JACS
JOURNAL OF THE AMERICAN CHEMICAL SOCIETY
Article
pubs.acs.org/JACS

Drug Delivery with a Calixpyrrole–*trans*-Pt(II) Complex

Grazia Cafeo,[†] Grazia Carbotti,[‡] Angela Cuzzola,[∥] Marina Fabbi,[‡] Silvano Ferrini,[‡] Franz H. Kohnke,*[,†] Georgia Papanikolaou,[†] Maria Rosaria Plutino,[⊥] Camillo Rosano,[‡] and Andrew J. P. White[§]

[†]Dipartimento di Scienze Chimiche, Università di Messina, viale F. Stagno D'Alcontres 31, 98166 Messina, Italy

[‡]IRCCS A.O.U. San Martino-IST, Istituto Nazionale per la Ricerca sul Cancro, Largo R. Benzi 10, 16132 Genoa, Italy

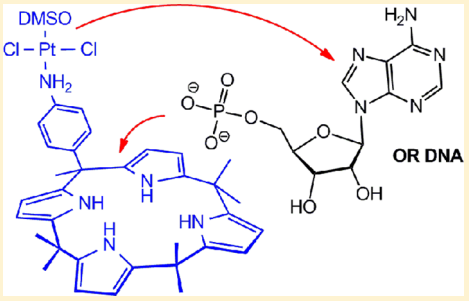
[∥]Dipartimento di Chimica e Chimica Industriale, Università di Pisa, via Risorgimento 35, 56126 Pisa, Italy

[⊥]Istituto per lo Studio dei Materiali Nanostrutturati, ISMN - CNR, OU Palermo, c/o Dipartimento di Scienze Chimiche, Università degli Studi di Messina, Viale F. Stagno d'Alcontres 31, 98166 Messina, Italy

[§]Chemical Crystallography Laboratory, Department of Chemistry, Imperial College London, South Kensington, London SW72AZ United Kingdom

Supporting Information

ABSTRACT: A *meso-p*-nitroaniline-calix[4]pyrrole derivative *trans*-coordinated to a Pt(II) center was synthesized and its structure solved by X-ray analysis. Adenosine monophosphate (AMP) was used as a model compound to evaluate the potential for the assisted delivery of the metal to the DNA nucleobases via the phosphate anion-binding properties of the calix[4]pyrrole unit. An NMR investigation of the kinetics of AMP complexation in the absence of an H-bonding competing solvent (dry CD_3CN) was consistent with this hypothesis, but we could not detect the interaction of the calix[4]pyrrole with phosphate in the presence of water. However, in vitro tests of the new *trans*-calixpyrrole–Pt(II) complex on different cancer cell lines indicate a cytotoxic activity that is unquestionably derived from the coexistence of both the *trans*-Pt(II) fragment and the calix[4]pyrrole unit.



INTRODUCTION

Traditional cancer chemotherapy is severely hampered by the toxic side effects of the drugs used for this purpose; hence, the search for ways to achieve selective drug delivery to the diseased tissues is a topic of considerable current activity. A number of novel drug-delivery methods have emerged over recent years that are based on the achievements of supramolecular chemistry and which include the use of dendrimers.[1] Passive targeting by the EPR effect[2] has been exploited using drug–nanoparticles and drug–polymer conjugates,[3] and active targeting has also been pursued, using chemical moieties that are preferentially recognized by cancerous cells as part of the drug's structure, so that these moieties can act as 'homing' devices.[4] The achievement of effective cancer regression using lower drug doses is also one of the objectives of selective drug-delivery systems.

Platinum complexes are a group of anticancer drugs with a long history, *cis*-$[PtCl_2(NH_3)_2]$ being the first of a large number of Pt(II) derivatives studied in this area, a few of which have reached and are still in clinical use.[5,6] Pt(II) complexes have recently received renewed attention with the introduction of a number of new-generation drugs containing carboxylate ligands associated with planar bulky amines and/or N-donor heterocycles.[7] Moreover, the initial assumption that *cis*-stereochemistry is an absolute prerequisite for anticancer activity, fostered by the observation that the *trans*-$[PtCl_2(NH_3)_2]$ is inactive, has been re-examined by the discovery of a number of *trans*-Pt(II) derivatives that were found to be effective and/or even complementary where *cis*-$[PtCl_2(NH_3)_2]$ resistance was observed.[5,8] Extensive research in this area has thus demonstrated that the nature of the ligands, along with the *cis*-/*trans*-stereochemistry, plays a crucial role in the cytotoxic activity of Pt(II) complexes.

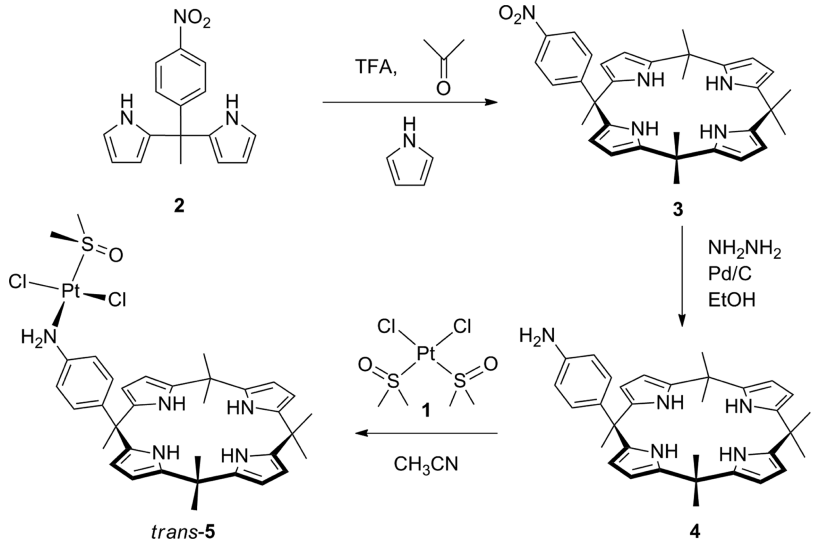
Calixpyrroles are a class of molecular receptors that have gained considerable interest due to their ability to bind anions[9] and to act as ditopic (ion-pair) receptors[10] as well as hosts for neutral molecules[11] that can accept NH hydrogen bonds.[12] In 1998, Sessler and co-workers[13] reported that SiO_2 stationary phase functionalized with calix[4]pyrrole units could be used in the HPLC separation of oligonucleotides and peptides with advantages over sapphyrin-modified silica gels, thus indicating the ability of the calix[4]pyrrole unit to recognize these anionic biomolecules.

Inspired by the above-mentioned results on the separation of oligonucleotides, we reasoned that, if an anticancer drug could be associated with the calixpyrrole unit, then its delivery to nucleobases (in DNA) could be improved as a result of the interaction of the calix with the anionic phosphate units.[14] With these thoughts in mind, we prepared the aminophenyl

Received: August 6, 2012

   dx.doi.org/10.1021/ja307791j | J. Am. Chem. Soc. XXXX, XXX, XXX–XXX

Scheme 1. Synthetic Pathway to *trans*-5



derivative of calix[4]pyrrole **4** (Scheme 1) to be used as a suitable ligand in Pt(II) coordination. Here we report our initial studies of a *trans*-Pt(II)-calixpyrrole derivative with potential application as an anticancer drug.

RESULTS AND DISCUSSION

meso-p-Aminophenyl derivative of calix[4]pyrrole **4** was synthesized as described in Scheme 1. The method is similar to that recently reported for a *meso*-ethyl-substituted analogue of **3**.[15] Calix[4]pyrrole **3** was obtained by the same method used for the cyclodimerization of **2**,[16] but in the presence of an excess of pyrrole (5 equiv). Mixing equimolar amounts of calix[4]pyrrole **4** and *cis*-$[PtCl_2(DMSO)_2]$ **1** in CH_3CN resulted in the formation of pale-yellow crystals (mp 166–168 °C).

The single-crystal structure of *trans*-**5** shows the platinum to have a distorted square planar coordination geometry [*cis* angles in the range 87.23(11)–94.41(4)°] with two *trans*-chloride ligands, the nitrogen of an aminophenyl moiety, and a sulfur-bound DMSO molecule comprising the coordination sphere (Figure 1). Hence, after the substitution, a configurational isomerization with respect to **1** has occurred.[17] The plane of the aminophenyl ring is inclined by ~106° to the mean plane of the calix[4]pyrrole macrocycle [as defined by the four quaternary carbon atoms C(6), C(14), C(22), and C(30)], and the Pt(II) coordination plane is inclined by ~110° to this former plane such that the complex forms a U-shaped cleft.

An included DMSO solvent molecule is held within this cleft by a series of N–H···O hydrogen-bonding interactions involving the N–H groups of three of the four pyrrole rings (interactions a–c in Figure 1). Whereas these three pyrrole rings are inclined to the mean plane of the calix[4]pyrrole macrocycle by between ~47° and 61° with their N–H groups pointing toward the trapped DMSO molecule, the fourth [based on N(18)] is inclined by ~73° with the N–H group pointing away from the hydrogen-bonded DMSO molecule. This N–H group is not involved in any significant intermolecular interactions. There is an approach of one of the methyl hydrogen atoms of the encapsulated DMSO molecule to the O(40) oxygen atom of its coordinated counterpart, but with an O···H separation of ~2.56 Å any interaction can only be weak. If one takes into account that

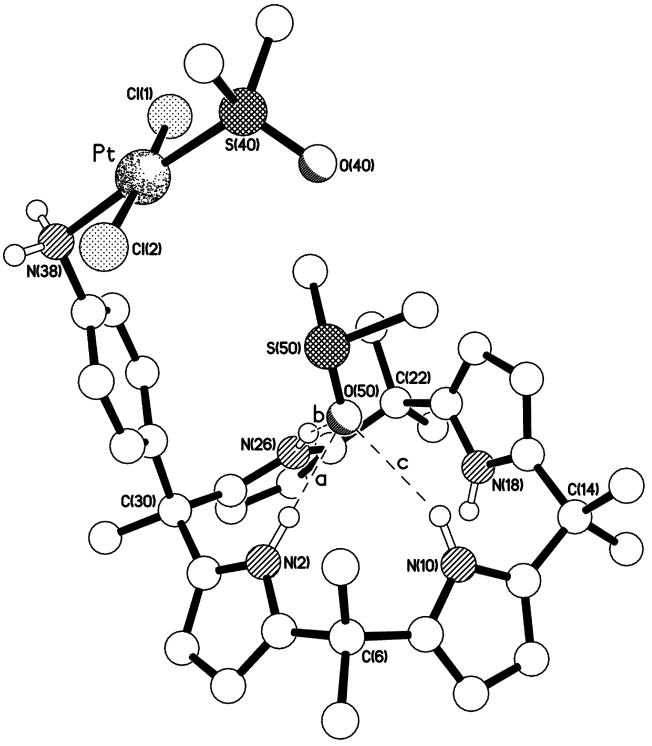


Figure 1. Crystal structure of *trans*-**5**. Selected bond lengths (Å) and angles (deg). Pt–Cl(1) 2.2980(11), Pt–Cl(2) 2.3090(11), Pt–N(38) 2.095(4), Pt–S(40) 2.2132(11), Cl(1)–Pt–Cl(2) 176.02(4), Cl(1)–Pt–N(38) 87.23(11), Cl(1)–Pt–S(40) 94.41(4), Cl(2)–Pt–N(38) 88.98(11), Cl(2)–Pt–S(40) 89.43(4), N(38)–Pt–S(40) 176.73(11). The N–H···O hydrogen bonds have [N···O] and [H···O] separations (Å), and [N–H···O] angles (deg) of (a) 2.878(5), 1.98, 178; (b) 3.041(6), 2.17, 164; (c) 3.215(6), 2.40, 150, respectively.

trans-**5** crystallizes from a CH_3CN solution (initial concentration of the two reagents 17 mM) in which the only 'available' DMSO is the one displaced from the Pt(II) center, the presence of the DMSO within the calixpyrrole moiety of *trans*-**5** indicates a selectivity of over $1:10^3$ (at least in the solid state) with respect to acetonitrile, which is also known to form hydrogen-bond complexes with calixpyrrole derivatives.[18] The other included solvent molecule (an acetonitrile molecule) is

dx.doi.org/10.1021/ja307791j | J. Am. Chem. Soc. XXXX, XXX, XXX–XXX

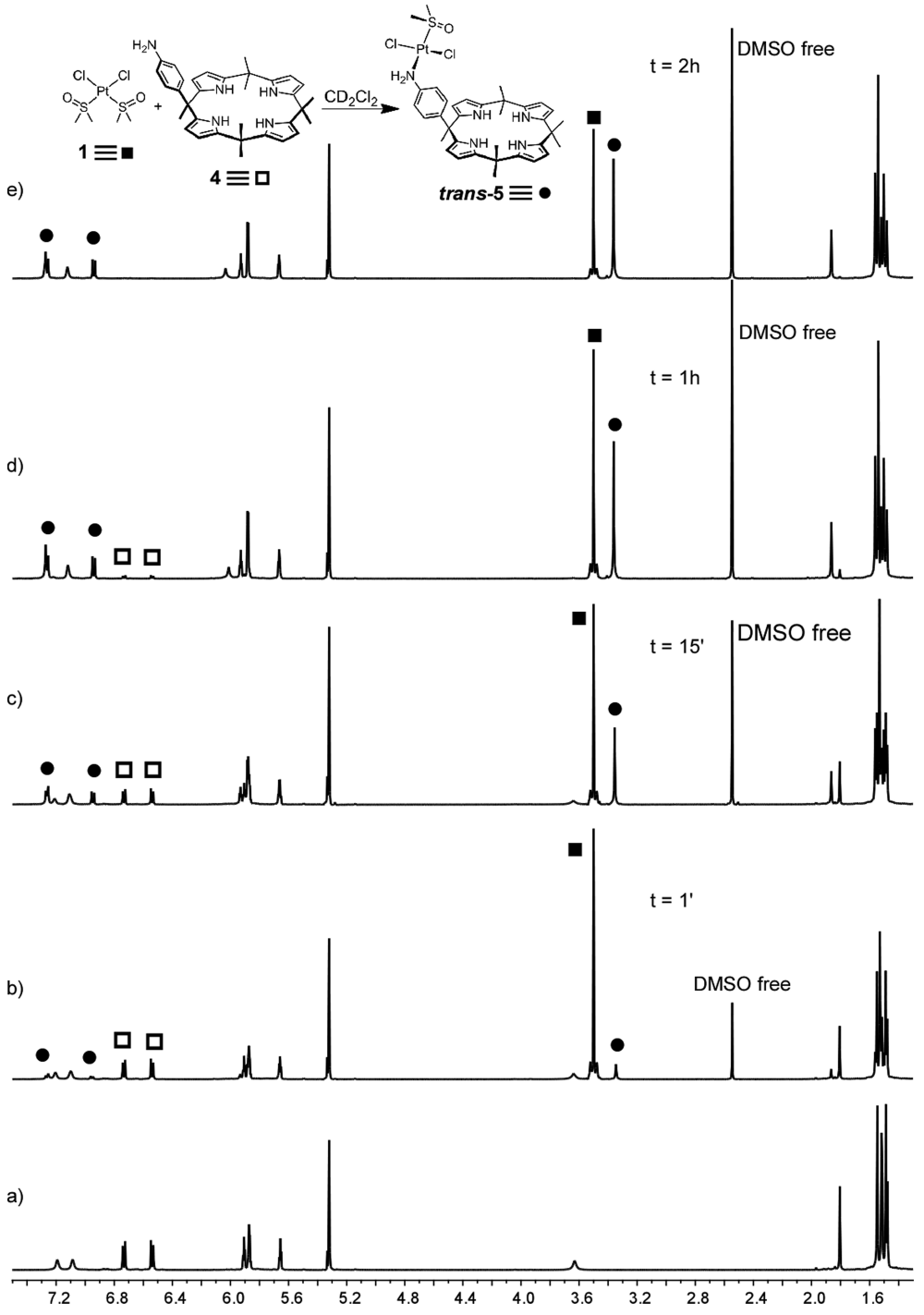


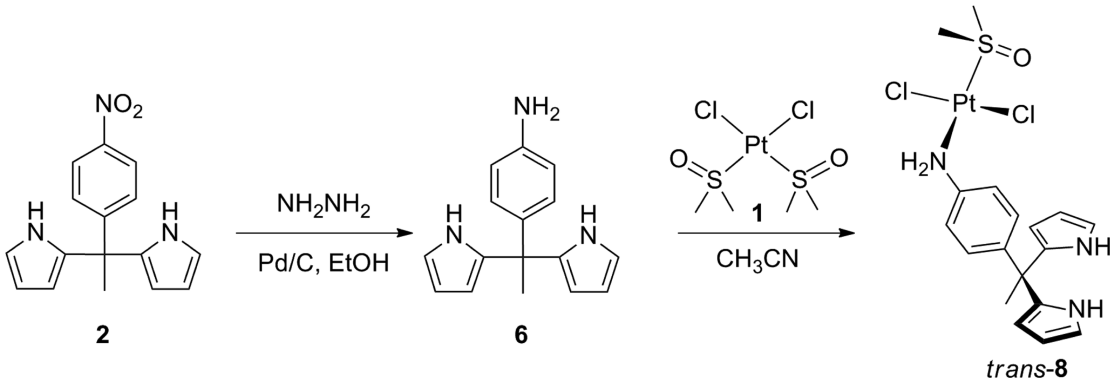
Figure 2. Partial ^{1}H MNR (500 MHz, CD_2Cl_2, 25 °C, 5 mM) spectra of **4** (a), and of the reaction mixture **4** + **1** (b–e) at the indicated times after the addition of 1 equiv of **1**. Selected signals are assigned using black squares and circles and white squares as shown in the reaction diagram.

linked to the outside of the complex by a hydrogen bond to the aminophenol nitrogen atom N(38) with N···O and H···O separations of 3.082(9) and ∼2.22 Å respectively, and an N–H···O angle of 159°.

The formation of *trans*-**5** was monitored by ^{1}H MNR also in CD_2Cl_2 solution (5 mM) at 25 °C where crystals were not formed at this concentration (see Figure 2). The displacement of DMSO from the Pt center was indicated by a reduction of intensity of the signal at δ 3.50 for the bound DMSO in *cis*-$[PtCl_2(DMSO)_2]$ **1** and the appearance of two new signals: one at δ 3.34 for the DMSO coordinated to the Pt(II) and one at δ 2.54 for the DMSO displaced from **1**. The ^{1}H NMR changes described for the DMSO signals were consistent with the spectral changes observed for the resonances pertaining to the calixpyrrole unit. Upon formation of *trans*-**5**, the resonance for the NH_2 unit at δ 3.63 disappeared to give a broad singlet at δ 6.02 for the newly formed Pt-NH_2 moiety. A new AA'BB' system centered at δ 6.95 and 7.27 was formed, while the resonances of the AA'BB' system of the uncoordinated calixpyrrole **4** at δ 6.55 and 6.74 disappeared. The general downfield shift relative to the calixpyrrole protons clearly indicated an happened Pt(II) coordination. On the other hand,

dx.doi.org/10.1021/ja307791j | J. Am. Chem. Soc. XXXX, XXX, XXX–XXX

Scheme 2. Synthetic Pathway to *trans*-8



the Pt-NH_2 amino and the bound DMSO protons did not show the expected diagnostic coupling constant ($^2J_{PtH}$ and $^3J_{PtH}$ respectively) with ^{195}Pt, even when lowering the magnetic field strength (300 vs 500 MHz) or increasing the sample temperature. The high molecular weight of *trans*-**5** caused a decrease of the Pt relaxation time (T_1) via the chemical shielding anisotropy (CSA), thereby resulting in an observed loss of the two typical satellite peaks.[19] Moreover, on standing, the compound developed a new resonance at δ 3.42 that increased gradually, while the resonance at δ 3.34 decreased by a corresponding amount (the sum of the intensities for the two signals remained constant). We assigned the new signal δ 3.42 to the DMSO unit of *cis*-**5**, formed by a rearrangement at the Pt(II) center of the initially formed *trans*-**5**. Concomitantly, a new AA'BB' system appeared at δ 6.99 and 7.37, and an NH_2 resonance at δ 5.80 increased in intensity over time, while the resonances for the initially formed *trans*-**5** decreased (Figure S2, Supporting Information). Within 77 h, the ratio between the initially formed and the rearranged isomers was 75:25. Even in the presence of an excess of calixpyrrole **4**, only one of the DMSO ligands of **1** was displaced from the Pt(II) center.

The formation of one *trans*-Pt(II) coordination compound that subsequently equilibrated with a rearranged *cis*-isomer generated concern regarding the correlation between the structure observed in the solid state and the stereochemical assignments proposed for the species in solution. To address this issue, a freshly prepared batch of the crystalline product obtained from CH_3CN was filtered from the crude mixture (the sample had the same melting point as that analyzed by X-ray diffraction), and it was immediately dissolved in CD_2Cl_2. The ^{1}H NMR spectrum was recorded and found to be identical to that of the coordination compound formed in the initial stages of the reaction (i.e., before *trans*/*cis* isomerization equilibrium). Free DMSO was also present in a 1:1 stoichiometric ratio with respect to *trans*-**5**. On standing, the spectral changes described above for the partial *trans*-**5**/*cis*-**5** equilibration was observed. However, all our attempts to isolate *cis*-**5** as a pure compound from CH_3CN solutions were unsuccessful, probably because it does not yield crystals and/or it equilibrates to *trans*-**5** as this is subtracted from the solution during its crystallization. Solutions of *trans*-**5** that did not contain free DMSO (displaced from **1**, see Supporting Information [SI]) were found to be configurationally stable (over 24 h at 25 °C in either CD_2Cl_2 or CD_3CN). Thus, the presence of 'free' DMSO plays a crucial role in the isomerization process described above. In fact, in separate experiments we observed that the *trans*/*cis* isomerization was accelerated on the addition of free DMSO to a solution of *trans*-**5**.

The DMSO molecule in the solid-state structure of *trans*-**5** prompted us to investigate whether the calixpyrrole moiety plays an accelerating role toward the formation of this compound. In fact, the calixpyrrole unit could either assist the departure of one DMSO molecule from the Pt(II) center in **1** through its complexation, or it could favor the approach of the NH_2 unit to the Pt(II) center by clamping to a bound DMSO moiety of the metal complex **1**. Coordination of DMSO to the calix moiety of *trans*-**5** could also accelerate the observed *trans*/*cis* isomerization. Calixpyrroles are also capable of complexing chloride.[9−12] We therefore compared the formation of *trans*-**5** with those of dipyrromethane derivative **6** (Scheme 2, Figure S3 in SI) and *p*-toluidine **7** (Figure S4 in SI), which both contain the NH_2 ligand but not the calixpyrrole moiety. The kinetic study of the substitution reactions of **1** with ligands **4**, **6**, and **7** (CD_2Cl_2 at 25 °C in a 1:1 molar ratio 10 mM) provided second-order rate constants k_2/s^{-1} M^{-1} (see SI) 0.311 (±0.006), 0.143 (±0.004), and 0.261 (±0.007) for *trans*-**5**, *trans*-**8**, and *trans*-**9**, respectively. The observed $t_{1/2}$ were 330, 750, 450 s, respectively (Figures S5−S7, SI).

The intensities of the diagnostic ^{1}H NMR signals were used to measure the progress of the reactions (see Figures S3−S4 in SI). Stereochemical assignments were based on analogies of the ^{1}H NMR resonances of these compounds with those of the corresponding substructure(s) present in *trans*-**5**. In all cases the *trans*/*cis* isomerization of the initially formed amino-Pt(II) complexes were marginal until nearly quantitative displacement of one mole of DMSO from **1** had occurred. However, while *trans*-**8** was sufficiently stable to be isolated as a pure isomer, stereochemically pure **9** could not be isolated. Interestingly, in the *p*-toluidine *trans*-**9**/*cis*-**9** derivatives, characterized by a lower molecular weight than *trans*-**5**/*cis*-**5**, the protons for the amino unit and the DMSO directly bonded to the platinum(II) center exhibited typical ^{195}Pt satellites (60 vs 70 Hz, and 20 vs 25 Hz, see SI). The difference in ^{195}Pt coupling constants for the two species was in agreement with the slightly different *trans* influence exerted by the dimethylsulfoxide/amino groups (*trans*-**9**) and the chloride moieties (*cis*-**9**).[20]

The amino-ligands **4**, **6**, and **7** have similar electronic requirements, but they have substantially different steric bulk. If we compare rates for *p*-toluidine, **7**, and dipyrromethane derivative **6** ($t_{1/2}$ = 450 and 750 s, respectively), we observe a difference that is qualitatively consistent with the larger steric hindrance of **6**. However, the bulkier **4** has a $t_{1/2}$ of 330 s, a value that suggests an active role for the calixpyrrole. However,

dx.doi.org/10.1021/ja307791j | J. Am. Chem. Soc. XXXX, XXX, XXX−XXX

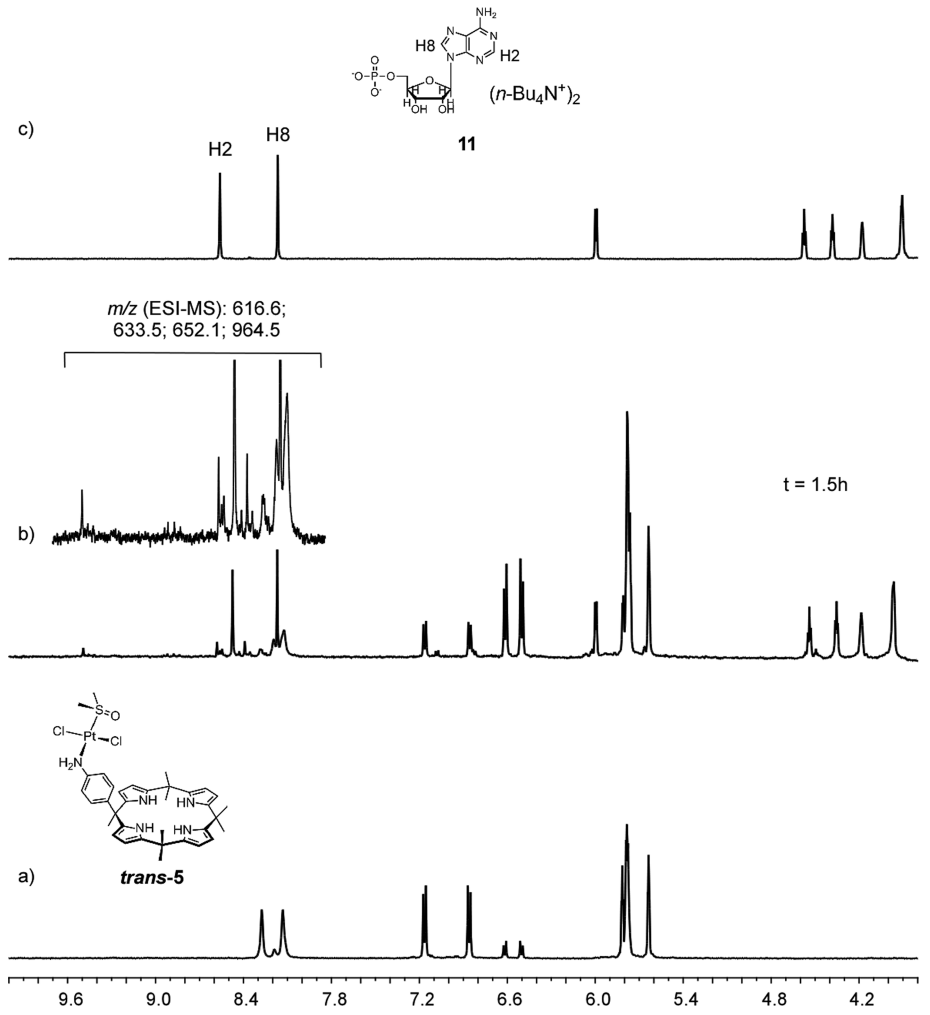


Figure 3. Partial ^{1}H NMR spectra (CD_3CN/D_2O 80:20 v/v, 1.5 mM, 25 °C) of (a) *trans*-**5**; (b) the reaction mixture of *trans*-**5** and $(TBA)_2AMP$ **11** (1:1) 1.5 h after mixing; the signals reproduced in the inset with expanded vertical scale are consistent with the presence of the indicated *m*/*z* values in the ESI-MS spectrum of this mixture; (c) $(TBA)_2AMP$ **11**.

when the concentration of **4** was doubled, $t_{1/2}$ for the formation of *trans*-**5** was 180 s (Figure S5b in SI). This further evidence of second-order kinetics suggests that the formation of *trans*-**5** does not occur within a supramolecular [**4** + **1**] complex.[21] However, complexation of the displaced DMSO by the newly formed *trans*-**5** (and possibly by unreacted **4**) would slow down the back re-entry reaction of DMSO, thus producing the observed acceleration compared with that in which *trans*-**8**, lacking the assisting calixpyrrole cavity, is formed.

As our objectives included an evaluation of the biological activity of *trans*-**5**, we tested its stability in the presence of two species that are ubiquitous in the biological environment: water and chloride. No significant spectral changes were observed in D_2O-saturated CD_2Cl_2 or in D_2O/CD_3CN (20:80, v/v) solutions after over 4 h. Appreciable stability was also observed in the presence of chloride [*trans*-**5**/TBACl 1:10, in D_2O/CD_3CN (20:80, v/v), 50 mM in chloride, i.e. ∼5 times the chloride concentration normally found in plasma].[22] Under these conditions only 30% of the Pt(II) in *trans*-**5** was displaced after 24 h. Although the formation of aqua species is believed to be a key step for the cytotoxic activity of Pt complexes,[8c] we were pleased with the observed stability of *trans*-**5**, as it supports the assumption that *trans*-**5** is, at least initially, the chemical species that interacts with the cells in the in vitro tests (vide infra). Similar considerations apply for the stability in the presence of chloride.

Since DNA is a polyphosphate, we then tested the ability of **4** and of *trans*-**5** to interact with $TBAH_2PO_4$ and $(TBA)_2HPO_4$. Significant complexation-induced shifts were observed in the ^{1}H NMR spectra (CD_2Cl_2) while *trans*-**5** was not decomposed by these anions. However, the measurement of association constants was hampered by the extensive broadening of the NMR resonances before an excess of salts was added (e.g. see Figure S8 in SI for *trans*-**5** + $TBAH_2PO_4$).

We arbitrarily selected adenosine monophosphate (AMP) as a DNA model that could be used in a preliminary investigation of the ability of *trans*-**5** to deliver the Pt(II) drug to a nucleobase,[23] and we tested the ability of **4** to form a supramolecular complex with the $(TBA)_2AMP$ **11**. The ^{1}H NMR spectrum of a mixture of **4** and $(TBA)_2AMP$ **11** (1.7:1 in CD_3CN, Figure S9 in SI) shows a broadening of all the calix resonances, especially for the pyrrole NH units that could not be located, even at −40 °C. The formation of a complex was also confirmed by a significant $\Delta\delta$ in the $^{31}P\{^1H\}$ NMR spectrum (4.20 to 3.10 ppm). The reaction of *trans*-**5** with $(TBA)_2AMP$ was investigated by ^{1}HNMR in CD_3CN (1:1; 1.5 mM, 25 °C). On mixing the reagents, the pyrrole NH units shifted and appeared as broad signals at higher ppm values.

dx.doi.org/10.1021/ja307791j | J. Am. Chem. Soc. XXXX, XXX, XXX−XXX

Table 1. In Vitro Cytotoxicity of Compounds 6 and 4 and of Their Pt(II) Derivatives *trans*-8 and *trans*-5, Compared with That of *trans*- and *cis*-$[PtCl_2(NH_3)_2]$, Oxaliplatin, and Carboplatin in a Human Cancer Cell-Line Panel As Assessed by the MTT Cell Viability Assay[a]

cmpd	A2774[b]	OVCAR3[b]	SKOV3[b]	MDA-MB-231[c]	MCF7[c]
6[d]	>100[e]	>100	>100	>100	>100
trans-**8**[d]	32 (8)	48 (13)	46 (16)	39 (8)	64 (51)
4[d]	98 (60)	103 (41)	106 (42)	89 (12)	114 (31)
trans-**5**[d]	23 (3)	35 (13)	28 (17)	26 (12)	40 (27)
trans-$[PtCl_2(NH_3)_2]$[d]	>100	>100	>100	>100	>100
cis-$[PtCl_2(NH_3)_2]$[f]	5 (4)	6 (2)	4 (1)	5 (1)	18 (12)
oxaliplatin[f]	1.3 (1)	0.5 (0.1)	0.7 (0.2)	1.7 (1)	1 (0.3)
carboplatin[f]	54 (1)	56 (2)	73 (6)	63 (13)	89 (7)

[a]Values are IC_{50}, in μM, expressed as mean values (±SD) of at least three independent experiments (72 h drug exposure). [b]Human ovarian cancer cell line. [c]Human breast cancer cell line. [d]Concentration range from 10 to 100 μM/L. [e]>100: no cytotoxic activity was detected within the concentration range tested. [f]Concentration range from 0.05 to 500 μM/L.

These changes are consistent with the formation of a complex between the calixpyrrole moiety of *trans*-**5** and the anionic component of the nucleotide. All of the nucleotide resonances were also considerably broadened. The substitution toward the Pt(II) complex by the nucleobase was indicated by the formation of uncoordinated **4**, while all of the AMP protons broadened, hampering any δ assignment (Figure S10 in SI). When the same experiment was performed in CD_3CN/D_2O, a number of signals could be detected for the adenine moiety (Figure 3) consistent with a mixture of complexation products which were not isolated.[22b] However, additional evidence for AMP coordination was provided by ESI-MS (negative ion mode) of the crude mixture (Figure S11 in SI), which contained signals at m/z values consistent with the presence of $[Pt(OH)_2(CH_3CN)AMP]$, $[PtCl(OH)(CH_3CN)AMP]$, $[PtCl_2(CH_3CN)AMP]$, and $[PtCl(CH_3CN)(AMP)_2]$. All of these peaks exhibited an isotopic pattern consistent with the indicated composition.

To investigate whether the calixpyrrole fragment played an active role for the transfer of Pt(II) from *trans*-**5** to the nucleobase, the kinetic behavior of the reaction was analyzed, and this could be fitted to a first-order model (the observed $t_{1/2}$ differences, i.e. 690 and 670 s for *trans*-**5**:**11** being 1:1 and 1:4, respectively, were within experimental errors, see Figure S12 in SI). These kinetic results are consistent with the coordination of the nucleobase taking place within the preformed [*trans*-**5**·**11**]complex. Although we could not measure the association constant for this complex by NMR titration, it is evident that at the concentration used for this study it is little dissociated. Increasing the concentration of **11** cannot significantly shift the equilibrium toward its formation any further, and if the reaction occurs mostly within the [*trans*-**5**·**11**] complex, the reaction rate is only marginally affected by changes in the concentration of **11**. Additional evidence for the 'intra complex' occurrence of the coordination is provided by the interference of TBAF, because fluoride can effectively compete with phosphate binding by the calixpyrrole unit (Figure S13 in SI).[24] In fact, the observed $t_{1/2}$ for the reactions [$(TBA)_2$AMP + *trans*-**5** (1:1)] and [$(TBA)_2$AMP + *trans*-**5** + TBAF (1:1:1.1)], at 25 °C, 1.5 mM *trans*-**5** in CD_3CN, were 690 and 1200 s (see Figure S14 in SI for the latter value).

Reactions of *trans*-**5** with **11** in CD_3CN/D_2O (80:20 v/v) could still be fitted to a first-order rate law, although they were slower than the analogous reactions in dry CH_3CN, with the only effect that the increased concentration of **11** moved the equilibrium toward the complete formation of the final species, suggesting the presence/contribution of a more complex model, but we did not investigate this further (Figure S15 in SI). Calixpyrroles have been shown to be able to bind anions with significant strength even in the presence of water,[25] although less effectively than in the absence of hydrogen-bonding competing solvents. However, water may also produce Pt(II) aqua species, and these would be reactive intermediates producing 'apparent' first-order kinetic behavior. However, *trans*-**8** reacted with **11** with a first-order kinetic behavior, at essentially the same rate in both dry CD_3CN and CD_3CN/D_2O (80:20 v/v) (Figures S16, S17 in SI, $t_{1/2}$ 1500 and 1400 s, respectively). The dipyrromethane unit of *trans*-**8** is expected to bind phosphate considerably less effectively than the calixpyrrole in *trans*-**5**, and for *trans*-**8** there was no evidence of an 'intracomplex' reaction of **11**. Since an intramolecular substitution could not take place, most probably in this case the first-order kinetic could be ascribed to a solvolytic pathway. Overall, the above kinetic studies indicate that the calixpyrrole unit is playing a role in the nucleobase coordination by means of *trans*-**5** under dry conditions, and it *might* still contribute to some extent in the presence of water.

Ligands associated with either *trans*- or *cis*-Pt(II) have been shown to play a crucial role in the cytotoxic activity of this metal.[7] We therefore tested *trans*-**5** and *trans*-**8** for their cytotoxicity toward a panel of human cancer cell lines (Table 1) using a cell viability assay based on tetrazolium reduction. A comparison between *trans*-**5** and *trans*-**8** was expected to define the importance of the whole calixpyrrole moiety, free ligands **4** and **6** were used as 'blanks', *trans*- and *cis*-$[PtCl_2(NH_3)_2]$, oxaliplatin, and carboplatin as benchmarks.[8c] The cytotoxic activity of the different compounds and complexes was first tested in dose−response experiments at different time points (24, 48, and 72 h) of continuous exposure. The representative experiments (see Figure S18 in SI) show that the cytotoxic activity of *trans*-**5** and *trans*-**8** is already observed at 24 h and reaches its maximal effect at 72 h. Interestingly, the cytotoxic effect of *trans*-**5** and *trans*-**8** was detectable earlier than that of the reference cytotoxic drug, *cis*-$[PtCl_2(NH_3)_2]$, which had limited effect at 24 h. As reported in Table 1, A2774, OVCAR-3, and SKOV-3 ovarian carcinoma cells were sensitive to the *trans*-**5** complex with an average IC_{50} of 23, 35, and 28 μM, respectively (at 72 h), while *trans*-$[PtCl_2(NH_3)_2]$ showed no toxicity in the concentration range tested (10−100 μM). *trans*-**5** was consistently more effective than carboplatin but less than cisplatin and oxaliplatin. In addition, the calix[4]pyrrole derivative **4** had very limited toxicity with IC_{50} close to 100

dx.doi.org/10.1021/ja307791j | J. Am. Chem. Soc. XXXX, XXX, XXX−XXX

μM in the three cell lines. Complex *trans*-**8** also showed cytotoxic activity with IC_{50} of 32, 48, and 46 μM, respectively whereas the dipyrromethane derivative **6** showed no toxicity. Since DMSO is a ligand that does not promote the activity of Pt(II) derivatives[26] these data indicate that ligands **4** and **6** confer cytotoxic activity to the otherwise inert *trans*-$[PtCl_2(NH_3)_2]$. Similar results were obtained with breast cancer cell lines MDA-MB-231 and MCF7 (Table 1).

To mimic the situation in vivo, where the drug exposure time is limited by its clearance, cytotoxic activity was evaluated by a short treatment (3 h) of the cells with the different compounds and complexes, followed by cell culture for 72 h. As shown in the representative experiment of Figure S19 in SI, *trans*-**8** and *trans*-**5** had significant cytotoxic activity (IC_{50} of 61 and 66 μM, respectively), which appeared more similar to that of *cis*-$[PtCl_2(NH_3)_2]$ (IC_{50} = 56 μM) under these experimental conditions.

To gain more insight into the cytotoxic mechanisms of *trans*-Pt complexes **5** and **8**, we evaluated apoptosis by the annexin-V/propidium iodide (annexin-V/PI) method in A2774 cells after a 24-h treatment. As shown in Figure 4 (and Figure S20 in SI), both *trans*-**8** and *trans*-**5** complexes induced apoptosis in ovarian cancer cell lines. In particular, *trans*-**8** efficiently induced an apoptotic response, whereas in samples treated with *trans*-**5** a considerable percentage of necrotic cells was also detected.

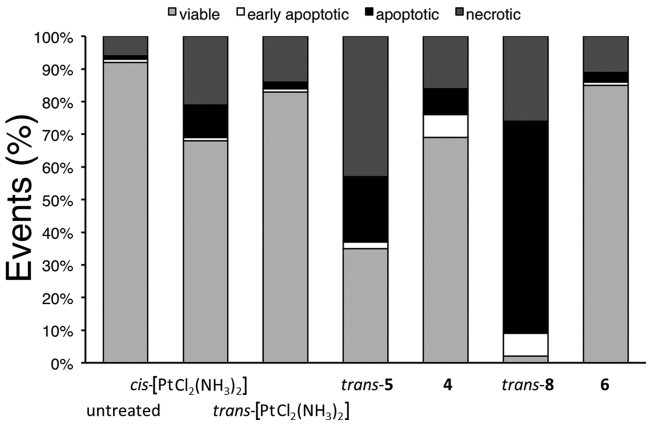


Figure 4. Flow-cytometric analysis of annexin-V/PI staining of A2774 ovarian cancer cells treated with the different compounds at their IC_{80} dose or their acetonitrile solvent for 24 h. Viable cells take up no stains. Cells stained with PI alone are necrotic, whereas cells stained with annexin V-FITC alone represent early apoptosis. Cells in the final stages of apoptosis take up both stains. Data are expressed as the percentage of cells in each category.

The antitumor activity of *cis*-$[PtCl_2(NH_3)_2]$ is related to the induction of apoptosis.[27] However, both apoptosis and necrosis have been found in the same population of *cis*-$[PtCl_2(NH_3)_2]$-treated cells,[28] possibly as a result of cisplatin-induced damage to the apoptotic program.[29]

CONCLUSIONS

This work was stimulated by the results of the kinetic studies discussed above and by the idea that calixpyrrole **4** could target the delivery of platinum drugs to DNA. We currently cannot provide irrefutable proof that the observed cytoxic activity of *trans*-**5** can be explained by a mechanism involving the initial formation of a complex between its calixpyrrole unit and the DNA phosphate groups under the conditions and in the presence of the complex chemical mixture that is used for cell cultures. Nevertheless, our hypothesis is supported by a molecular modeling and docking simulation (see SI and Figure S21). According to this model, *trans*-**5** forms hydrogen bonds between the pyrrole rings of the calyx and two oxygen atoms of a phosphate, consequently pointing the *trans*-Pt inside the minor groove toward two consecutive adenine residues. The results obtained to date indicate that the calixpyrrole unit has considerable potential for the development of new anticancer *trans*-Pt(II) drugs. In vitro *trans*-**5** outperforms the widely used carboplatin but lags behind oxaliplatin and the very toxic cisplatin. A comparison with the compounds used as blanks also indicates that *trans*-**5** and *trans*-**8** reach the biological site of action (most likely DNA) before losing the ligands **4** and **6** respectively. In this study we found that calixpyrrole **4** and dipyrromethane **6** have low cytotoxicity. We also observed that dipyrromethane **6** may produce proliferative effects (see Figure S18 in SI), and we plan additional studies to uncover the origins of this property. To the best of our knowledge, the work presented here is the first report on the biological activity of calixpyrroles, and it also shows that this class of receptor molecules, whose host guest chemistry was uncovered by the seminal work of Sessler and co-workers,[11] is still rich in potential applications even after 16 years of research.

ASSOCIATED CONTENT

Supporting Information

Experimental procedures, spectroscopic data, and crystallographic data for *trans*-**5**. This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author

franz@unime.it

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We thank Prof Luigi Monsù Scolaro for helpful discussions, Miss Silvia Carbone for helping with the biological assays, Mr. Gaetano Irrera (ISMN-CNR) for technical assistance, the Consiglio Nazionale delle Ricerche (CNR), AIRC Grant 5955, and the Compagnia di San Paolo di Torino and the Italian Ministry of Health for financial support.

REFERENCES

(1) (a) Venkataraman, S.; Hedrick, J. L.; Ong, Z. Y.; Yang, C.; Ee, P. L. R.; Hammond, P. T.; Yang, Y. Y. *Adv. Drug Delivery Rev.* **2011**, *63*, 1228−1246. (b) Yang, K.; Weng, L.; Cheng, Y.; Zhang, H.; Zhang, J.; Wu, Q.; Xu, T. *J. Phys. Chem. B* **2011**, *115*, 2185−2195. (c) Medina, S. H.; El-Sayed, M. E. H. *Chem. Rev.* **2009**, *109*, 3141−3157. (d) Tekade, R. K.; Kumar, P. V.; Jain, N. K. *Chem. Rev.* **2009**, *109*, 49−87.

(2) Fang, J.; Nakamura, H.; Maeda, H. *Adv. Drug Delivery Rev.* **2011**, *63*, 136−151.

(3) (a) Bei, D.; Meng, J.; Youan, B.-B. C. *Nanomedicine (London, United Kingdom)* **2010**, *5*, 1385−1399. (b) Rao, J. *ACS Nano* **2008**, *2*, 1984−1986. (c) Haag, R.; Kratz, F. *Angew. Chem., Int. Ed.* **2006**, *45*, 1198−1215.

(4) (a) Teow, Y.; Valiyaveettil, S. *Nanoscale* **2010**, *2*, 2607−2613. (b) Low, P. S.; Henne, W. A.; Doorneweerd, D. D. *Acc. Chem. Res.* **2008**, *41*, 120−129. (c) van Zutphen, S.; Reedijk, J. *Coord. Chem. Rev.* **2005**, *249*, 2845−2853. and references therein. (d) Ndinguri, M. W.; Solipuram, R.; Gambrell, R. P.; Aggarwal, S.; Hammer, R. P.

Bioconjugate Chem. **2009**, *20*, 1869−1878. (e) Mukhopadhyay, S.; Barnes, C. M.; Haskel, A.; Short, S. M.; Barnes, K. R.; Lippard, S. J. *Bioconjugate Chem.* **2008**, *19*, 39−49.

(5) Abu-Surrah, A. S.; Kettunen, M. *Curr. Med. Chem.* **2006**, *13*, 1337−1357. (b) van Zutphen, S.; Pantoja, E.; Soriano, R.; Soro, C.; Tooke, D. M.; Spek, A. L.; den Dulk, H.; Brouwer, J.; Reedijk, J. *Dalton Trans.* **2006**, 1020−1023.

(6) For several examples of supramolecular chemistry used for the 'drug delivery' of Pt compounds see: (a) Kapp, T.; Dullin, A.; Gust, R. *Bioconjugate Chem.* **2010**, *21*, 328−337. (b) Chen, G. J.; Kumar, J.; Gregory, A.; Stenzel, M. H. *Chem. Commun.* **2009**, 6291−6293. (c) Malik, N.; Evagorou, E. G.; Duncan, R. *Anticancer Drugs* **1999**, *10*, 767−776.

(7) (a) Harper, B. W.; Krause-Heuer, A. M.; Grant, M. P.; Manohar, M.; Garbutcheon-Singh, K. B.; Aldrich-Wright, J. R. *Chem.—Eur. J.* **2010**, *16*, 7064−7077.

(8) (a) Aris, S. M.; Farrell, N. P. *Eur. J. Inorg. Chem.* **2009**, 1293−1302. (b) Coluccia, M.; Natile, G. *Anti-Cancer Agents Med. Chem.* **2007**, *7*, 111−123. (c) Kelland, L. *Nat. Rev. Cancer* **2007**, *7*, 573−584. (d) Radulovic, S.; Tesic, Z.; Manic, S. *Curr. Med. Chem.* **2002**, *9*, 1611−1618. (e) Natile, G.; Coluccia, M. *Coord. Chem. Rev.* **2001**, *216−217*, 383−410.

(9) (a) Gale, P. A.; Anzenbacher, P.; Sessler, J. L. *Coord. Chem. Rev.* **2001**, *222*, 57−102. (b) Gale, P. A.; Sessler, J. L.; Kral, V. *Chem. Commun.* **1998**, 1−8.

(10) Custelcean, R.; Delmau, L. H.; Moyer, B. A.; Sessler, J. L.; Cho, W.-S.; Gross, D.; Bates, G. W.; Brooks, S. J.; Light, M. E.; Gale, P. A. *Angew. Chem., Int. Ed.* **2005**, *44*, 2537−2542.

(11) Allen, W. E.; Gale, P. A.; Brown, C. T.; Lynch, V. M.; Sessler, J. L. *J. Am. Chem. Soc.* **1996**, *118*, 12471−12472.

(12) Gale, P. A. *Acc. Chem. Res.* **2011**, *44*, 216−226.

(13) Sessler, J. L.; Gale, P. A.; Genge, J. W. *Chem.—Eur. J.* **1998**, *4*, 1095−1099.

(14) There are several examples in the literature of molecules (e.g., intercalators) that were linked to Pt(II) derivatives to target DNA. See for example Whan, R. M.; Messerle, B. A.; Hambley, T. W. *Dalton Trans.* **2009**, 932−939. For examples of anticancer activity of Pt(II) complexes of aromatic amines see: Osa, T.; Hino, H.; Fujieda, S.; Shimizu, F.; Arakawa, M. *Chem. Pharm. Bull.* **1986**, *34*, 1881−1887.

(15) Yang, W.; Yin, Z.; Wang, C.-H.; Huang, C.; He, J.; Zhu, Xi.; Cheng, J.-P. *Tetrahedron* **2008**, *64*, 9244−9252.

(16) Bruno, G.; Cafeo, G.; Kohnke, F. H.; Nicolo, F. *Tetrahedron* **2007**, *63*, 10003−10010.

(17) For examples of similar *cis*/*trans*-isomerisations see: (a) Kong, P.-C.; Rochon, F. D. *Inorg. Chim. Acta* **1982**, *61*, 269−271. (b) Szuecova, L.; Travnicek, Z.; Popa, I.; Marek, J. *Polyhedron* **2008**, *27*, 2710−2720. (c) Guido, E.; D'Amico, G.; Russo, N.; Sicilia, E.; Rizzato, S.; Albinati, A.; Romeo, A.; Plutino, M. R.; Romeo, R. *Inorg. Chem.* **2011**, *50*, 2224−2239 and refs therein.

(18) Anzenbacher, P., Jr.; Jursikova, K.; Lynch, V. M.; Gale, P. A.; Sessler, J. L. *J. Am. Chem. Soc.* **1999**, *121*, 11020−11021.

(19) (a) Pregosin, P. S. *Platinum NMR Spectrosc. Annu. Rep. NMR Spectrosc.* **1986**, *17*, 285−349. (b) Lippert, B. In *Progress in Inorganic Chemistry*; Lippard, S. J., Ed.; Wiley: New York, 1989; Vol. *37*, pp 1−97.

(20) (a) Monsù Scolaro, L.; Mazzaglia, A.; Romeo, A.; Plutino, M. R.; Castriciano, M.; Romeo, R. *Inorg. Chim. Acta* **2002**, *330*, 189−196. (b) Cafeo, G.; Lo Passo, C.; Monsù Scolaro, L.; Pernice, I.; Romeo, R. *Inorg. Chim. Acta* **1998**, *275−276*, 141−149.

(21) Cacciapaglia, R.; Di Stefano, S.; Mandolini, L. *Acc. Chem. Res.* **2004**, *37*, 113−122.

(22) The normal concentration of chloride in plasma should be between 8 and 16 mM; from http://www.medicalhealthtests.com/blog/pathology-test/chloride-plasma.html.

(23) Guanine N7 fragment in DNA is known to be one major target for *cis*-Pt(II) besides adenine N7; see: (a) Dijt, F. J.; Canters, G. W.; den Hartog, J. H. J.; Marcelis, A. T. M.; Reedijk, J. *J. Am. Chem. Soc.* **1984**, *106*, 3644−3647. (b) Martin, R. B. *Acc. Chem. Res.* **1985**, *18*, 32−38. (c) Navarro, J. A. R.; Lippert, B. *Coord. Chem. Rev.* **1999**, *185−186*, 653−667.

(24) The ^{1}H NMR titration of *trans*-5 with TBAF resulted in initial disappearance of the NH resonances which appeared as very broad signals at high ppm values in a 1:1 mixture (Figure S13 [SI]), thus hampering the measurement of a binding constant by this method.

(25) Buschmann, H.-J.; Schollmeyer, E.; Cleve, E.; Mutihac, L. *Anal. Lett.* **2011**, *44*, 2703−2710.

(26) (a) Yi, Y. W.; Insoo, B. *DNA Repair* **2011**, *10*, 1084−1085. (b) Fischer, S. J.; Benson, L. M.; Fauq, A.; Naylor, S.; Windebank, A. J. *NeuroToxicology* **2008**, *29*, 444−452.

(27) Eastman, A. The Mechanism of Action of Cisplatin: From Adducts to Apoptosis. In *Cisplatin. Chemistry and Biochemistry of a Leading Anticancer Drug*; Lippert, B., Ed.; Wiley-VCH: Basel, Switzerland, 1999; pp 111−134,

(28) Pestell, K. E.; Hobbs, S. M.; Titley, J. C.; Kelland, L. R.; Walton, M. I. *Mol. Pharmacol.* **2000**, *57*, 503−511.

(29) Gonzalez, V. M.; Fuertes, M. A.; Alonso, C.; Perez, J. M. *Mol. Pharmacol.* **2001**, *59*, 657−663.