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A first-in-class and a fished out anticancer platinum compound: *cis*-$[PtCl_2(NH_3)_2]$ and *cis*-$[PtI_2(NH_3)_2]$ compared in their reactivity towards DNA model systems

Domenica Musumeci,[1,2] Chiara Platella,[1] Claudia Riccardi,[1] Antonello Merlino,[1,2] Tiziano Marzo,[3,4] Lara Massai,[3] Luigi Messori,[3] Daniela Montesarchio[1*]

1 *Department of Chemical Sciences, University of Napoli Federico II, Via Cintia, 21, I-80126 Napoli, Italy*

2 *Institute of Biostructures and Bioimages, CNR, Via Mezzocannone 16, I-80100 Napoli, Italy*

3 *Department of Chemistry "Ugo Schiff", University of Firenze, Via della Lastruccia, 3-13, I-50019 Sesto Fiorentino, Italy*

4 *Department of Chemistry and Industrial Chemistry, University of Pisa, via Moruzzi, 13, 56124 Pisa, Italy*

*Corresponding Author's E-mail: daniela.montesarchio@unina.it;

Phone: +39 081 674126; Fax: +39 081 674090.

Abstract

Contrarily to what believed for many years, *cis*-$PtI_2(NH_3)_2$, the diiodido analogue of cisplatin, displays high *in vitro* antiproliferative activity toward a set of tumour cell lines, overcoming resistance to cisplatin in a platinum-resistant cancer cell line. In the context of a general reappraisal of iodinated Pt(II) derivatives, aiming at a more systematic evaluation of their chemical and biological profile, here we report on the reactivity of *cis*-$PtI_2(NH_3)_2$ with selected DNA model systems, in single, double strand or G-quadruplex form, using cisplatin as a control. A combined approach has been exploited in this study, including circular dichroism (CD), UV-visible spectroscopy and electrospray mass spectrometry (ESI-MS) analysis. Data reveal that *cis*-$PtI_2(NH_3)_2$ shows an overall reactivity towards the investigated oligonucleotides significantly higher than cisplatin.

1. Introduction

Cisplatin (*cis*-$[PtCl_2(NH_3)_2]$, hereafter indicated as CDDP) is one of the most widely employed chemotherapeutic agents, being highly effective in the treatment of several human tumors. Unfortunately, its use is restricted because of severe dose-limiting side effects such as nephrotoxicity, neurotoxicity, ototoxicity and myelosuppression.[1] Therefore, in the search of innovative metal-based drugs, great interest is devoted to the design and synthesis of novel and even unusual CDDP analogues displaying a wide range of anticancer activities and, concomitantly, overcoming CDDP side effects and resistance.[2]

Among the plethora of Pt-based potential drugs which have been described in the literature, the iodide platinum complexes represent a valuable example. Though strictly related to CDDP, these derivatives did not attract great attention as prospective pharmacological agents in early research studies on platinum chemistry. In fact, it was long believed that the iodide analogues of classical anticancer platinum(II) compounds had poor biological activity due to the higher stability in aqueous solution, and thus lower reactivity, of the Pt-I linkage compared to the Pt-Cl bond.[3] Moreover, in the first studies on the anticancer potential of *cis*-$[PtI_2(NH_3)_2]$, *i.e.*, the iodide analogue of CDDP, poor activity was reported in a standard animal model,[4] thus discouraging further investigations on this and related iodide platinum complexes for the successive 40 years.

Only recently these compounds have been reconsidered. Indeed, several *cis* and *trans*-configured iodide Pt(II) complexes, carrying two differently substituted amine ligands, have been investigated.[5] Interestingly, remarkable and unexpected *in vitro* antiproliferative properties, in some cases superior to that of CDDP, have been disclosed, associated with a peculiar mechanism of action towards representative biomolecules. For these Pt compounds, the nature of the amine ligands seem to play a crucial role in determining their reactivity with the biological targets, as well as their overall biological profile.

In this frame, *cis*-$[PtI_2(NH_3)_2]$ has proved to be an extremely intriguing compound. Indeed, some of us recently showed that it reacts with proteins or ammonia ligands.[6,7] The X-ray structure of the adduct formed in the reaction between the model protein hen egg white lysozyme and *cis*-$[PtI_2(NH_3)_2]$ also revealed peculiar structural features: the $[PtI_2(NH_3)]$ fragment binds close to the His15 side chains, adopting two different conformations.[7] This Pt(II) derivative has been therefore classified among the so-called "rule-breakers", *i.e.* CDDP analogues which seem to have a binding mechanism different from CDDP, even if they satisfy the general requirements for anticancer Pt(II) complexes deriving from the structure–activity relationships formulated shortly after the discovery of CDDP.[4]

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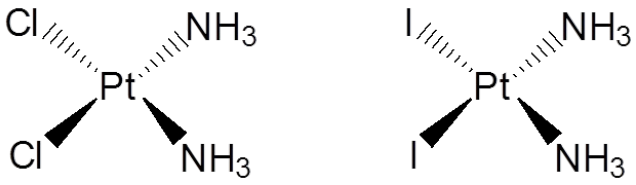
Conversely, biophysical and computational studies showed that the behavior of *cis*-$[PtI_2(NH_3)_2]$ towards DNA is analogous to that of CDDP and its activation occurs upon displacement of both iodido ligands with water molecules.[7] Notably, *in vitro* biological assays proved that *cis*-$[PtI_2(NH_3)_2]$ is able to produce cytotoxic effects comparable or even greater than those caused by CDDP, also overcoming resistance in a cisplatin-resistant cancer cell line, where it is internalised in far greater amounts than in healthy and cisplatin-sensitive cancer cells. When investigated in its interaction with calf thymus DNA using a variety of methods (*e.g.*, ethidium bromide-based fluorescence intensity assays, DNA unwinding experiments and UV-melting analysis), it showed a reactivity very similar to that documented for CDDP, distorting and destabilizing the double helix of DNA approximately to the same extent as cisplatin. Apparently, the major difference between these derivatives consists in a slower kinetics of platinum association to DNA, but it is suggested that the same adducts are formed.[7]

These findings have stimulated growing interest in understanding at a molecular level the mode of action of the iodide platinum complexes, studying in detail their interaction with various biological targets, and especially DNA, which is recognized as the main effective target for Pt(II)-based complexes. Aiming at a deeper insight into its chemical and biological profile, in the present work the binding of *cis*-$[PtI_2(NH_3)_2]$ with model single-strand, duplex and G-quadruplex DNA has been explored under pseudo-physiological conditions in comparison with CDDP by means of CD, UV-vis and ESI-MS data.



Scheme 1. Molecular structures of *cis*-$[PtCl_2(NH_3)_2]$ and *cis*-$[PtI_2(NH_3)_2]$.

2. Results and Discussion

2.1 Selection and preparation of the oligonucleotide model systems

In order to study the interaction of *cis*-$[PtI_2(NH_3)_2]$, hereafter indicated as PtI2, with different DNA models, and to compare its behaviour with that of CDDP, oligodeoxyribonucleotides (ODNs) with selected sequences, in single strand, duplex and G-quadruplex form, have been incubated in parallel experiments with the two metal complexes; the resulting mixtures have been then analysed over time using spectroscopic (CD, UV-vis) and spectrometric (ESI-MS) techniques.

Assuming that the mechanism of action of PtI2 is similar to that of CDDP, which is able to form stable covalent adducts with contiguous guanines on a DNA strand,[8] the following DNA model

systems have been here considered: a) a single strand 12-mer ODN, containing one "GG" box in the middle of the sequence; b) a 12-mer ODN, complementary to the previous sequence, containing two "GG" boxes, one at each end; c) a 12-mer duplex, overall containing three "GG" boxes; d) a single strand, guanine-free 24-mer, here selected as a negative control. Furthermore, a 26-mer G-rich ODN, able to fold into a stable unimolecular G-quadruplex structure, has been also included in this study to evaluate the behaviour of the here examined Pt(II)-based complexes towards this unusual nucleic acid structure, which is a potential target in anticancer strategies.[9]

In particular, the following sequences have been selected and synthesized:

- d($^{5'}$CCTCT**GG**TCTCC$^{3'}$) (**1**);
- d($^{5'}$**GG**A**G**ACCA**G**A**GG**$^{3'}$) (**2**);
- d($^{5'}$TCACACACACACACACACACTT$^{3'}$) (**3**);
- d[$^{5'}$(TTA**GGG**)$_4$TT$^{3'}$] (**tel$_{26}$**).

These synthetic ODNs have been chosen on the basis of their structural and/or biological relevance, and proved to be valuable models for the study of metal-DNA interactions also in recent works by our group.[10] In particular, the here studied duplex, obtained mixing the complementary sequences **1** and **2** in 1:1 ratio (**1/2**), was investigated in a previous crystallographic study, forming a stable 1:1 adduct with CDDP;[11] in turn, the ODN sequence **tel$_{26}$** is a well-studied model of the 3'-end single strand overhang of the human telomeric DNA, known to self-assemble into stable G-quadruplex conformations.[12]

In order to obtain the thermodynamically most stable conformations, the duplex structure **1**/**2** and the single strand **tel$_{26}$** have been first subjected to annealing (see Experimental Section). The formation of the expected secondary structures has been verified by CD, CD-melting and UV-melting analyses (data not shown).

In all our experiments, only neutral pH conditions have been analysed, considering that within nuclei the pH is strictly buffered at ca. 7.[13] Thus, in order to mimic a pseudo-physiological environment, in the spectroscopic studies the Pt/DNA mixtures have been investigated in a 10 mM phosphate buffer, 50 mM NaCl (or KCl for the G-quadruplex) solution taken at pH = 7.0 and 37 °C.

2.2 CD studies

2.2.1 Study on the interaction of PtI2 and CDDP with DNA single strands

In order to determine how and to what extent the platinum complexes PtI2 and CDDP interact with single-strand DNA, ODNs **1**, **2** and **3**, respectively containing one, two or no "GG" box, have been incubated in parallel experiments with each platinum compound in 2 and 10 mol excess with respect to the single strand, and the resulting systems analysed by CD spectroscopy.

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The CD spectrum of **1** dissolved in a pseudo-physiological solution shows a positive band at 275 nm and a negative one at 240 nm (Fig. 1); in the presence of 2 eq of CDDP, no relevant difference is found, whereas a decrease of the 275 nm positive band has been observed in the presence of the same amount of PtI2 (Fig. 1a). On increasing the Pt/DNA ratio from 2:1 to 10:1, major changes in the CD spectrum of **1** have been detected, especially upon treatment with PtI2, for which the net reduction of the 275 nm band is accompanied by a 6 nm bathochromic shift, while the negative band at 240 nm almost disappears (Fig. 1b).

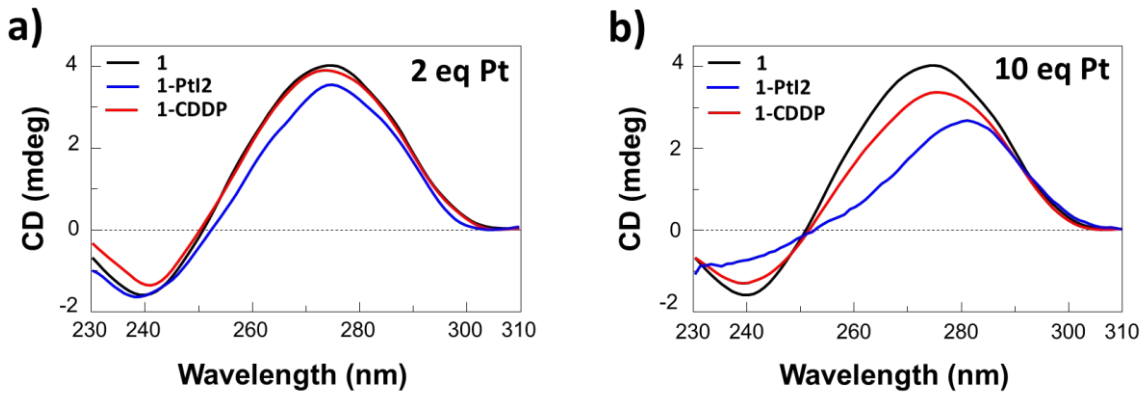

Figure 1. Overlapped CD spectra of ODN **1** (2 μM, 10 mM phosphate buffer/50 mM NaCl), in the absence (black lines) and presence of 2 eq (panel **a**) and 10 eq (panel **b**) of PtI2 (blue) or CDDP (red), recorded after 72 h incubation.

The effect of both Pt complexes on the conformation of ODN **2**, containing two "GG" boxes, is more relevant than on ODN **1** (Fig. 2). As a general behaviour, PtI2 is found to be more active than CDDP also in the reaction with ODN **2**. In particular, upon addition of 10 eq of PtI2, very marked changes in the CD profile of ODN **2** have been observed, with a peculiar inversion of the positive bands first present in the range 250-300 nm (Fig. 2b). To better investigate this process, a CD-monitored titration experiment has been carried out: upon increasing the PtI2/ODN ratio all the CD bands gradually decrease; the inversion of the 250-300 nm bands occurs when more than 4 eq of PtI2 are added, while the 240 nm band totally disappears upon addition of 6 eq of PtI2 (Fig. S1).

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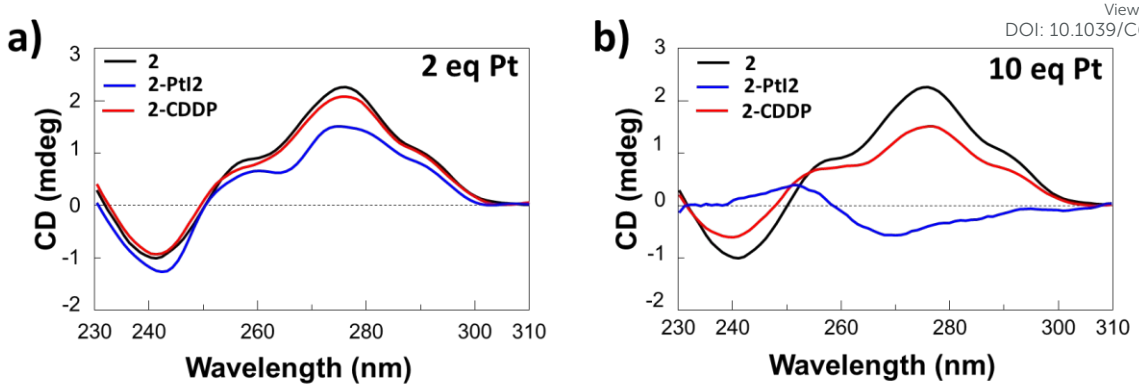

Figure 2. Overlapped CD spectra of ODN **2** (2 μM, 10 mM phosphate buffer/50 mM NaCl), in the absence (black lines) and presence of 2 eq (panel **a**) and 10 eq (panel **b**) of PtI2 (blue) or CDDP (red), recorded after stabilization of the CD signal (48 h incubation for PtI2 and 72 h for CDDP).

As in the case of ODN **1** and **2**, no appreciable changes in the spectra of ODN **3** are found when this ODN is mixed with 2 eq of CDDP and PtI2 (Fig. 3a), and, differently from the previous cases, only small effects are detected upon treatment of **3** with 10 eq of CDDP (Fig. 3b). On the contrary, relevant effects are observed upon addition of 10 eq of PtI2, which induces a significant decrease in the positive band of **3**, associated with a 4 nm red-shift (Fig. 3b). This evidence suggests that PtI2 and to a minor extent also CDDP, when added in large excess, are able to interact also with ODN sequences not containing guanines, probably exploiting the binding with adenines (10 adenine residues are indeed present in ODN **3**), as reported in the literature for other platinum complexes.[1] However, both PtI2 and CDDP are far less reactive towards ODN **3** than towards **1** and **2**, confirming that guanines are the preferred - although not the exclusive - binding sites of Pt(II)-based complexes.

Taken together, these experiments show that PtI2 is more reactive than CDDP towards all the investigated ODN single strands, but also less selective in recognizing guanine-containing ODNs.

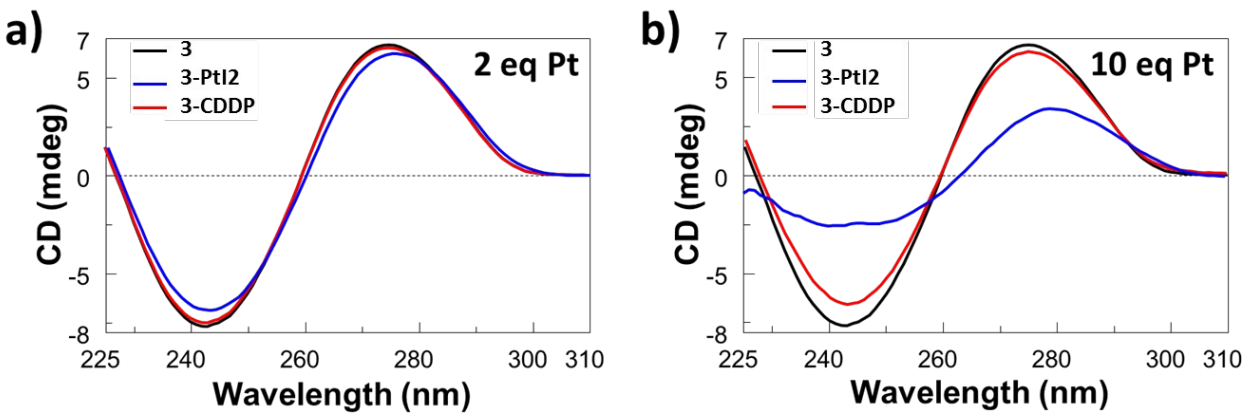

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Figure 3. Overlapped CD spectra of ODN **3** (2 μM, 10 mM phosphate buffer/50 mM NaCl), in the absence (black lines) and presence of 2 eq (panel **a**) and 10 eq (panel **b**) of PtI2 (blue) or CDDP (red), recorded after 72 h incubation.

Subsequently, the capability of platinated ODNs **1** and **2** to form duplex structures with their complementary strands has been also investigated. To this purpose, CD spectra have been carried out using a *tandem cell*, recording the *sum* and *mix* spectra of the two components (*i.e.*, respectively the spectrum obtained by maintaining the two systems, the platinated ODN and its complementary strand, in separate compartments of the cell, and the one obtained after mixing the two solutions). Considering on one side the platinated ODNs **1** or **2**, obtained by previous incubation of each single strand with 2 eq of PtI2, and, on the other, untreated ODNs **2** or **1**, respectively, as counterparts, the differences between the *sum* and *mix* spectra prove that duplex structures are formed in both cases (Fig. S2a and S2b). The two hybrid structures display conformations very similar to each other and to that of the preformed duplex incubated with PtI2 under the same conditions [here indicated as (**1**/**2**)-PtI2, described in section 2.2.2.], and only slightly different from the untreated duplex **1**/**2** (Fig. 4a and 4b).

Analysing the CD spectrum and CD-melting curve of untreated duplex **1**/**2** (black lines) in comparison with the various duplex structures obtained upon platination of either the single strands or the preformed duplex (Fig. 4a and 4c) with PtI2, it can be observed that all the platinated duplexes, and above all **2**-PtI2/**1**, are somehow destabilized with respect to the natural one (Fig. 4d). With a higher excess of the platinum complex PtI2 (10 eq), the platinated ODN **2** undergoes a deep conformational change (Fig. 2b, blue line). This behaviour could be attributed to the formation of multiple Pt-adducts, hampering the hybridization of **2** with its complementary strand, as apparent from the comparison of their *sum* and *mix* CD spectra, which are almost superimposable (Fig. S3a). Consistently, under these conditions, the CD-monitored melting curve for the system **2**-PtI2/**1** does not show the sigmoidal shape characteristic of a duplex structure, but only a drift of the CD signal (Fig. S3b).

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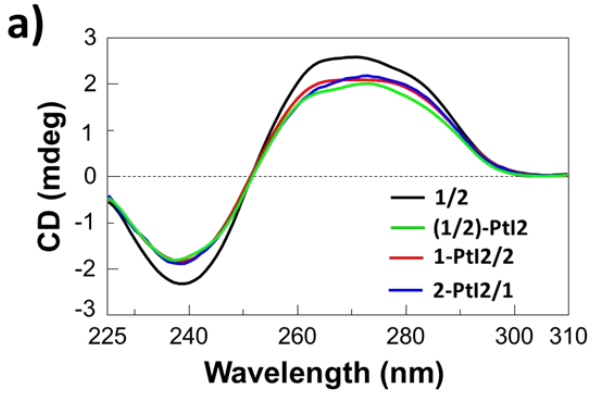
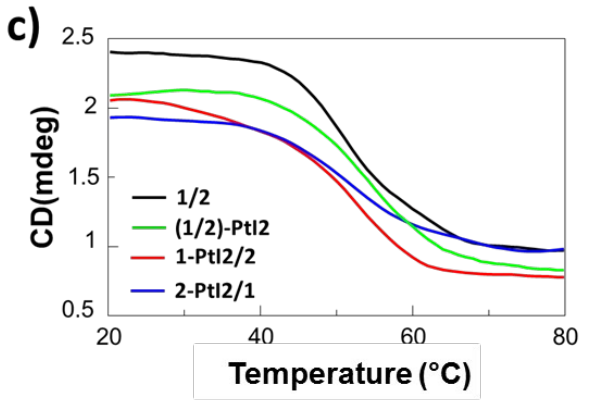


b)

	Positive band λ_{max}(nm)/CD (mdeg)	Negative band λ_{min}(nm)/CD (mdeg)
Untreated duplex **1**/**2**	270 / 2.57	239 / -2.34
(1/2)-PtI2	273 / 1.99	237 / -1.84
1-PtI2/2	270 / 2.08	238 / -1.85
2-PtI2/1	273 / 2.17	238 / -1.90



d)

	$\Delta CD_{(20\text{-}80°C)}$ (mdeg)	T_m (°C) ± 1
Untreated duplex **1**/**2**	1.46	54
(1/2)-PtI2	1.27	53
1-PtI2/2	1.28	53
2-PtI2/1	0.95	51

Figure 4. Overlapped CD spectra (panel **a**) and CD-melting curves (panel **c**) of the following duplex structures: untreated **1**/**2** (black lines), (**1**/**2**)-PtI2 (green), **1**-PtI2/**2** (red) and **2**-PtI2/**1** (blue), recorded at 1 μM ODN concentration and 2 eq of the platinum complex PtI2 under pseudo-physiological conditions. In the tables, CD (panel **b**) and CD-melting (panel **d**) data for the four duplex structures are reported.

The same hybridization experiments have been performed also on ODNs **1** and **2** platinated with 2 and 10 eq (Fig. S4) of CDDP. In all cases each platinated ODN preserves its ability to hybridize with the corresponding complementary strand; only if 10 eq of CDDP are used, the resulting duplex structures (**1**-CDDP/**2** and **2**-CDDP/**1**) are conformationally different from the untreated duplex **1**/**2** (Fig. 5a and 5b) and show lower T_m and ΔCD (*i.e.* difference between the CD band intensity determined at its maximum at 20 °C and that at 80 °C) values in CD-melting experiments (Fig. 5c and 5d); overall, these effects are comparable to those obtained treating the same systems with 2 eq of PtI2 (*cfr.* Fig. 4 and 5).

From these data, it appears that the reduction in the T_m values is strictly related to the decrease of the CD bands intensity. Therefore the lower thermal stability of all the platinated duplex structures here investigated, obtained either with PtI2 or CDDP, compared with the untreated duplex **1**/**2**, can be explained essentially in terms of loss of stacking interactions and/or partial unwinding of the double helical structure due to Pt-adducts formation. These destabilizing effects are clearly not fully

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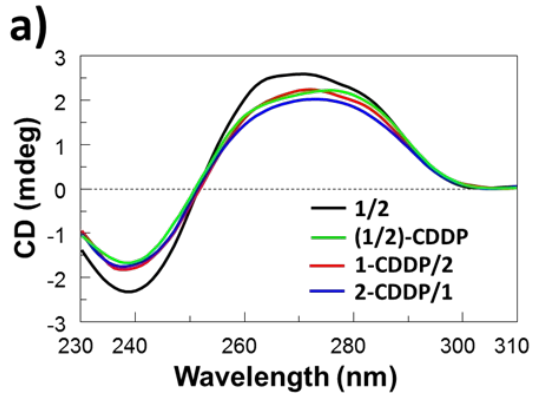
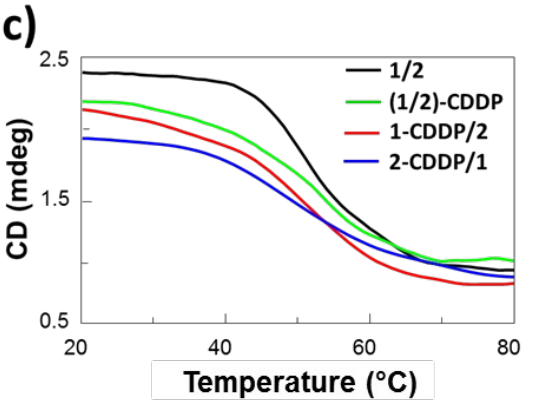
counterbalanced by other effects due to Pt coordination, *e.g.*, introduction of positive charges, hydrophobic interactions, etc., which, in turn, typically produce an overall stabilization.



b)

	Positive band λ_{max}(nm)/CD (mdeg)	Negative band λ_{min}(nm)/CD (mdeg)
Untreated duplex **1/2**	270 / 2.57	239 / -2.34
(1/2)-CDDP	276 / 2.21	238 / -1.69
1-CDDP/2	272 / 2.23	238 / -1.84
2-CDDP/1	273 / 2.02	238 / -1.77



d)

	$\Delta CD_{(20\text{-}80°C)}$ (mdeg)	T_m (°C) ± 1
Untreated duplex **1/2**	1.46	54
(1/2)-CDDP	1.19	52
1-CDDP/2	1.28	51
2-CDDP/1	1.00	53

Figure 5. Overlapped CD spectra (panel **a**) and CD melting curves (panel **c**) of the following duplex structures: untreated **1**/**2** (black lines), (**1**/**2**)-CDDP (green), **1**-CDDP/**2** (red) and **2**-CDDP/**1** (blue), registered in a *tandem cell* at 1 μM ODN concentration and 10 eq of CDDP in pseudo-physiological conditions. In the tables, CD spectral (panel **b**) and CD-melting (panel **d**) data for the four duplex structures are reported.

2.2.2 Study on the interaction of PtI2 and CDDP with a model DNA duplex

In order to compare the reactivity of PtI2 and CDDP towards a DNA double helix, a 2 μM solution of preformed duplex **1**/**2** has been incubated in parallel experiments with each platinum complex in 2 and 10 mol excess with respect to the duplex. CD spectra of the resulting mixtures have been recorded at different reaction times until stabilization of the signal, occurring after 48 h. PtI2 always proves to be more reactive than CDDP in our experiments: in fact, when 2 eq of this Pt complex are added to the duplex, a decrease in the intensity of the duplex CD bands is observed, whereas CDDP, under the same conditions, does not produce detectable changes (Fig. 6a). Upon addition of a higher excess (10 eq) of both Pt complexes, similar conformational changes – even if more marked for PtI2 - are produced on the duplex, suggesting that these metal complexes display similar interaction

modes with this target (Fig. 6b).

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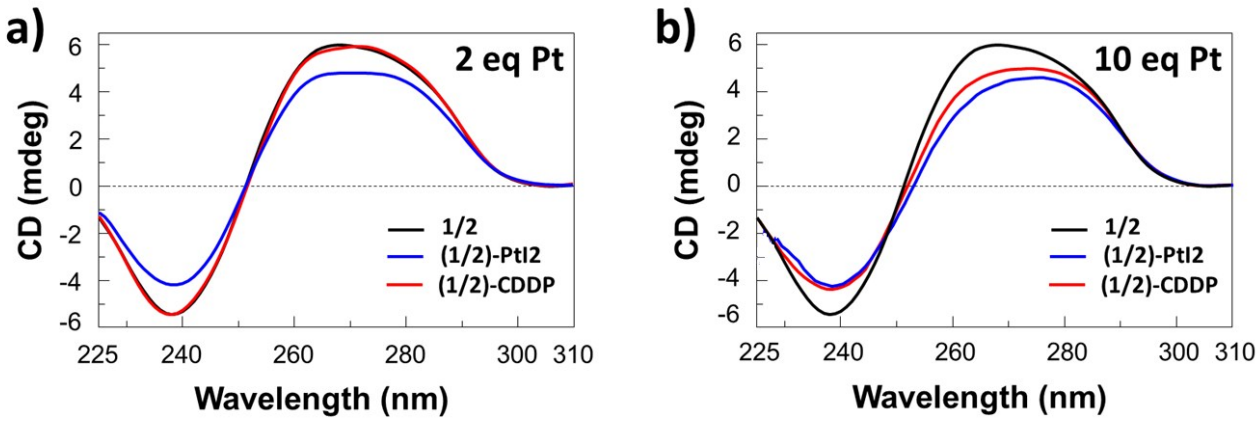
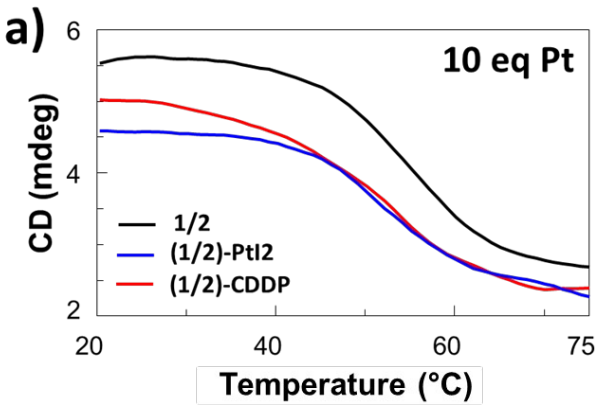


Figure 6. Overlapped CD spectra of duplex **1/2** (2 μM, 10 mM phosphate buffer/50 mM NaCl), in the absence (black line) and presence of 2 eq (panel **a**) and 10 eq (panel **b**) of PtI2 (blue) and CDDP (red), recorded after 48 h incubation.

For all the studied systems, *i.e.* the preformed duplex **1**/**2** incubated with either 2 or 10 eq of each platinum complex, CD-melting experiments have been carried out as well (Fig. S5 and Fig. 7, respectively). The obtained data show that these platination treatments lead to a small decrease of the T_m values for both complexes (for PtI2, observed with both 2 and 10 eq; for CDDP, only with the 10 eq treatment), thus producing a similar effect on the overall stability of the duplex structures. In addition, the variations of the CD signal at 270 nm (ΔCD) with the temperature relative to the platinated duplexes are lower than that of the untreated duplex, being always smaller for the PtI2 than for the CDDP treatment, and typically decrease as the Pt complexes concentration increases (Fig. S5b and Fig. 7b).



b)

	$\Delta CD_{(20\text{-}75\ °C)}$ (mdeg)	T_m (°C) ± 1
Untreated duplex **1/2**	2.84	55
(1/2)-PtI2	2.15	52
(1/2)-CDDP	2.63	53

Figure 7. Overlapped CD-melting curves of the untreated duplex **1/2** (black line) and of the duplex

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(2 μM) incubated with 10 eq of PtI2 (blue) and CDDP (red) in 10 mM phosphate buffer/50 mM NaCl, recorded at 270 nm with a temperature gradient of 1 °C/min (panel **a**); table of the resulting CD-melting data (panel **b**).

From this analysis, it can be deduced that the incubation with these platinum complexes generally causes a small, but detectable destabilization of the duplex **1/2**, with loss of some stacking interactions and/or partial unwinding of the double helical structure, especially when it is exposed to a large excess of Pt complex. This finding is in agreement with the formation of covalent Pt-DNA adducts, which produce local distortions in the double helix, as demonstrated in previous reports on CDDP and other platinum-based drugs.[8]

2.2.3 Study on the interaction of PtI2 and CDDP with the tel_{26} G-quadruplex

Following the experiments carried out on single strand and duplex DNA, we have also investigated the reactivity of PtI2 and CDDP towards the G-quadruplex (G4) structure adopted by an ODN sequence taken from the human telomeric DNA. To this purpose, a 2 μM solution of **tel_{26}** G4 has been incubated with each Pt complex at 2 and 10 mol excess, respectively, with respect to the G4. The CD spectra of the resulting mixtures have been recorded at different reaction times until stabilization of the signal, occurring after 48 h (Fig. 8).

The conformation adopted by **tel_{26}** G4, after annealing under the here used conditions, is essentially a hybrid-2 type structure, as confirmed by comparison of its CD spectrum with literature reports.[14] Its stability has been analysed by CD-melting experiments, monitoring the CD signal changes at 290 nm registered upon increasing the temperature in the range 20-85 °C (Fig. 9).

The effect of the two Pt complexes on the G-quadruplex structure is markedly different: while CDDP does not perturb the overall G-quadruplex conformation, even if added in large excess, PtI2 always causes significant conformational changes. Upon addition of 2 eq of the iodido complex, a moderate decrease of the maximum at 290 nm and of the shoulder at 270 nm is observed. With 10 eq added, a more marked decrease of the overall CD signal intensity, accompanied by a 2 nm red-shift of the 290 nm band, as well as appearance of a new, small positive band at 250 nm are detected, indicating that the initial conformation is sensibly perturbed, probably with formation of multiple, different G-quadruplex conformations at equilibrium (Fig. 8).

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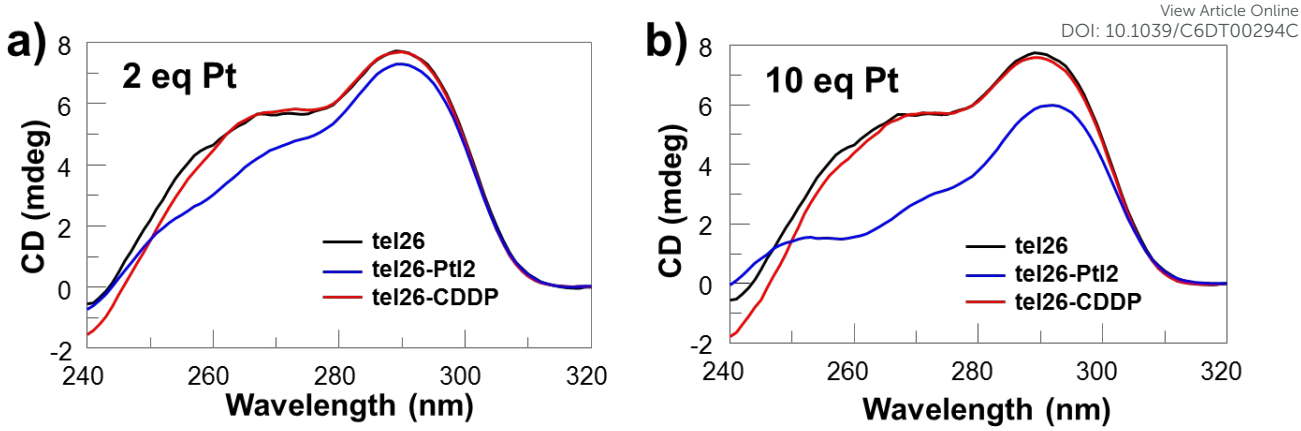

Figure 8. Overlapped CD spectra of **tel$_{26}$** (2 μM, 10 mM phosphate buffer/50 mM KCl) in the absence (black lines) and presence of 2 eq (panel **a**) and 10 eq (panel **b**) of PtI2 (blue) and CDDP (red), recorded after 48 h incubation.

When react with this ODN, CDDP and PtI2 are not able to coordinate the N7 atoms of guanines, involved in the Hoogsteen hydrogen bonds responsible for G-tetrads structuring. Thus it can be hypothesized that PtI2 – but not CDDP – coordinates alternative binding sites in the G-quadruplex, *e.g.* N1 or N7 of the adenines in the loops, indirectly interfering with G-tetrads stacking[15] and leading to a mixture of different conformations.

After 48 h incubation, CD-melting experiments (Fig. 9) have been performed on the systems G4/CDDP and G4/PtI2, both mixed in 1:10 ratio; also these results show that PtI2 interacts with its target causing major perturbations with respect to CDDP. In fact, while CDDP does not affect the CD-melting curve of the G-quadruplex, PtI2 causes a slight stabilization (ΔT_m = +2 °C) even if its ΔCD is reduced from 7.26 to 4.33. These data can be explained considering that the adduct formation produces relevant distortions in the G-quadruplex conformation, with loss of some stacking interactions in the G-tetrads assembly which is reflected in the lower ΔCD value compared with the untreated G4. In the case of PtI2, this destabilization is however fully counterbalanced by other contributions, such as the binding of the metal complex to adenines in the loops and/or partial neutralization of the negative charges of the ODN phosphates by the positive charge on Pt(II) centers, thus leading to more thermally stable structures.

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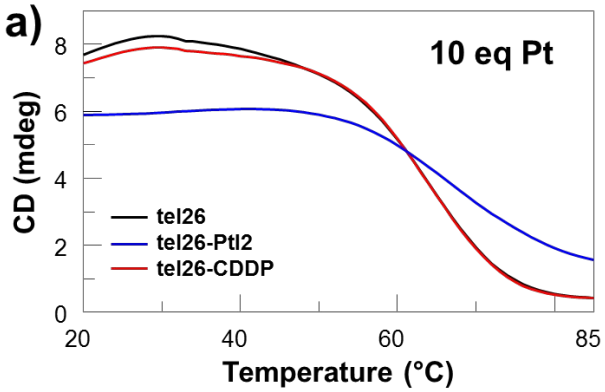

b)

	$\Delta CD_{(20\text{-}85\ °C)}$ (mdeg)	T_m (°C) ± 1
tel26	7.26	64
tel26-PtI2	4.33	66
tel26-CDDP	7.02	64

Figure 9. Overlapped CD-melting curves of **tel$_{26}$** (2 μM, 10 mM phosphate buffer/50 mM KCl) in the absence (black line) and presence of 10 eq of PtI2 (blue) and CDDP (red) after 48 h incubation, recorded at 290 nm with a temperature increase of 1 °C/min (panel **a**); table of the resulting CD-melting data (panel **b**).

Subsequently, CD experiments have been performed to determine whether the Pt complexes are able to induce G-quadruplex formation in the absence of G4-stabilising monovalent cations (Na^+ or K^+). Thus 10 eq of each Pt(II) complex have been added to a 2 μM solution of **tel$_{26}$** dissolved in 20 mM Tris-HCl (pH 7.2), which is a Na^+/K^+-free buffer unable to promote G-quadruplex self-assembly, and the mixtures analysed over time for 72 h. The CD spectra of the resulting mixtures have showed that none of the two complexes is able to promote, *per se*, G-quadruplex formation, in all cases causing only some conformational changes on **tel$_{26}$** (Fig. 10a and 11a).

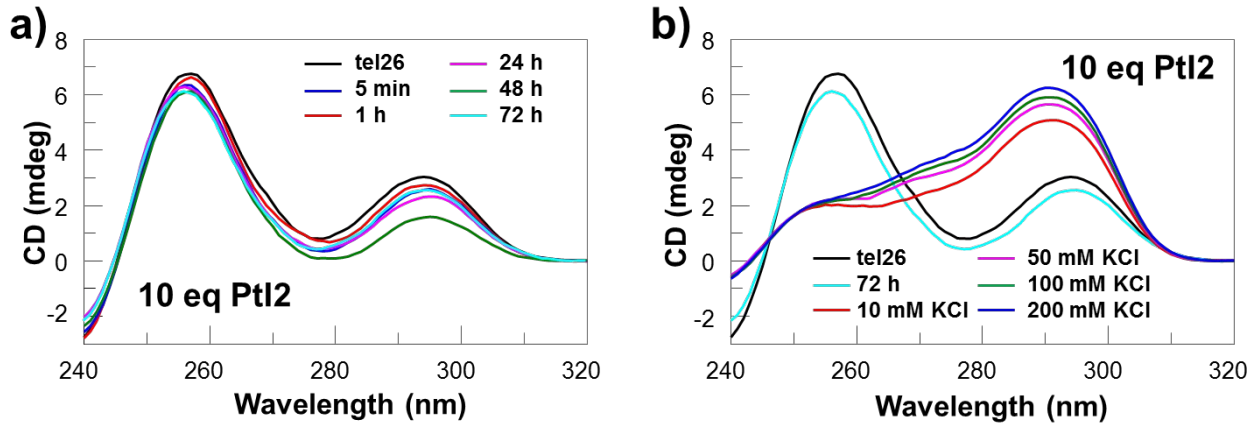

Figure 10. Overlapped CD spectra of **tel$_{26}$** (2 μM, 20 mM Tris-HCl pH=7.2) in the absence (black line) and presence of 10 eq of PtI2 (panel **a**). Spectra have been recorded 5 min (blue), 1 h (red), 24 h (magenta), 48 h (green) and 72 h (light blue) after addition of the complex; overlapped CD spectra of **tel$_{26}$** (2 μM, 20 mM Tris-HCl pH=7.2) in the absence (black line) or presence of 10 eq of PtI2 after 72 h incubation (light blue), recorded upon addition of increasing KCl amounts (panel **b**, see legend).

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Furthermore, to evaluate if the platinated **tel$_{26}$** single strand is still able to fold into a G-quadruplex structure when, successively, K^+ ions are added to the Tris-HCl solution, CD titration experiments with increasing amounts of K^+ have been carried out. Interestingly, for both the Pt(II) complexes, a G4 structure having a CD spectrum with a maximum at 291 nm (for PtI2) or at 290 nm (for CDDP) and a shoulder at 250 nm (for PtI2) or at 258 nm (for CDDP) is formed (Fig. 10b and 11b). Remarkably, the **tel$_{26}$** structures thus obtained at high K^+ concentration (100-200 mM) largely differ from the untreated **tel$_{26}$** G4, as well as from the structures resulting after incubation of the preformed **tel$_{26}$** G-quadruplex with PtI2 or CDDP (Fig. S6). These results suggest that PtI2 is able to interact with **tel$_{26}$** more markedly than CDDP, in a manner that sensibly depends on whether the platination initially occurs on the preformed G4 or on the unstructured strand; in the latter case, the platination deeply affects the G4 folding of the G-rich ODN, which, if left in a K^+-containing buffer, probably forms different G4 conformations (Fig. S6a).

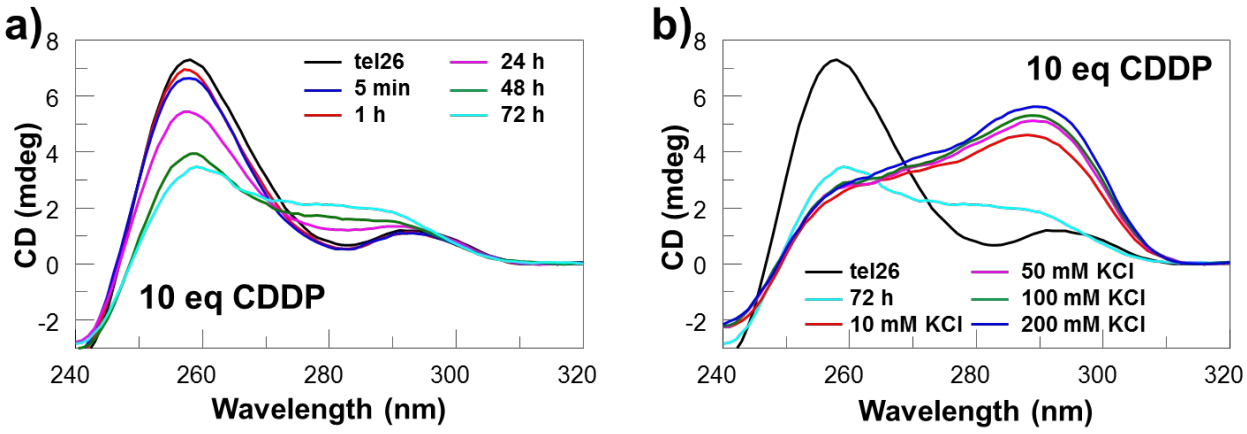

Figure 11. Overlapped CD spectra of **tel$_{26}$** in "non-G4" form (2 μM, 20 mM Tris-HCl pH=7.2) in the absence (black line) and presence of 10 eq of CDDP (panel **a**). Spectra have been recorded 5 min (blue), 1 h (red), 24 h (magenta), 48 h (green) and 72 h (light blue) after the addition of the complex; overlapped CD spectra of **tel$_{26}$** (2 μM, 20 mM Tris-HCl pH=7.2) in the absence (black line) or presence of 10 eq of CDDP after 72 h incubation (light blue), recorded upon addition of increasing KCl amounts (panel **b**, see legend).

2.2.4 CD data overview

From an overview of the CD data, it emerges that PtI2 is more reactive than CDDP, inducing higher conformational changes in all the investigated ODN systems in a dose-dependent manner (Fig. 12). From the comparison of the % variation of the CD signal at its maximum (ΔCD), indicative of the conformational changes showed for the studied ODNs, the following relative reactivity order can be drawn:

ODN **2** > ODN **1** ≈ duplex **1**/**2** > ODN **3** > **tel$_{26}$** for CDDP (both at 2 and 10 eq);

ODN **2** > duplex **1**/**2** ≈ ODN **1** > **tel$_{26}$** ≈ ODN **3** for PtI2 (at 2 eq);

ODN **2** > ODN **3** > ODN **1** > duplex **1**/**2** ≈ **tel$_{26}$** for PtI2 (at 10 eq).

In all our experiments CDDP and PtI2 show higher affinity for ODN **2** (containing 2 sets of 2 contiguous guanines in the sequence, plus 2 isolated guanines) than for ODN **1** (2 contiguous guanines in the sequence) and ODN **3** (0 guanines). If added in a large excess, PtI2 displays a strong affinity also towards the guanine-free ODN **3**, thus showing a lower sequence selectivity than CDDP. Moreover, both the platinum complexes prove to be very poorly reactive towards **tel$_{26}$** G4 (having the N7 of guanines involved in hydrogen bonds) unless, in the case of PtI2, the complex is added in a large excess. All these findings confirm that the preferred binding sites for the two Pt(II)-based complexes are guanines; when these are not present, or are not available for coordination (as in the case of the G4-structured, G-rich **tel$_{26}$**), alternative nucleobases can be targeted, as adenines or even cytosines. This clearly explains the higher reactivity of PtI2 and CDDP for ODN **3** - with a large content in adenines and cytosines – than for G4 **tel$_{26}$**.

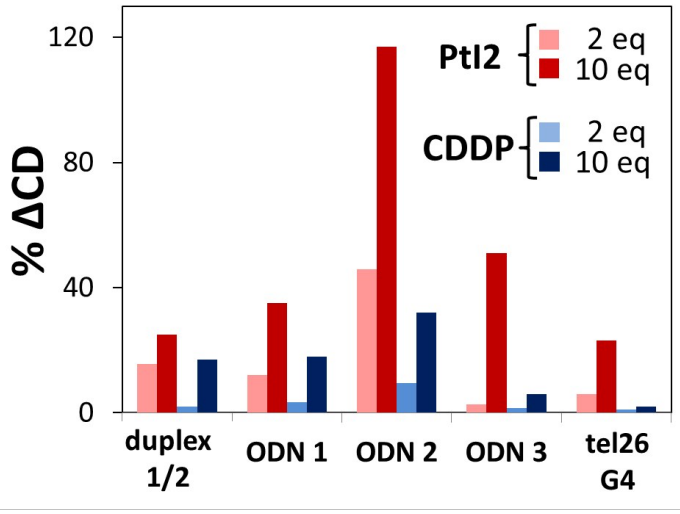

Figure 12. Reduction (in %) of the ODNs CD signals at their maxima, calculated with respect to the CD values of the untreated ODN (2 μM), in the presence of 2 and 10 eq of the Pt complex here investigated. The errors associated to the percentage values are estimated to be within ±3%.

2.3 UV studies on the interaction of PtI2 and CDDP with DNA model systems

UV-vis spectroscopy has been used first to explore the behaviour of PtI2 and CDDP alone in the adopted experimental conditions, monitoring the evolution over time of their characteristic UV-vis absorption bands. This allows to obtain information about the ligands exchange processes.

UV-vis spectra of the complexes freshly dissolved in a 10 mM phosphate buffer/50 mM NaCl, pH=7.0 solution show characteristic bands in the region 210 - 400 nm (Fig. S7). In particular, PtI2

and CDDP display strong absorption bands, centred at 236 and 217 nm, respectively identified as charge-transfer bands, and a weaker band at ca. 300 nm originated from the d-d transitions of the square planar Pt^{2+}, in accordance with literature studies.[5b,5c,6,16]

Immediately after the dissolution of the two Pt complexes, a small hypsochromic shift of the long wavelength bands occurs, ascribable to the progressive replacement of the halide ligands with water molecules. Notably, in the first 3 h monitoring, both the 236 and 299 nm bands of PtI2 show a hyperchromic effect, accompanied by a 2 nm bathochromic and hypsochromic shift, respectively (Fig. S8). In contrast, both the 217 and 301 nm bands of CDDP show hypochromic effects, also undergoing a 1 nm hypsochromic shift (Fig. S9), thus revealing a somehow different behaviour of the two Pt complexes. This difference could be also attributed to the presence of 1% DMSO in the PtI2 solution, introduced to fully dissolve the complex at the studied concentration, which can compete with water in the ligand exchange process. After 24 h incubation, increasing scattering phenomena in the UV-vis absorption spectra have been observed in both cases.

In order to confirm the interaction of the platinum complexes with DNA, UV-vis spectra of the ODNs, either as single strands (**1**, **2** and **3**) or in a structured form (duplex **1**/**2** and G4 $\mathbf{tel_{26}}$), incubated in parallel with PtI2 and CDDP, have been registered (Fig. S10 and S11).

The Pt-complex/ODN solutions (Pt/DNA, 10:1 molar ratio) have been analysed over time, monitoring the evolution of the characteristic ODN band at ca. 260 nm. As a general trend, the UV spectra of these ODN systems showed very small hypochromic effects upon Pt treatment, slightly more pronounced in the case of PtI2.

In order to investigate the thermal stability of the platinated duplex and G-quadruplex systems, UV-melting experiments have been carried out on the ODNs treated with 10 eq of either PtI2 or CDDP under the same experimental conditions (Fig. 13). The products of the reaction of these systems with CDDP show small-to-null differences in the Tm values when compared to the untreated duplex [Tm for (**1**/**2**)-CDDP = 54 °C; Tm for the untreated duplex = 55 °C; Tm for $\mathbf{tel_{26}}$-CDDP and for the untreated $\mathbf{tel_{26}}$ were both equal to 64 °C], essentially in accordance with the corresponding CD-melting experiments (*cfr*. Fig. 7 and 9, respectively). In contrast, large differences have been observed with the ODNs treated with PtI2, which caused a large loss in cooperativity on the resulting duplex and G-quadruplex structures (Fig. 13). These effects could be attributed to platination of multiple ODN sites, which would produce families of differently multiplatinated structures, partially unfolded and with significant loss of base-stacking, in agreement with the overall picture emerging from the CD studies.

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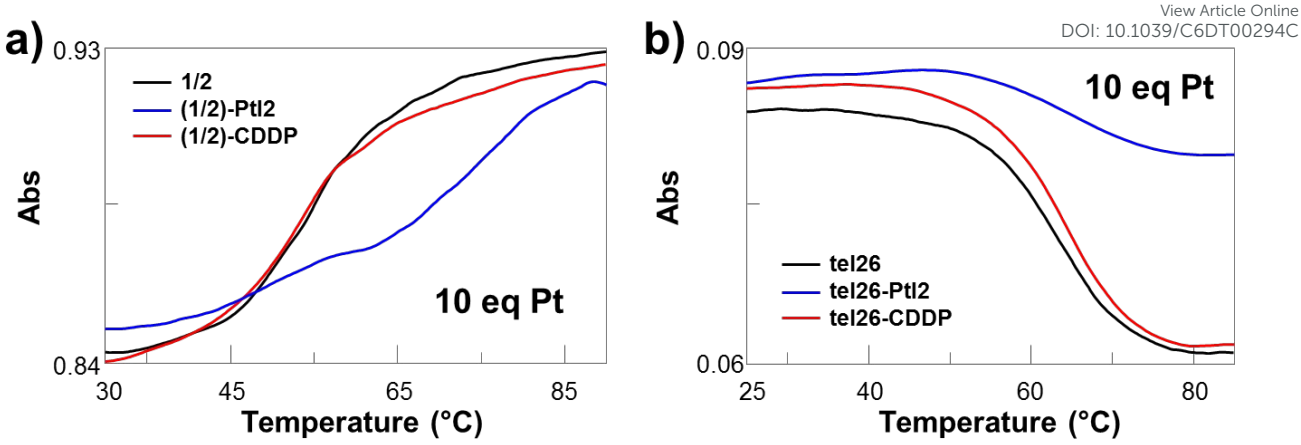

Figure 13. Overlapped UV-melting curves of: duplex **1**/**2** (panel **a**, 4 μM, 10 mM phosphate buffer/50 mM NaCl), and **tel$_{26}$** G4 (panel **b**, 2 μM, 10 mM phosphate buffer/50 mM KCl) in the absence (black lines) and presence of 10 eq of PtI2 (blue) and CDDP (red) after 48 h incubation, recorded, respectively, at 260 and 295 nm with a temperature increase of 1 °C/min.

2.4 ESI-MS studies

The interaction of the platinum complexes PtI2 and CDDP with the single strand ODNs **1**, **2**, **3** and **tel$_{26}$** has been then studied also by means of ESI mass spectrometry. Each ODN, with 10 μM concentration in LC-MS grade water, has been incubated with 1 and/or 3 eq of the metal complexes at 37 °C and, after 48 h incubation, negative ion mode mass spectra of the mixtures have been recorded.

In these experiments, mass peaks corresponding to metal-ODN adducts have been observed for the reaction on ODN **1** (Fig. 14), ODN **2** (Fig. 15) and **tel26**, when not structured into a G4 conformation (Fig. S12 and S13), but not in the case of ODN **3** (spectra non shown) and **tel$_{26}$** G4 (Fig. S14 and S15). Therefore ESI-MS analysis unambiguously proves that the presence of guanines in the sequence (unless unavailable for Pt(II) coordination, as in the case of an ODN folded in a G4 conformation) is a main requisite for the formation of stable adducts between the metal complexes and the oligonucleotides in the here investigated conditions.

In agreement with previous studies,[6] the two metal complexes show in general a similar reactivity towards the selected single strand ODNs (*cfr.* panel **A** with panel **B** of Fig. 14 and 15, and Fig. S12 with S13), highlighted by the presence of ODN-platinum adducts in the mass spectra in which both the chloride (for CDDP) and iodide ligands (for PtI2) are lost, while ammonia ligands are retained. Indeed, in all cases exclusively adducts with the coordinating unit $[Pt(NH_3)_2]^{2+}$ have been found.

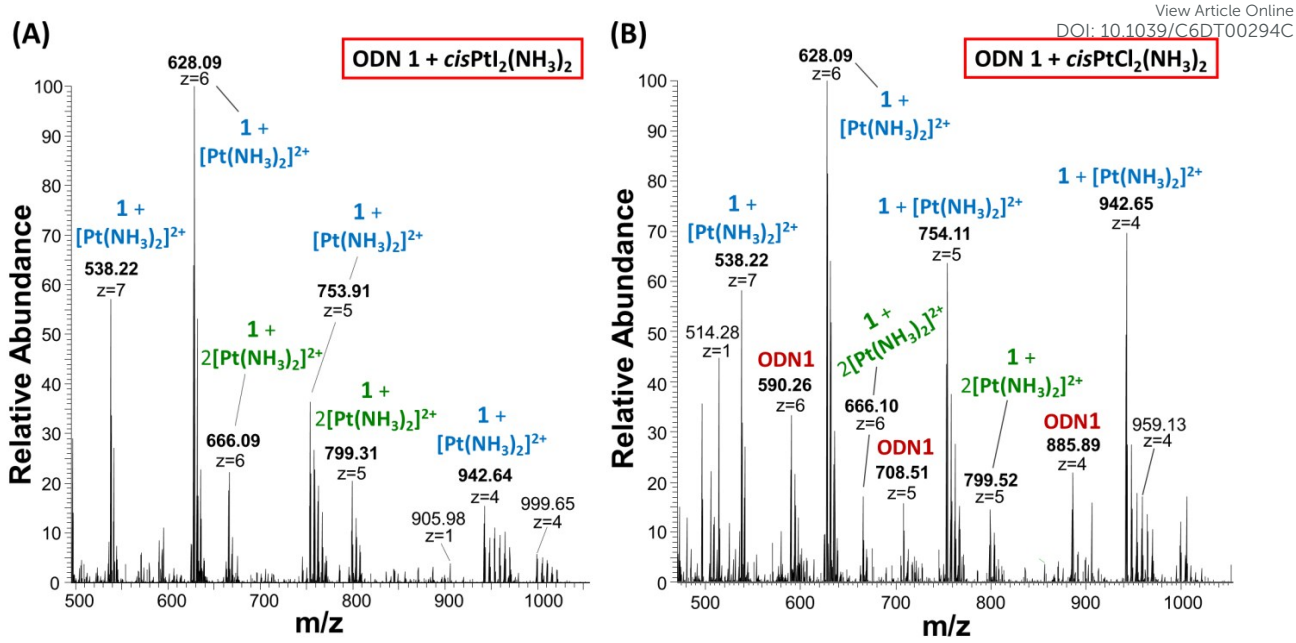

Figure 14. ESI-MS spectra of ODN **1** incubated in water at 37 °C for 48 h at 10 μM concentration with 3 eq of the platinum complexes PtI2 (panel **A**) and CDDP (panel **B**). The analysis has been carried out at final concentration of 5 μM ODN in CH_3OH/H_2O, 1:1 (v/v).

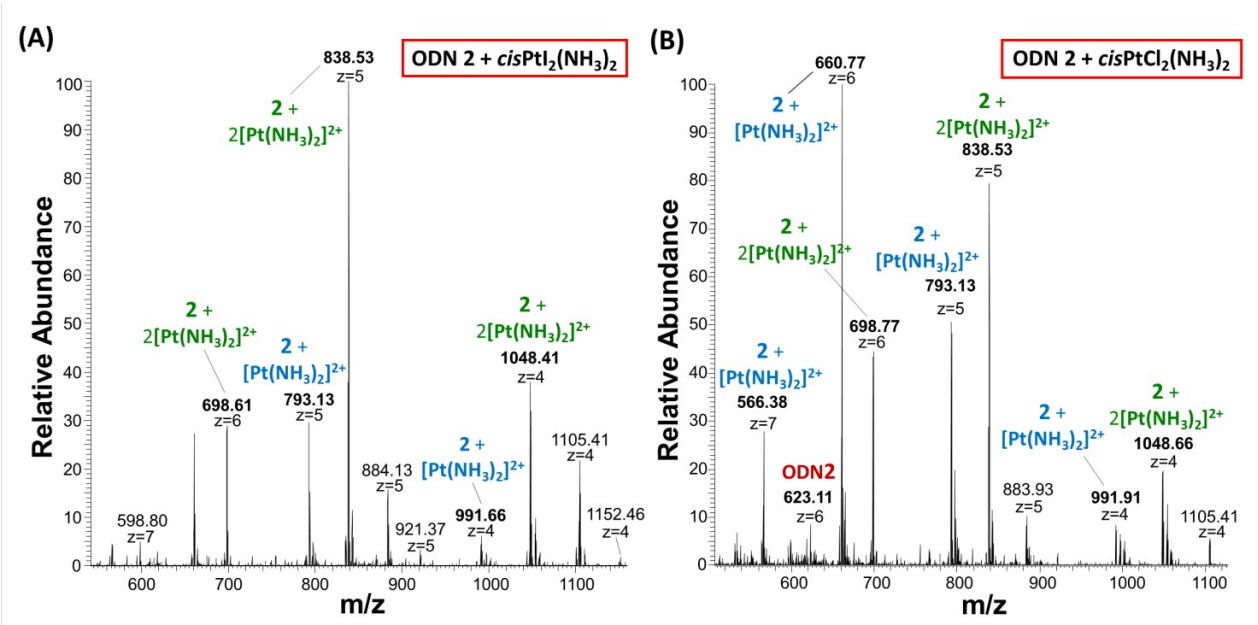

Figure 15. ESI-MS spectra of ODN **2** incubated in water at 37 °C for 48 h at 10 μM concentration with 3 eq of the platinum complexes PtI2 (**A**) and CDDP (**B**). The analysis has been carried out at final concentration of 5 μM ODN in CH_3OH/H_2O, 1:1 (v/v).

Notably, also bis-adduct peaks have been observed. These are more abundant for ODN **2** than for ODN **1**, incubated with 3 eq of PtI2 or CDDP (*cfr*. Fig. 14 and 15): this result is consistent with the

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higher reactivity of the Pt(II) complexes towards ODN **2**, evident also in the CD spectroscopy experiments, attributed to the presence of 2 "GG" (and 3 "GA") potential binding sites.

Furthermore, only the mono-adduct has been found in the experiments with **tel$_{26}$** in the absence of Na^+/K^+ ions (Fig. S12 and Fig. S13): the absence of bis-adducts, despite the high number of guanines within the sequence, is probably attributable to some kind of structuration of the 26-mer **tel$_{26}$** also in pure water. This partially limits the accessibility of the N7 atoms of guanines to the platinum centre, in agreement with the CD data here obtained (see Fig. 10a and 11a). Then, in accordance with CD data (see Fig. 8), when **tel$_{26}$** has been first pre-structured in G4 and then incubated with either PtI2 or CDDP, no detectable peak relative to the formation of metal-ODN adducts is observed in the ESI-MS spectra (Fig. S14 and Fig. S15).

3. Conclusions

In the context of a general reconsideration of iodido platinum complexes as potential anticancer drug candidates, some of us have recently demonstrated that PtI2, in contrast to early claims of inactivity, is even more cytotoxic than cisplatin towards a few solid tumor cell lines *in vitro*. Proved to be internalised by cells in a far greater amount than cisplatin, this compound has also been found to interact with calf thymus DNA through an interaction mode very similar to that well documented for CDDP, though showing a slower reactivity.[6]

Following these intriguing results, we have here investigated the interaction of *cis*-diamminediiodoplatinum (II), the closest iodido analogue of CDDP, with selected DNA models; thus several oligonucleotide sequences, either in unstructured or structured (duplex and G-quadruplex) form, have been reacted under pseudo-physiological conditions with this Pt complex, using CDDP as a control, and then studied by CD and UV-vis spectroscopy, as well as by ESI-MS analysis.

CD experiments suggest that PtI2 is more reactive than CDDP towards all the investigated DNA models. Indeed, the two complexes produce adducts of similar overall conformation, but some differences emerge: for example, the DNA duplex treated with PtI2 shows higher distortions and lower thermal stability than that platinated with CDDP. In analogy with CDDP, PtI2 shows a marked preference for ODN sequences containing contiguous guanines, if not self-assembled in superstructures as the G4 conformations; however, if added in large excess, it proves to be less selective than CDDP towards guanine-free sequences, being able to interact also with other potential binding sites, as for example adenines.

Remarkably, ESI-MS data confirm the similar reaction mode of the two platinum complexes towards the selected single strands, showing in both cases the formation of stable adducts on the

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ODN sequence containing the $[Pt(NH_3)_2]^{2+}$ coordinating unit. Stable metal-coordinated ODN species are observed in ESI-MS only for the guanine-containing strands, unless some intrinsic structuration limits the accessibility of the N7-guanine atoms to the platinum centre. Thus not only guanine-free single strands, but also G-rich ODNs, if pre-structured in a G-quadruplex conformation, cannot be easily platinated upon treatment with both the metal complexes.

Taken together, these findings clearly demonstrate that, when reacting with DNA targets, PtI2 exhibits an interaction mode very similar to CDDP. Interestingly, it always proves to be more reactive than CDDP, in line with the results of *in vitro* antiproliferative assays on cancer cell lines.

In revisiting an "old" platinum complex, this work provides additional, valuable information for a deeper knowledge on iodinated Pt(II) complexes. These research efforts can be useful to provide new clues for the design of optimized Pt-based anticancer agents, taking into account that also tiny structural modifications may result into markedly different reactivity, eventually determining very different *in vivo* bioactivity.

4. Experimental section

Abbreviations: CDDP = *cis*-diamminedichloroplatinum (II); PtI2 = *cis*-diammineiodoplatinum (II); CD = Circular Dichroism; ESI-MS = ElectroSpray Ionization Mass Spectrometry; ODN = OligoDeoxyriboNucleotide; G4 = G-quadruplex.

(**1**/**2**)–PtI2 and (**1**/**2**)–CDDP stand for: preformed duplex mixed with the selected platinum complex. **1**–PtI2/**2**, **1**–CDDP/**2**, **2**–PtI2/**1** and **2**–CDDP/**1** stand for: the duplex formed between the platinated ODN, **1** or **2**, and the corresponding complementary strand.

4.1 Preparation of the ODN model systems and platinum complexes

The following ODN sequences have been synthesized in the solid phase following a standard phosphoramidite protocol (1 μmol scale) using an ABI Expedite 8909 synthesizer:

-d($^{5'}$CCTCT**GG**TCTCC$^{3'}$) (**1**);

-d($^{5'}$**GG**A**G**ACCA**G**A**GG**$^{3'}$) (**2**);

-d($^{5'}$TCACACACACACACACACACACTT$^{3'}$) (**3**);

-d[$^{5'}$(TTA**GGG**)$_4$TT$^{3'}$] (**tel$_{26}$**).

After an aq. ammonia treatment (55 °C, 12 h) for deprotection and detachment from the solid support, the ODNs have been purified by using GlenPack cartridges (C18), desalted by dialysis vs. H_2O and lyophilized. Quantification of the ODNs has been performed by UV measurements (λ =

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260 nm, 85 °C) of a stock solution obtained by dissolving the oligomers in a known volume of bidistilled H_2O.

The duplex structure, formed by hybridizing **1** and **2** in 1:1 ratio (**1**/**2**), and the ODN sequence **tel$_{26}$** have been subjected to an annealing procedure in pseudo-physiological conditions, consisting in taking the samples, dissolved in a 10 mM phosphate buffer solution at pH = 7.0, also containing 50 mM NaCl or 50 mM KCl, respectively, at high temperature (95 °C) for 5 min and then leaving them to slowly cool to room temperature.

Stock solutions of CDDP (3.3 mM) and PtI2 (2 mM) have been freshly prepared by dissolving the solid complexes, respectively, in a 0.9% NaCl (154 mM NaCl) solution and in an aqueous solution containing 20% DMSO to ensure the complete Pt complex dissolution. Indeed, 20% DMSO was the minimal amount which allowed us to completely dissolve PtI2 in an aq. solution at mM conc., necessary to properly perform the titration experiments on the oligonucleotide samples. In order to minimize the ligand exchange process,[17] in all our experiments this stock solution has always been freshly prepared and, immediately after the dissolution, very small volumes of this sample have been taken and rapidly left in contact with the ODN solutions, thus being rapidly diluted to final DMSO conc. of 0.04 and 0.3% in volume (for the experiments with 2 and 10 eq. PtI2, respectively). The whole process (dissolution + addition to the ODN solution) has typically taken less than 3 min, including the time required for spectra recording.[18]

4.2 CD experiments

CD spectra have been recorded on a Jasco J-715 spectropolarimeter equipped with a Peltier-type temperature control system (model PTC-348WI), using a quartz cuvette with a path length of 1 cm or a tandem cell (2 × 0.4375 cm). The spectra have been registered at 37 °C in the range 220-320 nm with response of 1 s, scanning speed of 100 nm/min and 2.0 nm bandwidth and corrected by subtraction of the background scan with buffer. All the spectra have been averaged over 3 scans and each experiment was performed in triplicate.

CD analysis has been performed as follows: 2 μM solutions of the ODNs **1**, **2** and **3** in 10 mM phosphate buffer/50 mM NaCl (pH = 7.0) have been prepared upon dilution of a 400 μM stock solution of each ODN. The 2 μM solution of duplex **1**/**2** has been obtained by mixing in 1:1 ratio ODNs **1** and **2**, withdrawn from the corresponding 400 μM stock solutions, in 10 mM phosphate buffer/50 mM NaCl (pH = 7.0), followed by an annealing procedure. For each ODN solution, an aliquot has been used as a reference during the analysis. 2 or 10 eq of PtI2 (withdrawn from the 2 mM stock solution) have been added to the remaining part, and then the resulting solutions have been left in the dark at 37 °C. CD spectra of the platinum-treated solutions have been then recorded

at 24, 48 and 72 h, and compared with the corresponding ODN reference solutions at the same incubation times. Analogous experiments have been carried out also with CDDP (addition of 2 or 10 eq of the complex withdrawn from a 3.3 mM stock solution). The CD experiments carried out to assess the formation of a duplex structure between the platinated ODNs and the corresponding complementary strands have been performed in a tandem cell by placing, in one chamber, the metallated ODN and in the other the untreated, complementary ODN sequence, each at 2 μM concentration in 10 mM phosphate buffer/50 mM NaCl (pH = 7.0). Analogously to previous studies,[10,19] two CD spectra have been registered for each experiment: the first one recorded as the sum of the separate components, here named *sum* spectrum, and the second one recorded after mixing the two solutions and stabilization of the signal, here named *mix*. Upon mixing the two solutions, the ODN concentration resulted to be reduced by a factor of 2, going from 2 to 1 μM, and the solution path length increased from 0.437 to 0.875 cm.

For the CD experiments with **tel$_{26}$**, the selected oligonucleotide (taken from a 73 μM stock solution in water) has been dissolved in a 10 mM phosphate buffer/50 mM KCl (pH = 7.0) solution, so to obtain a 2 μM solution. 2 or 10 eq of PtI2 (withdrawn from the 2 mM stock solution) have been added to the **tel$_{26}$** solution. CD spectra of the platinum-treated solutions have been then recorded over 48 h incubation. Analogous experiments have been carried out in parallel also with CDDP, involving the addition of 2 or 10 eq of the complex withdrawn from the 3.3 mM stock solution.

For the G4-induction studies, carried out in the absence of Na^+ or K^+ cations, a 2 μM **tel$_{26}$** solution in 20 mM Tris-HCl (pH 7.2) buffer has been used. 10 eq of PtI2 (withdrawn from the 2 mM stock solution) have been added to the **tel$_{26}$** solution. CD spectra of the platinum-treated solutions have been then recorded over 72 h incubation. The same experiments have been carried out also with CDDP: 10 eq of the complex, added to the 2 μM **tel$_{26}$** solution, have been withdrawn from the 3.3 mM stock solution containing 154 mM Na^+; this addition has led to a solution with an approximately 1 mM final concentration of Na^+: this concentration is not sufficient to induce **tel$_{26}$** G4 self-structuring, thus this ODN is still present in the form of a poorly structured coil.[20]

For the CD-monitored melting experiments, the ellipticity has been recorded at 270 nm and at 290 nm (for duplex and G-quadruplex structures, respectively) with a temperature scan rate of 1 °C/min in the range 20-85 °C. T_m values of 54 °C and 64 °C have been determined, respectively, for the duplex and the G-quadruplex model systems in our working conditions. All the CD melting experiments, relative to the reference duplex **1**/**2** and all the platinated duplexes, have been performed in triplicate in a tandem cell at 1 μM concentration in 10 mM phosphate buffer/50 mM NaCl or 50 mM KCl (pH = 7.0). The errors associated with the T_m have been determined on the basis of three independent experiments and reported as the mean values ± SD.

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4.3 UV-vis experiments

UV measurements have been recorded on a JASCO V-550 UV/vis spectrophotometer equipped with a Peltier block (PTC-348WI) by using 1 cm quartz cells of 1 ml internal volume (Hellma). The spectra have been registered at 37 °C in the range 210-450 nm with response of 1 s, scanning speed of 100 nm/min and bandwidth of 2.0 nm, and corrected by subtraction of the background scan with buffer. All the spectra have been averaged over 3 scans and each experiment has been performed in triplicate. UV-melting curves have been recorded by following the absorbance changes at a determined wavelength on increasing the temperature (heating rate 1 °C/min, monitoring wavelength 260 nm for the duplex, 295 nm for tel_{26}). The Tm values have been calculated as the maxima of the plots of the first derivative of the absorbance *vs.* temperature (associated error: ±1 °C).

4.4 ESI-MS experiments

The ESI-MS spectra have been recorded by direct injection at 5 µl min^{-1} flow rate (or 20 µl min^{-1} for pre-structured **tel_{26}** in order to optimise signals) in an Orbitrap high-resolution mass spectrometer (Thermo, San Jose, CA, USA), equipped with a conventional ESI source.

Stock solutions of the Pt complexes have been prepared at 100 µM concentration in LC-MS grade water. Stock solutions of the tested oligonucleotides have been prepared at 100 µM in LC-MS grade water.

For the reactions of the Pt complexes with oligonucleotides, the substances have been combined in a 1:1 or 3:1 metal/oligonucleotide ratio in LC-MS grade water to give a final concentration for the tested oligonucleotides of 10 µM. The samples have been kept at 37 °C for 48 h before measurement.

To record the ESI mass spectra, the incubated samples or the free oligonucleotides have been diluted to 10 µM in LC-MS grade water.

The mass spectra have been recorded in the range 300 - 2000 m/z values. The following standardized working conditions have been applied: spray voltage 2.7 kV, tube lens voltage -113 V, capillary voltage -20 V and capillary temperature 280 °C. Sheath and auxiliary gases have been set at 23 a.u. and 4 a.u., respectively.

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18. Although the ligand exchange process with DMSO is very fast for cisplatin, taking into account: *i*) the higher chemical inertness of PtI2 compared with cisplatin (t1/2 value for the hydrolysis of PtI2 is 556 min, for cisplatin 259 min6); *ii*) Hall *et al*.'s conclusion that if the DMSO amount is <3%, the biological effects of cisplatin are unaltered,[17b] we considered that the tiny amount of

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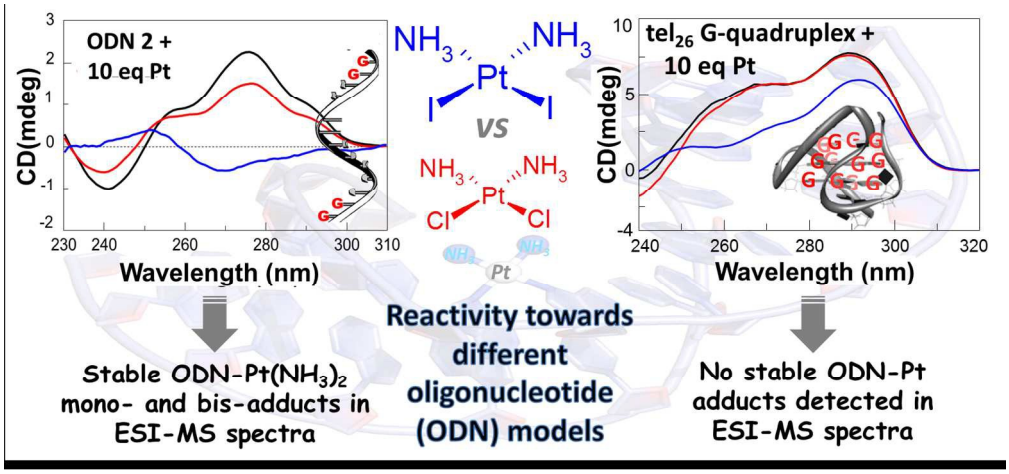

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