, only weak through-space coupling between the ferrocene protons and those of the cyclodextrin host was evidenced by ROESY NMR spectral analyses (Supporting Information, Figure S14). This could reflect a high degree of complex lability, a weak binding of **2** by β -CD, or both. DOSY experiments revealed only a modest decrease in the diffusion coefficient for **2** upon the addition of β -CD (Supporting Information, Figure S15).

The inclusion complex formation and its effect on the water solubility of **2** and **11** were evaluated quantitatively using UV–visible spectroscopy (Figure 3). Complex **2** or its congener **11** was added independently as solids to deionized (DI) water in the presence and absence of HP- β -CD. Upon sonication and filtration, spectra were recorded and compared. For both **2** and **11**, the presence of the cyclodextrin allowed the dissolution of essentially 100% of the compound. In contrast, only 5.4% of **2** and 1.5% of **11** were dissolved in pure DI water. This result provides support for our contention that cyclodextrins are useful solubilizing agents for complexes such as **2** and **11**.

Several attempts were made to grow crystals of the putative inclusion complex formed from **2** and β -CD. Unfortunately, these two species were found to crystallize separately, forming both orange needles and colorless cubes, respectively, within the same crystallization vial. This was taken as further support for the notion that the inclusion complex between **2** and β -CD undergoes rapid exchange. As reflected in the structures shown in Figure 4, diffraction-grade crystals could be obtained by the slow evaporation of a concentrated solution of **1** in methanol and by the vapor diffusion of acetone in a solution of **2** + β -CD in a methanol/water mixture. The resulting structures confirmed what was inferred on the basis of the spectroscopic analyses discussed above.

To evaluate the strength of the interactions between **2** and β -CD, the heat changes that were produced as **2** were titrated with 2-hydroxypropyl- β -CD (HP- β -CD) and were monitored using isothermal titration calorimetry (ITC; see SI Figure S16). Here, HP- β -CD was chosen instead of β -CD because of its higher solubility in water. The curve resulting from this titration was best fitted with a sequential model involving two binding sites. The resulting association constants provide support for the conclusion that the formation of the first Fc- β -CD complex is much more favorable ($K_a = 8 \times 10^3 \text{ M}^{-1}$) than the ensuing formation of a 2:1 (receptor/guest) complex ($K_a = 30 \text{ M}^{-1}$). The association constant corresponding to the first inclusion event is lower by roughly a factor of 2 than the association constant reported in the literature for ferrocene and β -CD ($K_a = 1.65 \times 10^4 \text{ M}^{-1}$). We attribute this difference to the fact that in **2** the Fc moiety is not free but is tethered to a Pt(IV) center; this could preclude an optimal binding orientation within the β -CD cavity.

Additional evidence in support of the proposed formation of host–guest complexes involving the present Fc-Pt conjugates came from the finding that the highly hydrophobic compounds **2** and **11** could be purified in an aqueous medium on a reversed-phase silica gel (C18) column when β -CD (commercial price: ca. \$5/kg) was added directly to the mobile phase.¹⁷ In fact, the use of β -CD in this way allowed for the rapid purification of **2**. However, difficulties in purification were encountered in the absence of β -CD as a result of the poor solubility of conjugate **2** in water.

On the basis of the above findings, it was envisioned that a multifunctional Pt(IV) complex containing β -CD and Fc on the same molecule might confer higher water solubility. These modifications were expected to modify the biological activity of the resulting Pt(IV)-based prodrug, perhaps as a consequence of self-assembly as seen for a previously reported β -CD system containing a tethered Fc moiety.¹⁸ Hence hybrid **3**, incorporating one Fc unit and one β -CD unit in the respective axial ligand positions of the Pt(IV) complex, was synthesized

(Scheme 1B) and purified by reverse-phase HPLC. The resulting hybrid, conjugate **3**, proved to be soluble in water. Moreover, low field shifts were seen for the signals corresponding to protons H_b and H_c in the ¹H NMR spectrum; this is as would be expected for an inclusion complex (Supporting Information, Figure S6).

The self-assembling properties of **3** were investigated using microscopic means. Specifically, a solution of **3** in water was placed on a glass coverslip and evaporated slowly under a stream of nitrogen. Both optical and scanning electronic microscopy revealed the formation of fibers (Figure 5). We thus infer that at high concentrations, such as those pertaining to the conditions of air drying, interactions between the Fc and β-CD subunits present in **3** favor self-assembly, as demonstrated by the formation of fibers.

Recent electrochemical studies of Fc-based anticancer drugs have provided support for the suggestion that the cytotoxic effects are correlated with the ease of oxidation of the ferrocene group.¹⁹ It was also demonstrated in a side-by-side experiment that ferrocenium is the active species that promotes oxidative damage to DNA.²⁰ Hence, the redox properties of the Pt(IV)-Fc conjugates in the present study were investigated using cyclic voltammetry, in both methanol and aqueous solutions. As expected, **2** and **11** show reversible features in their respective cyclic voltammograms. The redox potentials for these two species were separated by ca. 60 mV (*E*₀(**11**) = 422 mV; *E*₀(**2**) = 362 mV). The amide analogue **2** was seen to undergo oxidation at a less positive potential than for **11**, but the redox value is nevertheless shifted anodically by 160 mV relative to Fc (Figure 6C). Electrochemical studies of **2** were also carried out in an aqueous medium (Figure 6A,B) in an effort to probe the effect of β-CD on the redox behavior of **2**. Upon addition of β-CD (up to 4.3 equiv), the redox potential of **2** is shifted by about 50 mV toward more positive potentials.²¹ This finding is rationalized in terms of the positively charged ferrocenium form being destabilized in the hydrophobic cavity.²² The oxidation wave also became broader, a finding ascribed to a change in the diffusion features of the system. On the other hand, the reduction potential associated with the irreversible Pt⁴⁺ → Pt²⁺ reduction varied little upon addition of β-CD.

Hybrid **3** was also tested by cyclic voltammetry (Supporting Information, Figure S8), and the redox potential for the Fc⁺/Fc oxidation wave proved to be consistent with what would be expected for Fc constrained within a β-CD cavity. The reduction potential ascribed to Pt(IV) reduction proved to be similar to that of compound **2**.

The redox cycling ability of the ferrocene moiety in **2**@HP-β-CD and **11**@HP-β-CD was probed using fluorescence spectroscopy.²³ More precisely, in the presence of H₂O₂ (10 mM), glutathione (5 mM), and EDTA (10 mM) in PBS, the fluorescence at 531 nm (excitation at 501 nm) resulting from the oxidation of 2',7'-dichlorofluorescein by reactive oxygen species was monitored over time (Figure S17). It was found that the fluorescence increase for **2**@HP-β-CD was consistently higher (about 2.2-fold) than that for **11**@HP-β-CD. We believe that this difference in reactivity might result from differences in the redox potentials of **2** and **11** as noted above. Of note is that the presence of HP-β-CD does not prevent **2** from producing ROS as effectively as L3.

The in vitro activity of new compounds **2** and **3** as well as previously reported compound **11** was measured using MTT assays. In previous cell culture studies carried out by Keppler and co-workers, the medium used to dissolve compounds **11** and **12** contained low levels of DMSO (i.e., 0.5% v/v after final dilution). DMSO can be toxic and has been found to negatively affect the activity of some platinum drugs in vitro.¹⁰ On one hand, recent results have led to the suggestion that cyclodextrins can enhance transport and cytotoxicity by forming inclusion complexes with various drugs.^{24–26} However, a strong interaction between the drug of interest and cyclodextrin can lead to a decreased cellular uptake and a loss of activity.²⁷ We thus decided to test **2** and **11** against A549 cells using stock solutions prepared with either dimethyl sulfoxide (DMSO) or water containing β -CD as a formulating agent. In the case of **2** and **11**, the results of the MTT assays in DMSO (Figure 7C) revealed a >2.8-fold lower IC₅₀ value for **2** relative to that of **11** (i.e., greater antiproliferative potency). The difference in the redox potential ($E_0(\mathbf{2})$ being 60 mV less positive than $E_0(\mathbf{11})$) and the lower solubility and greater instability of ester-linked system **11** relative to that of **2** likely plays a role in this difference. Indeed, a stock solution of **11** in DMSO (50 mM) left on the benchtop turns dark brown-black in 1 h, presumably because of the release of Fe³⁺. It is noteworthy a brownish precipitate formed rapidly (within a few hours) when L3 was prepared in water. In contrast, **2** was found to be stable for several hours under the same conditions. The lower activity of **2** and **11** when compared to that of oxaliplatin may result from an incomplete reduction of the Pt(IV) core in these two complexes. Indeed oxaliplatin Pt(IV) derivatives bearing carboxylates as axial ligands are known to be particularly stable to reduction.²⁸ A statistically significant increase in toxicity was observed for **2** when β -CD was used instead of DMSO (Figure 7A, *p* value from the *t* test <0.05); however, this change in cytotoxicity is much more moderate than that reported for oxaliplatin encapsulated within cyclodextrins.²⁵ On the other hand, β -CD does not affect the cytotoxicity of **11** at all.

The concentration of β -CD with respect to the antiproliferative activity of **2** was also investigated. A V-shaped curve was obtained when potency was plotted against [β -CD] (Supporting Information, Figure S26). This finding leads us to suggest that an appropriate amount (around 3 equiv) of cyclodextrin is beneficial, whereas too much can cause a decrease in potency, perhaps as the result of overeffective sequestration, as observed for oxaliplatin encapsulated within cucurbit[7]uril.¹⁶

It was hypothesized that the slight increase in toxicity against A549 when β -cyclodextrin was used to formulate **2** might be due to an increase in intracellular Pt concentration. In an effort to test this latter supposition, the platinum uptake of **2** (DMSO) versus **2**@ β -CD into A549 cells at various concentrations was measured by inductively coupled plasma mass spectrometry (ICP-MS, Figure 7B). At each of the concentrations tested (40 and 80 μ M), the Pt uptake for **2**@ β -CD was found to be higher than that for **2**. This higher Pt level might be at the origin of the increased cytotoxicity obtained with **2**@ β -CD. On the other hand, these results show that the use of β -CD as an adjuvant does not prevent the Pt(IV), presumably in the form of the conjugate, from passing through the cell membrane. It is worth mentioning that usually with this kind of system the benefit of the drug inclusion phenomenon is observed in vivo, mainly as a result of increased drug stability in the bloodstream.²⁹

Unlike **2**@ β -CD, hybrid **3**, which has a cyclodextrin molecule covalently bound, was found to display lower antiproliferative activities against the A549 cell line than the other compounds tested in this study. This finding could reflect its higher polarity and correspondingly lower cell membrane permeability.

In an attempt to visualize drug uptake, fluorescence microscopy techniques were used. Previous studies of platinum(IV) drugs have taken advantage of the axial ligands to incorporate a fluorophore.³⁰ Even though this strategy has been proven successful, it is also recognized that the covalent attachment of a probe to a drug can modify its inherent properties, including activity. In an attempt to obtain insights into the intracellular localization of **2**, β -CD was functionalized with SiR, a silicon-rhodamine B derivative (Scheme 2).³¹ As is true for native β -CD, this modified system (referred to as SiR- β -CD) was able to dissolve **2** when the latter was added as a solid in pure water. We thus infer that the presence of the tethered SiR dye does not prevent Fc from interacting with the β -CD and promoting solubilization. In addition, fluorescence spectroscopy shows a decrease in the emission at 693 nm upon addition of **2** (Supporting Information, Figure S24). Accordingly, A549 cells were incubated in the presence of either SiR- β -CD alone or a mixture of **2** and SiR- β -CD (designated as complex **2**@SiR- β -CD; Figure 8).

The red fluorescence emitted from SiR- β -CD was detected for the cyclodextrin alone and the mixture of **2** and SiR- β -CD (presumed inclusion complex **2**@SiR- β -CD) after 4 h with localization predominantly in the cytosol, around the nucleus. Co-staining experiments using mitochondria and endoplasmic reticulum (ER) trackers did not reveal evidence of merged (overlapping) signals. We thus suggest that **2**@SiR- β -CD does not accumulate appreciably within mitochondria or ER (cf. SI, Figure S29). After 24 h, the observed levels of red fluorescence were higher, as would be expected were more of the SiR- β -CD being internalized. Side-by-side comparison of SiR- β -CD (left panels) and **2** + SiR- β -CD (referred to as **2**@SiR- β -CD, right panels) leads to the conclusion that the presence of **2** improves the cellular uptake of SiR- β -CD, presumably through the formation of an inclusion complex. In addition, when **2** was present, some blue and red merged fluorescence was detected. This is taken as evidence that a fraction of SiR- β -CD is localized within the nucleus in the presence of **2**, albeit not in its absence. We thus conclude that **2** has a significant impact on the cellular uptake and intracellular localization of SiR- β -CD. This provides support for the notion that the formation of an inclusion complex between **2** and SiR- β -CD is possible even in cell culture media.

CONCLUSIONS

We have demonstrated through a comparison of **2** and **11** that a minor modification to the linker (ester vs amide) used to prepare Pt-Fc conjugates can produce major changes in the chemical properties of the resulting constructs. As inferred from ¹H NMR spectroscopic analyses, these modifications allow for the formation of different hydrogen bond networks, which in turn leads to differences in the polarity and water solubility. The switch from an ester to amide linkage on going from **11** to **2** also affects the stability of the complex as well as the antiproliferative activity as determined by MTT assays using the A549 cancer cell line. In the context of the present study, we have also found that β -cyclodextrin can be used

to improve the water solubility of Pt-Fc conjugates as well as their stability in aqueous environments. In fact, β -CD was found to facilitate the purification of the title Fc-Pt(IV) complexes under aqueous reverse-phase chromatography conditions. Finally, the combination of Pt(IV)-Fc construct **2** and SiR β -CD resulted in improved intracellular absorption of SiR β -CD. The present study thus illustrates how a combination of rational design and the judicious formation of host–guest complexes can be used to create platinum-based drug candidates with improved chemical features and in vitro anticancer activities. The present work thus sets the stage for further advances in drug discovery and delivery.

EXPERIMENTAL SECTION

General Considerations.

Starting materials were purchased from Fisher Scientific, Sigma-Aldrich, Ark Pharm, Inc. (oxaliplatin), and TCI (cyclodextrins) and used without further purification unless otherwise specified. Solvents were purified using a solvent purifier system (Vacuum Atmospheres). Dichloromethane was freshly distilled after being dried over CaH₂ under argon. Reaction progress was monitored by thin-layer chromatography (TLC silica gel 60 F₂₅₄, Silicycle UltraPure Silica Gels). Analytical RP-HPLC and semipreparative RP-HPLC were performed on a Thermo Scientific Dionex Ultimate 3000 equipped with a PDA detector (set at 254 nm for Pt compounds). The analytical column was a Synchronis C18 column, 5 μ m, 4.6 $\times$ 250 mm² (Thermo Scientific); the mobile phase consisted of an increasing gradient (from 10 to 99% in 10 min) of acetonitrile in water, both containing 0.1% acetic acid. In this case, the flow rate was set to 1.2 mL/min. The semipreparative column was a Hypersil gold C18, 5 μ m, 10 $\times$ 150 mm² (Thermo Scientific). The gradient was the same as that for the analytical studies; however, a flow rate of 5 mL/min was used. Mass spectrometric analyses were carried out at the University of Texas at Austin Mass Spectrometry Facility. Low-resolution and high-resolution electrospray mass spectrometry (ESI-MS) analyses were carried out using a Thermo Finnigan LTQ instrument and a Qq-FTICR (7 T) instrument, respectively. ¹H NMR spectra were recorded using a Varian 400 MHz instrument. Two-dimensional correlation experiments were recorded using a Bruker AVIII HD 500 equipped with a Prodigy liquid-nitrogen cryoprobe. ROESY spectroscopic studies were carried out on a Varian 600 MHz instrument (relaxation delay 2 s, flip angle 45°, line broadening 200 Hz, 3500 ppm wide). UV–vis spectra were recorded on a Varian 5000 UV–vis–NIR spectrophotometer. LC-MS analyses were carried out using an Agilent 6120 Quadrupole instrument, starting the elution at 5% MeOH (with 0.1% formic acid) in water (with 0.1% formic acid) and increasing the polarity of the eluent up to 100% MeOH over the course of 12 min. Isothermal calorimetry experiments were carried out on a VP-ITC MicroCal microcalorimeter. SEM experiments were carried out on a Hitachi S-5500 SEM/STEM instrument. ICP-MS measurements of intracellular Pt levels were carried out on an Agilent 7500ce ICP-MS at the Jackson School of Geosciences at the University of Texas at Austin. Cyclic voltammetry was carried out using a CV-50W voltammetric analyzer. Electrodes were purchased from CH Instruments, Inc. The working electrode was made of glassy carbon, the counter electrode was a platinum wire, the reference electrode was Ag/AgCl, and in some cases, a simple Ag wire was used as a pseudoreference electrode.

Microscopy.

A drop of a solution of **3** in water (ca. millimolar concentration) was placed onto a microscope glass slide, and the solvent was removed under a gentle flow of nitrogen. The resulting dried sample was first observed by means of optical microscopy. Thereafter, the sample was frozen and dried to obtain a powder that was placed onto a carbon double-sided conductive tape to carry out SEM analyses.

Cell Culture.

A549 cells were grown in RPMI containing 10% heat-inactivated fetal bovine serum (FBS) and antibiotics (100 mg/mL streptomycin and 100 U/ml penicillin) and subcultured every 3 days when reaching around 80% confluency.

Viability Tests.

The proliferation of exponential phase cultures of A549 cells was assessed by tetrazolium dye reduction (the so-called MTT assay). In brief, tumor cells were seeded in 96-well microliter plates at 400 cells per well and allowed to adhere overnight in culture medium. After 15 h, the agent under study was added via serial dilution (from 200 to 0.01 μM) and the plate was incubated at 37 °C in 5% CO₂. After incubating for 5 days with the complex in question (6 days overall), 50 μL of a solution of tetrazolium dye at 3 mg/mL was added to each well. The plate was incubated for 4 more hours, whereupon the medium was removed and the formazan product was dissolved in 50 μL of DMSO. The absorbance at 570 nm was measured using a microplate reader (Molecular Devices, Sunnyvale, CA), corrected for background and then normalized to wells containing untreated cells to allow plate-to-plate comparisons. The growth inhibition data were fitted to a sigmoidal dose-response curve (logistic function, Origin Software) to generate IC₅₀ values (defined as the concentrations of drug inhibiting cell growth by 50%). The IC₅₀ is presented as the mean $\pm$ standard deviation.

Pt Cellular Uptake Determined by ICP-MS.

A549 cells were grown until confluent in T-75 flasks (around 6 million cells). Drugs were added (100 μM) and left for the desired times. Cells were then washed twice with PBS, detached by being submitted to trypsin (1X), counted, and pelleted by centrifugation (3 min at 2000 rpm). After removal of the supernatant, the pellet was suspended/washed one more time in/with PBS (5 mL) and reformed by centrifugation (3 min at 2000 rpm). It was then digested in nitric acid (100 μL) for 15 h at 60 °C and diluted with Milli-Q water to a final concentration of 2% HNO₃ before being analyzed by ICP-MS.

Confocal Fluorescence Microscopy.

A549 cells were incubated at 50 000 cells per well per mL ($V = 1 \text{ mL}$) in 24-well plates for 24 h. Note that a round glass coverslip was placed in each well, onto which cells were adhered and allowed to grow. Drugs were added to cells, and after a defined amount of time, cells were washed with PBS three times. Then, 500 μL of paraformaldehyde (4% in PBS) was added to each well, and the plate was held at 37 °C for 10 min. Cells were washed three times with PBS before removing the immersed glass coverslip, which was transferred onto a microscope slide with the cells facing up. A drop of Hoechst dye solution was added to the

coverslip before turning it upside down. As a result, the cells were “sandwiched” between the slide and the slip. Nail polish was applied around the slip to immobilize it. The slides produced in this way were kept in the dark for 10 min before being placed inside the microscope chamber at 37 °C for observation.

For the costaining experiments (Figure S29), confocal fluorescence microscope studies were carried out using live A549 cells. The cells were incubated with 2@- β -CD for 24 h at 50 μ M. ER (0.5 μ M), mitochondria (50 nM), and nucleus trackers (1 μ g/mL) were added and incubated at 37 °C for the last 20 min prior to observations. These observations were made using a Leica SP5 X white light laser confocal microscope. Images were taken with a 63 $\times$, NA 1.4 lens.

Synthesis and Characterization.

Please refer to the Supporting Information for the synthetic experimental and supporting analytical data for the new compounds prepared in the context of the present study (cf. part 2, pp S2–S18). Complex **11** was prepared following the protocol described in ref 9. As noted in the main text, compound **11** could be readily purified by reverse-phase chromatography (C18 column, Sep-Pack) when β -CD was present in the aqueous mobile phase.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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REFERENCES

- (1). Rosenberg B; VanCamp L; Trosko JE; Mansour VH Platinum compounds: a new class of potent antitumour agents. *Nature* 1969, 222, 385–386. [PubMed: 5782119]
- (2). Martins I; Kepp O; Schlemmer F; Adjemian S; Tailler M; Shen S; Michaud M; Menger L; Gdoura A; Tajeddine N; Tesniere A; Zitvogel L; Kroemer G Restoration of the immunogenicity of cisplatin-induced cancer cell death by endoplasmic reticulum stress. *Oncogene* 2011, 30, 1147–1158. [PubMed: 21151176]
- (3). Göschl S; Schreiber-Brynzak E; Pichler V; Cseh K; Heffeter P; Jungwirth U; Jakupec MA; Berger W; Keppler BK Comparative studies of oxaliplatin-based platinum(IV) complexes in different in vitro and *in vivo* tumor models. *Metallomics* 2017, 9, 309–322. [PubMed: 28205649]
- (4). Zeeshan HMA; Lee GH; Kim H-R; Chae H-J Endoplasmic reticulum stress and associated ROS. *Int. J. Mol. Sci* 2016, 17, 327. [PubMed: 26950115]
- (5). Shi Y; Tang B; Yu P-W; Tang B; Hao Y-X; Lei X; Luo H-X; Zeng D-Z Autophagy protects against oxaliplatin-induced cell death via ER stress and ROS in Caco-2 cells. *PLoS One* 2012, 7, e51076. [PubMed: 23226467]

- (6). Patra M; Gasser G The medicinal chemistry of ferrocene and its derivatives. *Nat. Rev. Chem* 2017, 1, 0066.
- (7). Tabbi G; Cassino C; Cavigliolo G; Colangelo D; Ghiglia A; Viano I; Osella D Water stability and cytotoxic activity relationship of a series of ferrocenium derivatives. ESR insights on the radical production during the degradation process. *J. Med. Chem* 2002, 45, 5786–5796. [PubMed: 12477361]
- (8). Reshetnikov V; Daum S; Mokhir A Cancer-specific, intracellular, reductive activation of Pt^{IV} prodrugs. *Chem. - Eur. J* 2017, 23, 5678–5688. [PubMed: 28319647]
- (9). Banfi J; Legin AA; Jakupec MA; Galanski M; Keppler BK Platinum(IV) complexes featuring one or two axial ferrocene bearing ligands-synthesis, characterization, and cytotoxicity. *Eur. J. Inorg. Chem* 2014, 2014, 484–492.
- (10). Hall MD; Telma KA; Chang K-E; Lee TD; Madigan JP; Lloyd JR; Goldlust IS; Hoeschele JD; Gottesman MM Say no to DMSO: dimethylsulfoxide inactivates cisplatin, carboplatin, and other platinum complexes. *Cancer Res.* 2014, 74, 3913–3922. [PubMed: 24812268]
- (11). Kang D; Ricci F; White RJ; Plaxco KW Survey of redox-active moieties for application in multiplexed electrochemical biosensors. *Anal. Chem* 2016, 88, 10452–10458. [PubMed: 27659949]
- (12). Srinivas P; Prabhakar S; Chevallier F; Nassar E; Erb W; Dorcet V; Jouikov V; Krishna PR; Mongin F Synthesis of ferrocene amides and esters from aminoferrocene and 2-substituted ferrocenecarboxylic acid and properties thereof. *New J. Chem* 2016, 40, 9441–9447.
- (13). Hapiot F; Tilloy S; Monflier E Cyclodextrins as supramolecular hosts for organometallic complexes. *Chem. Rev* 2006, 106, 767–781. [PubMed: 16522008]
- (14). Loftsson T; Duchêne D Cyclodextrins and their pharmaceutical applications. *Int. J. Pharm* 2007, 329, 1–11. [PubMed: 17137734]
- (15). Nakahata M; Takashima Y; Hashidzume A; Harada A Redox-generated mechanical motion of a supramolecular polymeric actuator based on host-guest interactions. *Angew. Chem., Int. Ed* 2013, 52, 5731–5735.
- (16). Jeon YJ; Kim S-Y; Ko YH; Sakamoto S; Yamaguchi K; Kim K Novel molecular drug carrier: encapsulation of oxaliplatin in cucurbit[7]uril and its effects on stability and reactivity of the drug. *Org. Biomol. Chem* 2005, 3, 2122–2125. [PubMed: 15917899]
- (17). Bulk price; see <https://www.alibaba.com/showroom/cyclodextrin.html>; Last downloaded February 22, 2019.
- (18). Casas-Solvas JM; Ortiz-Salmerón E; Fernández I; García-Fuentes L; Santoyo-González F; Vargas-Berenguel A Ferrocene- γ -cyclodextrin conjugates: Synthesis, supramolecular behavior, and use as electrochemical sensors. *Chem. - Eur. J* 2009, 15, 8146–8162. [PubMed: 19637257]
- (19). Hillard E; Vessières A; Le Bideau F; Pla uk D; Spera D; Huché M; Jaouen G A series of unconjugated ferrocenyl phenols: prospects as anticancer agents. *ChemMedChem* 2006, 1, 551–559. [PubMed: 16892391]
- (20). Osella D; Ferrali M; Zanello P; Laschi F; Fontani M; Nervi C; Cavigliolo G On the mechanism of the antitumor activity of ferrocenium derivatives. *Inorg. Chim. Acta* 2000, 306, 42–48.
- (21). Matsue T; Evans DH; Osa T; Kobayashi N Electron-transfer reactions associated with host-guest complexation. Oxidation of ferrocenecarboxylic acid in the presence of M-cyclodextrin. *J. Am. Chem. Soc* 1985, 107, 3411–3417.
- (22). Osella D; Carretta A; Nervi C; Ravera M; Gobetto R Inclusion complexes of ferrocenes and C-cyclodextrins. Critical appraisal of the electrochemical evaluation of formation constants. *Organometallics* 2000, 19, 2791–2797.
- (23). Hagen H; Marzenell P; Jentzsch E; Wenz F; Veldwijk MR; Mokhir A Aminoferrocene-based prodrugs activated by reactive oxygen species. *J. Med. Chem* 2012, 55, 924–934. [PubMed: 22185340]
- (24). Prashar D; Shi Y; Bandyopadhyay D; Dabrowiak JC; Luk Y-Y Adamantane-platinum conjugate hosted in, -cyclodextrin: enhancing transport and cytotoxicity by noncovalent modification. *Bioorg. Med. Chem. Lett* 2011, 21, 7421–7425. [PubMed: 22050886]

- (25). Zhang D; Zhang J; Jiang K; Li K; Cong Y; Pu S; Jin Y; Lin J Preparation, characterization and antitumor activity of P-, – and HP—cyclodextrin inclusion complexes of oxaliplatin. *Spectrochim. Acta, Part A* 2016, 152, 501–508.
- (26). Petrovski Ž.; Norton de Matos MRP; Braga SS; Pereira CCL; Matos ML; Goncalves IS; Pillinger M; Alves PM; Romão CCJ Synthesis, characterization and antitumor activity of 1,2-disubstituted ferrocenes and cyclodextrin inclusion complexes. *J. Organomet. Chem* 2008, 693, 675–684.
- (27). Yong JH; Barth RF; Rotaru JH; Wyzlic IM; Soloway AH Evaluation of in vitro cytotoxicity of carboranyl amino acids, their chemical precursors and nido carboranyl amino acids for boron neutron capture therapy. *Anticancer Res.* 1995, 15, 2039–2043. [PubMed: 8572599]
- (28). Zhang JZ; Wexselblatt E; Hambley TW; Gibson D Pt(IV) analogs of oxaliplatin that do not follow the expected correlation between electrochemical reduction potential and rate of reduction by ascorbate. *Chem. Commun* 2012, 48, 847–849.
- (29). Li BL; Meng Z; Li Q; Huang X; Kang Z; Dong H; Chen J; Sun J; Dong Y; Li J; Jia X; Sessler JL; Meng Q; Li C A pH responsive complexation-based drug delivery system for oxaliplatin. *Chem. Sci* 2017, 8, 4458–4464. [PubMed: 28970876]
- (30). Song Y; Suntharalingam K; Yeung JS; Royzen M; Lippard SJ Synthesis and characterization of Pt(IV) fluorescein conjugates to investigate Pt(IV) intracellular transformations. *Bioconjugate Chem.* 2013, 24, 1733.
- (31). Lukinavičius G; Umezawa K; Olivier N; Honigsmann A; Yang G; Plass T; Mueller V; Reymond L; Corrêa IR Jr.; Luo Z-G; Schultz C; Lemke EA; Heppenstall P; Eggeling C; Manley S; Johnsson K A near-infrared fluorophore for live-cell super-resolution microscopy of cellular proteins. *Nat. Chem* 2013, 5, 132–139. [PubMed: 23344448]

Figure 1. Partial ¹H NMR spectra (DMSO-*d*₆, 400 MHz, 300 K) of **11** (ester/amide linker, top) and **2** (amide/amide linker, bottom).

Figure 2.

Partial ^1H NMR spectra (for a–c: 400 MHz, 300 K, $[\mathbf{2}] = 5.9$ mM, $\text{D}_2\text{O}/\text{DMSO-}d_6 = 9:1$) of $\mathbf{2}$ before (a), after addition of 2 equiv of β -CD (b), after adding 3.9 equiv of β -CD (c), and in the presence of $[\mathbf{2}] = 3.3$ mM and $[\beta\text{-CD}] = 9$ mM, D_2O , giving what is inferred to be $\mathbf{2}@\beta\text{-CD}$ (d). *Residual solvents. Note that the spectra in (d) were recorded at 300 K using a 500 MHz instrument equipped with a cryoprobe.

Figure 3.

UV-visible spectra (H_2O , 300 K) of **2** (left, 1.75 mM) and **11** (right, 1.27 mM) added separately as solids to DI water solutions in the presence (orange, 7.16 mM) and absence of HP- β -CD (blue). The solutions were filtered ($0.22\ \mu\text{m}$) before spectra were recorded. The gray curve is the spectrum of HP- β -CD alone. Percentages represent the amounts of **2** and **11** dissolved.

Figure 4. Single-crystal X-ray diffraction structures. View of the Pt(IV) complexes in **1** and **2** showing the heteroatom labeling scheme. Displacement ellipsoids are scaled to the 50% probability level. Further details of these structures may be obtained from the Cambridge Crystallographic Data Centre by quoting CCDC numbers 1890548 and 1890547, respectively.

Figure 5. Images obtained using optical (A) and scanning electronic microscopy (B) of the residue formed upon slow evaporation of a solution of **3** in water under a flow of nitrogen.

Figure 6.

Cyclic voltammograms (CVs) of **2** (1 mM) in PBS/DMSO (9:1); four scans (A); **2** upon addition of 0, 1, 2, 3.3, and 4.3 equiv of β -CD (B); scan rate, 200 mV/s; reference electrode, Ag/AgCl (1 M KCl); working electrode, glassy carbon (GC); auxiliary electrode, platinum wire; electrolyte, KCl (0.1 M). (c) CVs of **2** (1 mM), **11** (0.94 mM), Fc, and Fc-COOH (1 mM) in methanol, 200 mV/s, a Ag wire, GC, and a Pt wire were used as the pseudoreference, working, and auxiliary electrodes, respectively, with tetrabutylammonium perchlorate (0.1 M) as the electrolyte.

Figure 7.

(A) Antiproliferative activities measured by MTT assay (five-day incubation), here, **2**@ β -CD to compound **2** studied in the presence of 3 equiv of β -CD, with * representing the Student *t* test (unpaired) with a *p* value of <0.05. (B) Pt cellular uptake (A549) measured by ICP-MS after 4 h of incubation at 40 and 80 μM . (C) Summary of the antiproliferative activities against A549 cells. Note that L3 was not toxic within the concentration range used for the other compounds ($\leq 100 \mu\text{M}$).

Figure 8.

Fluorescence confocal microscopy images obtained upon the incubation of A549 cells for 4 or 24 h with SiR β -CD or 2@SiR β -CD, [SiR β -CD] = 100 μ M (red). Thereafter, cells were fixed and Hoechst stained (blue, nuclei). The white arrow shows the merged red and blue fluorescence.

Scheme 1. (A) Structures of Oxaliplatin and of the Compounds Studied by Keppler and Coworkers^a and (B) Syntheses of New Analogues 2, 2@β-CD, and Hybrid 3

^a11 and 12 following the numbering scheme in the original report.