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Speciation of Phenanthriplatin and its Analogs in the Core of Tobacco Mosaic Virus

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Abstract

Efficient loading of drugs in novel delivery agents has the potential to substantially improve therapy by targeting the diseased tissue while avoiding unwanted side effects. Here we report a first systematic study of the loading mechanism of phenanthriplatin and its analogs into tobacco mosaic virus (TMV), previously used by our group as an efficient carrier for anticancer drug delivery. A detailed investigation of the preferential uptake of phenanthriplatin in its aquated form (~2000 molecules per TMV particle versus ~1000 for the chlorido form) is provided. Whereas the net charge of phenanthriplatin analogs and their ionic mobilities have no effect on loading, the reactivity of aqua phenanthriplatin with the glutamates, lining the interior walls of the channel of TMV, has a pronounced effect on its loading. MALDI-MS analysis along with NMR spectroscopic studies of a model reaction of hydroxy-phenanthriplatin with acetate establish the formation of stable covalent adducts. The increased number of heteroaromatic rings on the platinum ligand

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Associated Content

Supporting Information

MS of reaction mixture, ¹H and ³¹P NMR spectra, a table showing calculated and observed m/z values for various fragment ions obtained by ETD-MS/MS analysis, and a supporting video.

Notes

The authors declare no competing financial interest.

appears to enhance loading, possibly by stabilizing hydrophobic stacking interactions with TMV core components, specifically Pro102 and Thr103 residues neighboring Glu97 and Glu106 in the channel. Electron transfer dissociation-MS/MS fragmentation, a technique that can prevent mass condition-vulnerable modification of proteins, reveals that Glu97 preferentially participates over Glu106 in covalent bond formation to the platinum center.

Graphical abstract

Introduction

Platinum-based anticancer drugs such as cisplatin, carboplatin, and oxaliplatin are used in the treatment of nearly 50% of cancer patients undergoing chemotherapy.^{1,2} These drugs, however, lack selectivity and have dose-limiting toxicities, low bio-availability, rapid clearance from the body, and may encounter drug resistance mechanisms, leading to the recurrence of cancer.^{3,4} To circumvent these challenges, drug delivery systems have been devised to provide selective tissue-specific targeting and intracellular delivery, while decreasing side effects. Given that the development of new drugs is expensive, reducing toxicity caused by existing drugs by packaging them into delivery vehicles is an attractive strategy and can be achieved by controlled and targeted methodologies.

To address this problem, drug formulations in nanoparticle forms have been explored as carriers.^{5–8} Liposome- and albumin-based drug formulations such as Doxil® and Abraxane® are FDA-approved nanoparticle-based drug formulations in this class. A few platinum-based nanoparticle formulations such as Lipoplatin,^{9–13} SPI-077,^{14–18} LiPlaCis,¹⁹ Lipoxal,^{20,21} Aroplatin,^{22–24} and MBP-426,^{25,26} have been in clinical trials, yet currently no platinum drug-based nanoparticle formulations are widely applied. Although presently available nano-sized delivery agents impart safety, their limited efficacy and inability to penetrate tissue adequately are some of the limitations. Consequently, development of new drug delivery methods has been a subject of recent burgeoning interest.

Viruses are simple in structure and composition, and they can be considered as biology-derived nanocarriers, naturally evolved to carry and deliver cargos to target cells. Mammalian viruses have been developed for gene and drug delivery applications.^{27–31} The

major concern, however, is the inherent immunogenic nature, unwanted side effects, and biosafety of viral vectors.^{30,32,33} Several artificial viruses lacking harmful genes have been proposed for delivering drugs,^{33,34} but their polydispersity of molecular weight is a serious limitation that affects its clinical application. Plant viruses are an emerging platform technology that offer advantages over mammalian vectors, namely, 1) scalable production through molecular farming in plants or fermentation in yeast/bacteria, 2) non-infectivity toward mammals, 3) a high degree of stability and tunability through genetic and chemical modification, and 4) tubular and filamentous nucleoprotein shapes. Whereas most mammalian viruses, in particular those used for nucleic acid and oncolytic virus therapy, are spherical in nature,³⁵ data indicate that non-spherical nanoparticles may offer advantages for drug delivery, such as immune system evasion and enhanced margination. Like any biological material, plant viruses are immunogenic, but this property can be overcome through stealth coating (PEG, POx) or self-shielding (albumin coating).^{36–38}

Tobacco mosaic virus (TMV), which has a broad host range infecting close to 200 species from 30 families of the angiosperms,³⁹ consists of 2130 identical coat protein subunits assembled around a single stranded RNA to form a right-handed helix. It has a rod-like structure, 300 nm long and 18 nm wide with a 4 nm-wide internal channel,^{40,41} that can be useful for the packaging of drugs.^{42–45} The inner lining of the channel is highly negative, consisting of 4260 glutamate residues and its exterior provides a suitable platform for conjugating cancer cell targeting agents.^{43,46}

Recently, we reported a simple strategy to prepare TMV loaded with phenanthriplatin (**1**, Figure 1A), a drug candidate having 40-fold higher potency than the classical platinum-based anticancer drugs, and we successfully delivered it to cancer cells.⁴² TMV efficiently releases phenanthriplatin in tumors, while rendering stability at physiological pH and prevent off-target release of phenanthriplatin which may cause toxicity. Furthermore, treatment of tumor using phenanthriplatin encapsulated in TMV exhibited exceptional activity compared with free phenanthriplatin and also inhibited tumor growth rates. Aquated phenanthriplatin obtained after AgNO₃ treatment was proposed to bind carboxylate groups lining the inner core of the virus and to be released at acidic pH conditions. Complex **1** and its hydroxo derivative (**4**, Figure 1A) are differentially taken up in the channel of TMV. Whereas ~1000 molecules of **1** are loaded in TMV, **4** is taken up in higher quantities (~2000 molecules per TMV particle). These values indicate impressive loading capacity and can also contribute to higher uptake of drugs in cells in comparison to free drugs. TMV delivers cisplatin to cisplatin sensitive and resistant ovarian cancer cell lines, and the cisplatin-loaded TMV exhibited superior activity over cisplatin alone.^{47,48} To gain a better understanding of the mechanism involved in the uptake of **1** and its analogs, we undertook the present investigation. Here we report the influence of net charge of **1** and its analogs (Figure 1) on their uptake into the TMV channel. The effect of the aromatic ring size of the ligand coordinated to Pt was also studied. To evaluate the formation of covalently platinated glutamate adducts, kinetic studies of a model reaction involving acetate were performed using NMR spectroscopy. The site of platination, Glu 96 or Glu 107, was also determined, by electron transfer dissociation (ETD)-MS/MS analysis of trypsin-digested **4**-treated TMV samples.

Results and Discussion

Wild-type TMV samples used for this study were obtained and purified from *Nicotiana benthamiana* plants by a previously reported method.⁴⁹ Phenanthriplatin and its analogs were synthesized as described.^{50,51}

The fact that ~1000 molecules of phenanthriplatin (**1**) and ~2000 molecules of its aqua form are taken up in TMV⁴² suggests that uptake may be determined at least in part by the net charge. To check this possibility, we prepared quinoplatin (**2**), pyriplatin (**3**), and aquated derivatives of **1** (deprotonated forms **4** and **5**, Figure 1) as well as the dicationic complex **6**, and compared their uptake with those of **1** and **4** into the TMV core by atomic absorption spectroscopy (AAS). A greater positive charge suggested better stabilization inside the TMV channel by electrostatic interactions, but organic portions of the ligands may also influence their binding. The uptake of complexes **2**, **3** and **5** is similar to that of **1**, i.e., ~1000 molecules per TMV, indicating that there is no difference in the loading of complexes bearing the same net charge. The dicationic complex **6**, however, showed slightly higher uptake than **1**. This result is probably due to increased stability attained by the higher charge interactions between this complex and the glutamate residues inside the TMV channel. Although, like **4**, complex **5** has a coordinated hydroxyl group, its loading was only ~1250 molecules per TMV. This result suggests that the ammine groups in **4** better satisfy the spatial constraints for the proper interaction with glutamates compared with the bulkier 1,2-diaminocyclohexane group in **5**, which has a lower uptake in TMV. The greater uptake of **4** may also be mediated by more effective hydrogen bonding and by covalent and noncovalent interactions with the interior of TMV.

We next evaluated whether ionic mobility plays any role in the uptake of **1** vs. **4** into TMV. Ionic mobility in this case refers to the time required to migrate charged Pt complexes from the solution to the TMV channel. Complexes having lower positive charge may take longer to migrate. In this study, **1** was incubated with TMV in phosphate buffer (10 mM, pH 7.8) for time periods ranging from 5 to 60 min. Because the uptake was similar at these time points (Supplementary Information, Figure S1), we conclude that ionic mobility has no significant effect on uptake. Incubation of **1** with TMV for longer time periods (270 and 480 min), however, revealed relatively higher uptake, owing to formation of **4** by aquation. We next investigated certain physical properties of **4**. The pK_a value was measured to be 5.57 ± 0.04 by potentiometric titration (Figure 2), consistent with the knowledge that coordination to a metal center increases the acidity of aqua ligands. Hence, a very small amount (~1/1000) of the aquated species is expected to be present at pH 7.8.

To simulate conditions in the TMV interior, a reaction between **4** and sodium acetate in phosphate buffer (10 mM, pH 7.8) was carried out, and the formation of acetate-bonded phenanthriplatin (**7**) was revealed by mass spectrometry (MS, Supplementary Information, Figure S2). Formation of a covalent adduct was also confirmed by MS when the same reaction was carried out using *N*-benzyloxycarbonyl-L-glutamic acid 1-methyl ester for 15 min instead of acetate (Supplementary Information, Figure S3). To further confirm the interaction between the glutamate residues of TMV and **4**, we recorded a MALDI-TOF mass spectrum (Figure 3) that showed the formation of adducts. These adducts probably involve

substitution of aqua ligands by glutamate residues, maintaining the equilibrium (Figure 2) toward the protonated form of **4**. To confirm this idea, we performed loading at pH 6 for a short duration of 5 min. The reaction was faster under these conditions, resulting in loading of 1831 ± 43 phenanthriplatin molecules per TMV particle. Formation of Pt-TMV adducts increased with the incubation time (15 min to 1080 min, Figure 3). In contrast, TMV treated with **1** under identical conditions did not reveal the formation of covalent adducts, confirming that the interaction between **1** and TMV is of a purely ionic nature (Figure 3). To further investigate whether the peak appearing at 408 mass units higher than the mass of the TMV coat protein is not due to ion pair formation in the mass spectrometer, we recorded the same spectra immediately after mixing TMV with **4**. There was no peak corresponding to the Pt-TMV adduct (Supplementary Information, Figure S4), reinforcing the conclusion that adducts form in the other cases. This conclusion is also supported by an increase in peak intensity corresponding to the adducts obtained at longer incubation times.

We further considered the possibility that the local environment inside the TMV channel might have a different pH than that of the bulk solution and thereby influence the relative proportions of **4** and protonated **4**. To probe the pH value inside the channel, we attempted to use ^{31}P NMR spectroscopy, which is known to be a useful tool for probing local pH in confined volumes.^{52–54} The nature of an intracellular environment, for example, can significantly differ from that of the bulk solution.⁵⁴ Phosphate speciation can be monitored through ^{31}P NMR spectroscopy and inform through chemical shifts the relative proportion of phosphate species and, thereby, the pH. ^{31}P NMR spectroscopy has also been used to estimate the cytoplasmic and vacuolar pH in higher plant cells.⁵⁵ In our experiment, we expected that the phosphate ions present in the TMV channel can undergo protonation due to the local pH and that this reaction can result in a different chemical shift of phosphorus nuclei apart from the signals arising from the phosphate ions present in the bulk medium. But the singlet arising at 2.84 ppm for the ^{31}P nuclei of phosphate buffer alone shifted minimally, to 2.78 ppm, without showing any additional signal, when TMV was incubated in it for 15 min (Supplementary Information, Figure S5). There was a negligible shift to 2.71 ppm even after incubation of TMV in phosphate buffer for 21 h (Supplementary Information, Figure S5). Given the dimensions of the channel and phosphate ions, the latter should be able enter the virus interior. The near zero difference in ^{31}P chemical shifts (0.13 ppm) indicates that the pH inside the TMV channel is essentially the same as that in the external buffer, i.e., 7.8. This experiment supports the conclusion that, if protonated forms of **4** are generated by the shift of equilibrium from **4**, they rapidly convert to covalent adducts.

We next investigated the kinetics of the reaction between **4** and carboxylates, to gain a better understanding of the relative extent of direct conjugation versus non-bonded interactions during the loading of **4** in TMV. The reaction kinetics between **4** and acetate may serve to model the reaction of **4** with the carboxylate side chains of glutamate residues 97 and 106. Cation **4** was first prepared by the quantitative precipitation of silver chloride upon addition of aqueous silver nitrate to **1**. The rate constants of the forward aquation reaction and its reverse are reported to be $6.2 \times 10^{-5} \text{ s}^{-1}$ and $1.4 \times 10^{-4} \text{ s}^{-1}$, respectively, with an equilibrium constant $K_{\text{eq}} = k_1/k_{-1} = 0.43$ (Scheme 1).⁵⁷

To determine k_2 , the rate constant of interest, **4** was allowed to react with 10-, 20-, 30-, 40-, and 50-fold excess amounts of potassium acetate in D₂O, and the reaction was monitored by ¹H NMR spectroscopy. The starting complex (**4**) was tracked by integration of the NMR resonance at δ 8.2 (1H, Supplementary Information, Figure S6), and the acetate-bound form (**7**) by the resonance at δ 1.7 (3H, Figure 4). Assuming a second-order process, the formation of **7** and disappearance of **4** were modeled by the following differential rate equation:

$$v = -\frac{d[\mathbf{4}]}{dt} = k_2[\mathbf{4}][\text{OAc}] \quad (1)$$

The reactions were analyzed by pseudo first-order kinetics, because $[\text{OAc}] \gg [\text{phentdPtAq}]$:

$$v = -\frac{d[\mathbf{4}]}{dt} = k_{\text{obs}}[\text{OAc}], \text{ where } k_{\text{obs}} = k_2[\text{OAc}] \quad (2)$$

The integrated rate equation was then used to experimentally determine k_{obs} , which is the negative slope of the linear regression when plotting $\ln X_4$ against time (Figure 5B). The k_{obs} values were further plotted against acetate concentration (Figure 5C), and the second-order constant k_2 was determined from the slope of the linear regression to be $0.016 \pm 0.001 \text{ M}^{-1} \text{ min}^{-1}$ ($= 2.7 \pm 1 \times 10^{-4} \text{ M}^{-1} \text{ sec}^{-1}$).

$$\ln \frac{[\mathbf{4}]_t}{[\mathbf{4}]_0} = \ln X_4 = -k_{\text{obs}} t \quad (3)$$

The derived rate constant was then used to extrapolate the rate of reaction under experimental TMV-loading conditions.⁴² Native TMV has a 4 nm-wide interior channel and a length of 300 nm.⁵⁸ Accounting for the 4260 glutamate residues 97 and 106 in the 300×4 nm TMV channel, the local concentration of carboxylate side chains in the channel was calculated to be $\sim 2 \text{ M}$. From the measured second-order rate constant and assuming 2 M acetate, the relative amounts of **4** and **7** over time were plotted as shown in Figure 5D. After the 30-minute loading protocol, this model calculation predicts that $\sim 60\%$ of the starting **4** would be converted to glutamate-bound material, analogous to **7**. The primary sources of error in the model are the unknown difference in reactivity between TMV-glutamate residues and free acetate and whether both glutamate residues are able to bind Pt. The second-order rate constant k_2 is, however, expected to serve as a reasonable estimate for glutamate reactivity.

TMV uptake studies of **4** for various times (1-480 min, Figure 5E) were also performed. At shorter incubations of 1 and 5 min, the uptake was similar to that obtained for **1**. This result is probably a consequence of noncovalent interactions between **4** and the glutamate residues of TMV. Uptake greatly increases after 15 min incubation and remains constant thereafter for longer incubation times (270 and 480 min), revealing the maximum possible loading of **4**

in the TMV channel, i.e. ~2500 Pt per TMV particle. This value is probably due to formation of stable covalent adducts, in agreement with MALDI-MS and NMR spectroscopic data.

To reveal which glutamate residue might better react with **4**, we carefully trypsinized TMV that had been treated with **4** in an excess acetonitrile over aqueous triethylammonium acetate (TEAA) buffer before subjecting it to LC-MS analysis, to prevent hydrolysis of any covalent adducts. The mass spectra of tryptic digests obtained after treatment of TMV with **4** show peaks at m/z 865 corresponding to the platinated peptide (IIEVENQANPTTAETLDATR) of interest, having a +3 charge (Supplementary Information, Figure S7); such signals are not seen in unmodified TMV. To get a clearer picture and better resolution, targeted MS analysis of the tryptic digest of **4**-treated TMV was carried out, resulting in the appearance of the typical isotopic abundance pattern in the region m/z 864.40- m/z 866.40, which is consistent with the platinated peptide (Figure 6A), confirming the formation of covalent adducts. To further check which Glu residue participates in the covalent binding to **4**, we employed the ETD-MS/MS technique, which is well known to reveal mass condition-vulnerable post-translational modifications of proteins.^{59,60} Further fragmentation of the tryptic digest of **4**-treated TMV in the ETD mode revealed that Glu97 is preferentially platinated over Glu106 (Figure 6B). The increase in the mass of 'y' fragment ions by 407 units after the position of Glu97 ultimately confirms that this Glu participates in covalent binding to **4**. This result was also verified by checking the mass of resulting 'a' and 'c' ions after fragmentation (Supplementary Information, Figure S8).

Carboxylate ligands are generally labile and therefore the stability that is attained in the case of TMV treated with **4** may have contributions from other interactions in the viral interior. To check whether the phenyl aromatic rings are important for stabilization of **4** inside the TMV channel, we studied the uptake using hydroxido-pyriplatin (**8**) and hydroxido-quinoplatin (**9**) (Figure 7).⁵⁰ Under identical reaction conditions, uptake of **9** was greater than that of **1** (~1400 molecules per TMV), whereas for **8** it was similar to that of **1**. These results indicate that the size of the aromatic ligand affects loading. Larger aromatics lead to greater hydrophobic interactions and better stability, perhaps because of hydrophobic/stacking interactions with a neighboring amino acid chain. Pro102 and Thr103 are reasonable candidates for such if **4** interacts preferentially with exposed Glu97 (Figure 7). Although **9** has intermediate number of aromatic hydrophobic fused rings, the additional stability due to hydrogen bonding from hydroxide group in **9** with neighboring amino acid residues may account for its higher uptake in the TMV channel than that of **1**. It appears that the hydrophobic interactions do not play a major role in stabilization of chlorido complexes (**1-3**, Figure 1), and, therefore, loading of these complexes is almost the same. Besides hydrophobic interactions, hydrogen bonding from the coordinated ammine groups may also play an important role in the overall stability of the platinum/TMV-**4** conjugate. Such an interaction was already evoked to explain the relatively low loading of **5** into TMV (Figure 1). In a reaction where external chloride was added, the loading of **4** in the TMV channel was affected. In this case, 1785 ± 44 Pt atoms load per TMV particle, less than the uptake of **4** itself (2300 ± 343 Pt per TMV particle). This result is probably due to formation of **1**, which does not bind the viral interior as efficiently.

Classical molecular dynamics simulations were performed to assess and visualize the interaction of the charged **4** complex with the interior surface of TMV (Supplementary Information, Figure S9). The simulation starts with the complex away from the surface of the virus, but is rapidly driven to the negatively-charged regions where the glutamate residues lie (ca. 4 ns), where it remains bound to a glutamate residue for the remainder of the simulation (to 10 ns). The final view reveals H-bond interactions with Glu106, while the aromatic rings interact with Thr103 and Pro102 (Supplementary Information, Figure S9B and Video S1). A pictorial representation of **1** resulting from molecular docking in the Glu97 region shows the noncovalent interaction with the amino acid residues in the TMV channel (Figure 8).

Conclusion

Systemic toxicity arising from chemotherapy with platinum anticancer agents can be attenuated by shielding them in delivery agents. Even though nanoparticle-based delivery vehicles offer significant advantages, there are several limitations including decreased efficacy and design complexity of synthetic nanoparticles as well as infection risk for mammalian viral vectors. Plant viruses, non-infectious to animal cells, are a viable alternative to mammalian viruses for drug delivery. They can be easily produced in a highly monodisperse form and have precisely controlled pattern-like structures made up of identical subunits. They also form an excellent platform for the modification of the viral interior and exterior surfaces. We systematically deciphered the mechanism for the loading of a promising platinum complex, phenanthriplatin, into viral nanoparticles. Following electrostatic interactions within the TMV core, phenanthriplatin preferentially binds to Glu97 residues to yield a covalent Pt-TMV complex. The current investigation informs the design of future drug analogs than can be efficiently encapsulated, stabilized in TMV, and delivered to the tumor site.

Experimental Section

General information

All reagents were purchased from Strem Chemicals, Sigma-Aldrich, or Alfa Aesar and used without further purification. Reactions were carried out under normal atmospheric conditions. ^1H NMR and ^{31}P NMR spectra were recorded at 22 °C on a Bruker AVANCE 400 NMR spectrometer and Varian 300 NMR spectrometer at the Massachusetts Institute of Technology Department of Chemistry Instrumentation Facility (MIT DCIF). Chemical shifts for ^1H NMR spectra were referenced to the residual signals of deuterated solvents (^1H NMR: DMSO at $\delta = 2.50$ ppm; D_2O , 4.79 ppm). The chemical shifts for ^{31}P NMR spectra were referenced to 85% phosphoric acid in a coaxial NMR tube. The spectrometer was used in *no lock* mode with the internal reference. Electrospray ionization mass spectrometry (ESI-MS) spectra were obtained on an Agilent Technologies 1100 Series liquid chromatography/MS instrument. Graphite furnace atomic absorption spectroscopic (GFAAS) measurements were taken on a Perkin Elmer Analyst 600 spectrometer. Distilled water was purified by passage through a Millipore Milli-Q Biocel water purification system (18.2 M Ω) with a 0.22 μm filter. MALDI mass spectra were obtained with a Bruker

Microflex MALDI-TOF mass spectrometer. MS/MS analysis was performed on an Orbitrap Fusion Lumos Tribrid Mass Spectrometer (Thermo Scientific) equipped with an EASY-nLC 1200 system (Thermo Scientific). Chromatographic separation was carried out using PepMap RSLC C18, 2 μm particle size, 100 $\text{\AA}$ pore size, 50 μm ID $\times$ 15 cm length column.

Uptake of **1 and its analogs by TMV**

Established protocols were used to produce wild-type TMV in *Nicotiana benthamiana* plants.⁴¹ **1** was allowed to react with AgNO_3 to give the aquated species. To 695 μL of a TMV solution (0.0376 μM) in potassium phosphate buffer (pH 7.8, 10 mM), were added 55 μL of 10 mM solution of **1** and its analogs with vortexing. The final concentration of Pt species was 640 μM (~17,000 fold higher than the concentration of TMV). The reaction mixture was incubated for 15 min in the dark, spin-dialyzed using a MWCO (30,000 Da), and washed three times with 0.5 mL potassium phosphate buffer (pH 7.8, 10 mM). The retained viral suspensions were diluted to 1 mL using potassium phosphate buffer (pH 7.8, 10 mM) and then analyzed by AAS to estimate the amount of Pt per TMV. The concentration of TMV was determined by UV-vis spectroscopy, and the platinum content was measured by using AAS. Kinetics of uptake measurements were performed by a similar procedure.

Determination of pK_a by potentiometry

Potentiometric studies were carried out using a Mettler Toledo potentiometer fitted with a combined microglass pH electrode. The electrode was calibrated with pH standards. The NaOH aqueous solution, standardized against potassium hydrogen phthalate, had a concentration of 0.102 M. In a typical determination, an aqueous solution (30 mL) of **4** (1 mm) in 0.058 M HNO_3 was titrated with the standardized NaOH solution. The temperature of the solutions (298 K) in the covered water-jacketed cell was maintained constant by a circulating water bath.

Reaction of **4 with NaOAc**

The reaction was carried out with an 8 mM solution of **4** and a 2 mM solution of NaOAc in phosphate buffer (10 mM, pH 7.8). The reaction mixture was analyzed by mass spectrometry to check for the formation of any adducts.

Molecular dynamics

Classical molecular dynamics simulations were performed by using the GPU-accelerated Desmond code (Schrodinger, LLC).⁶² Parameters were kept standard as in the OPLS force field, including for the platinum complex, which means the use of zero-order bonds to the metal center to constrain the input geometry during simulations, and this input geometry originated from a QM run. The structure was explicitly solvated in a water box (using the TIP3P model), then relaxed while the backbone was constrained. The integration step was set to 2 fs and the system was simulated for 10 ns. A Langevin thermostat was used and set to 300 K. The system was neutralized with chloride ions and the total size was about 120,000 atoms. A single starting position, remote from any glutamate residue, was used. Charges were neutralized using chloride ions.

Substitution kinetics

A 10.1 mg portion of **1** (0.020 mmol, 1 equiv) and 3.7 mg AgNO₃ (0.022 mmol, 1.1 equiv) were dissolved in D₂O (2 mL) and stirred for 40 h at room temperature. The mixture was then filtered through a 0.45 µm polyethersulfone filter to remove the AgCl precipitate. Various amounts of KOAc (10, 20, 30, 40, or 50 equiv) were dissolved in deuterated potassium phosphate buffer pD = 8.2 (1 mL, 20 mM). A 300 µL portion of the solution was quickly added to 300 µL KOAc/phosphate buffer, resulting in final reaction concentrations of 5 mM of **4**, 50-250 mM KOAc, and 10 mM phosphate buffer. The reaction progress was then monitored by ¹H NMR spectroscopy.

MALDI-MS analysis

A 2 µL amount of TMV or TMV treated with **1** and its analogs was drop-cast on a grid and mixed with sinapinic acid as a matrix. It was then air-dried quickly before placing in the mass spectrometer. To verify that the signal obtained for adducts is not due to ion-pairs in the chamber, the mass spectrum was recorded immediately after mixing **4** with TMV.

³¹P NMR Spectroscopic studies

A ³¹P NMR spectrum of phosphate buffer (10 mM, pH 7.8) was recorded first. To probe the interior pH of TMV, a 0.0376 µM TMV solution in phosphate buffer (10 mM, pH 7.8) was used. Spectra were recorded for incubation times of 15 min and 12 h.

Tryptic digestion MS and ETD-MS/MS analysis

To 695 µL of a TMV solution (0.0376 µM) in potassium phosphate buffer (pH 7.8, 10 mM) was added 55 µL of 10 mM solution of **4** while vortexing. The final concentration of **4** was 640 µM (~17,000-fold higher than the concentration of TMV). The reaction mixture was then incubated for 5 to 480 min in the dark and then spin-dialyzed using MWCO (30,000 Da) and washed three times with 0.5 mL potassium phosphate buffer (pH 7.8, 10 mM). The retained solutions were diluted to 1 mL using potassium phosphate buffer (pH 7.8, 10 mM). To a 50 µg portion of **4**-treated TMV solution was added 1 µg trypsin and MeCN at a final concentration of 75%. The solution was incubated for 30 min and evaporated for 20 min under vacuum centrifugation followed by resuspension in 50 µL of 10 mM triethylammonium acetate (TEAA) buffer (pH 6.5). The solution was further desalted using spin tips, where 50 µL of sample was washed with 100 µL TEAA and then with 50 µL 99% MeCN/TEAA. The sample was washed again with 50 µL of TEAA and finally eluted with 50 µL 99% MeCN/TEAA. The sample was then dried for 20 min under vacuum centrifugation. A similar procedure was used for digesting TMV alone. For MS analysis, the obtained samples were each diluted with 250 µL H₂O, shaken to dissolve the contents, and centrifuged to remove any large particles. The supernatants were further diluted 25 times with H₂O and a 50 µL portions were loaded in HPLC vials before being analyzed. A gradient mode elution was set up using as mobile phase 80% MeCN, 20% H₂O, and 0.1% formic acid. Whereas the gradient used for non-targeted mode was 2-60 %, 70 min, 300 nL/min, the gradient used for targeted mode was 2-60 %, 40 min, 300 nL/min. The peptides were further fragmented using ETD-MS/MS mode and the sequencing analysis was carried out using PEAKS studio 8 software.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

(A) Structures of phenanthriplatin (1) and its analogs used in this study (2-6). (B) Graphic representation of TMV showing longitudinal and cross-sectional orientations (images were created using PDB entry 2TMV in the Chimera software) (C) Uptake of 1-6 inside TMV measured by AAS.

Figure 2.

(A) Protic equilibria of **4**. (B) Potentiometric pH titration curve of **4** in 30 mL 0.058 M HNO_3 solution. The inflexion points for both steps (strong and weak acids, HNO_3 and **4**) were determined by the second derivative method and the half equivalence for step 2 (i.e., titration of the weak acid) gave a pK_a value of 5.57 ± 0.04 .

Figure 3.

MALDI mass spectra of TMV treated with **1** and **4** for 15, 270 and 1080 min. The parent peak due to the TMV coat protein appears at different m/z values; accurately determining masses of macromolecules is difficult by MALDI-TOF MS. The m/z values may also vary depending on instrument calibration. The coat proteins are assembled by noncovalent interactions that are broken up by laser irradiation, giving rise to mass values for individual coat proteins, which are all identical in the case of TMV (only one type of coat protein). The higher mass fragments, dimers or trimers, are not detected due to difficulty of ionization and limitation of mass detection range. The modification of TMV, however, can be readily determined by the relative increase in the mass. A peak appearing 224 mass units higher near the parent TMV coat protein is due to the formation of an ionic complex of coat protein with sinapinic acid, which is used as a matrix.

Figure 4.

The reaction between **4** and potassium acetate (here with a 30-fold excess) as monitored by ^1H NMR spectroscopy, showing the appearance of **7** over time, highlighted by the methyl resonance of the Pt-bound acetate group.

Figure 5.

(A) Interconversion of **1** and its derivatives. The reaction between **4** and KOAc was selected as a model for the loading of **1** in TMV at glutamate residues 97 and/or 106. (B) Time dependence of the reaction of **4** to form of **7** in the presence of 10, 20, 30, 40, and 50 equivalents acetate, plotted as the natural log of the molar fraction of **4** (X_4). Observed rate constants, k_{obs} , are calculated from the slope of linear regression. (C) k_{obs} at five concentrations used to calculate k_2 . Error bars, mean $\pm$ SD ($n = 3$). (D) Predicted concentration profiles of platinum-species using phenanthriplatin-TMV loading conditions. Experimental loading time is 30 min. Under the assumption that glutamate and acetate reactivities are equal, with $[\text{Glu}] = 2 \text{ M}$ and $[\text{Pt}]_0 = 0.46 \text{ mM}$, $k_2 = 0.016 \text{ M}^{-1}\text{min}^{-1}$, RT. (E) TMV uptake of **4** incubated for 1-480 min.

Figure 6.

(A) Targeted mass spectrum of the tryptic digest of **4**-treated TMV showing platinated 20-mer peptide, IIEVENQANPTTAETLDATR, for which signals from m/z 864.40 to m/z 866.40 are obtained. This peptide has a charge of +3. (B) ETD-MS/MS spectra of the peptide of interest showing the selective platination of Glu97, as correlated by the appearance of peaks corresponding to the peptide fragments.

Figure 7.

(A) Structures of hydroxido-quinoplatin and hydroxido-pyriplatin and their loading into TMV. (B) Loading of **8** and **9** in TMV. For comparison, loading of **1** and **4** in TMV is also shown. (C) The interior part of the coat protein showing hydrophobic Pro102 and Thr103. These amino acid residues can interact non-covalently with the phenyl ring in **4**, which can contribute to the stabilization of phenanthriplatin in viral nanoparticles.

Figure 8.

Graphic representation of **4** non-covalently bound on the inner surface of TMV, through interaction with Glu97. The illustration was obtained by molecular docking of **4** to a Glu97 residue belonging to a partial model of TMV (nine CPs (3×3) taken from PDB entry 2TMV), using Glide from Maestro Schrodinger.⁶¹ Visualization and electrostatic potential map were obtained using the same software (electronegative regions in red, electropositive ones in blue). A close-up on the right panel highlights the negative region due to the Glu97 residues, for which the upper row is boxed. The close-up also shows a row of Glu106 residues, which are more deeply buried in the structure.

Scheme 1.

Aquation of **1** and the subsequent reaction with acetate. phentd = phenanthridine