Increasing DNA reactivity and *in vitro* antitumor activity of *trans* diiodido Pt(II) complexes with UVA light

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To Professor Giovanni Natile on the occasion of his 70th birthday

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1. ABSTRACT

Trans diiodido platinum(II) complexes bearing the same as well as different aliphatic amines (mixed-amines) have interesting biological activity; cytotoxicity and interactions with some important biological models have already been demonstrated. Herein we described the interaction of such compounds with ct-DNA, supercoiled and linearized plasmid DNA and 5-GMP. Interestingly, UV irradiation of these compounds results in an increase in reactivity towards DNA and 5-GMP in such model systems. Additionally, the cytotoxicity of the *trans*-Pt(II) complexes towards human cancer cells is noticeably increased when treatment is combined for 90 min with UVA-irradiation. With this work we provide evidence that *trans* diiodido compounds can be activated by UV-light over relatively short treatment times.

2. INTRODUCTION

The design of antitumor metallodrugs has been strongly guided by the structure of cisplatin for many years [1]. However, despite the clinical successes of this drug as well as the analogues carboplatin and oxaliplatin there are still many drawbacks to their use in anticancer treatment; such as adverse side- effects, the development of resistance to the therapy, and the relatively narrow spectrum of antitumor activity.[2] The discovery of the anti-tumor potential shown by non-conventional metallodrugs, for example *trans* Pt-complexes [3], has opened up new possibilities for metallodrug development.

There are multiple advantages of using transition metal-based complexes in treating cancer. For example, many key biological structures such as DNA form strong coordination complexes with transition metal ions, which can have an anti-tumor effect. Different metal ions adopt diverse geometries for a given ligand (Ru, Pt, Au, Os, Ir are examples), thus the electronic configuration of the metals can introduce a new shape in the complex. Metal ions also give the ligands more diverse reactivity, including photochemical reactions that promote unusual reactivity, for example activating platinum anticancer complexes with visible light.[4]

An important strategy in this trend is to reevaluate accepted rules, based on chemical and biological features, by using novel techniques and new approaches that were not available at the time the original rules were formulated. A reevaluation of some complexes previously discarded as inactive could provide new insights into the design of novel drug candidates. For example, pioneering work with cisplatin derivatives appeared to rule out iodido as a leaving ligand in classical structure-antitumor activity relationships [5]. Our reevaluation of the diiodido Pt(II) complexes began when we identified unexpected in vitro cytotoxicity for the complex *cis*-Pt(isopropylamine)I_2, which also showed a particular strong reactivity versus S-donors such as cytochrome c and N-acetylmethionine. [6] The studies with the *cis* configured series bearing aliphatic amines showed much lower reactivity towards sulfur donors and a clear retention of the amine ligands in the overall adducts studied [7]. On the other hand, the *trans* series appeared more affected by the size of the amine ligand, showing particular differences in cytotoxicity in cancer cell lines when the size of the amine was varied.[8].

The elucidation of the mechanism of action of these complexes, possible targets and specificity versus cancer cell lines is important for the reevaluation of these complexes as potential antitumor drugs. We are particularly interested in using irradiation with UVA light to increase the reactivity of these molecules towards the biological targets in order to create a more powerful and selective metallodrug. The use of UVA radiation in photoactivation of platinum drugs has been described previously for Pt^{IV} complexes to produce photoreduction of the Pt and activation of the prodrug compounds [9, 10], and in Pt^{II} complexes to promote hydrolysis and increased reactivity towards model bases [11]. The UVA irradiation of diiodido Pt(IV) complexes has been previously studied as an activation process, but these were *cis* platinum complexes bearing stabilizing chelate ligands such as ethylenediamine [12, 13].

3. RESULTS AND DISCUSSION

The synthesis and characterization of **1-5** were performed according to our previous work[7, 8] and the information can be found in the experimental section of those publications. The complexes studied in this work were selected according to their varying reactivity toward biological model nucleophiles: a) *cis*-Pt(ipa)$_2$I$_2$ (Figure 1, compound **1**) showed adduct formation with S-containing model compounds with only Pt-I fragments where no trace of the spectator ligand was present. Moreover the reactivity of **1** towards DNA, observed in DNA model systems (5-GMP), supercoiled pBR322 and oligonucleotides, resulted in the same binding pattern as cisplatin [14, 15]. b) The parent *trans* complex (Figure 1, compound **2**) showed a very similar pattern to cisplatin and compound **1**, but compound **2** produces noticeable changes in the mobility of supercoiled DNA at lower concentrations than cisplatin. c) the *trans* complexes series (Figure 1, compound **3, 4** and **5**) showed an apparent correlation between the produced cytotoxic effects and the modification of supercoiled DNA mobility. The reactivity towards sulfur donors was similarly low or even

non-reactive in some of the cases (compound **4**). DNA was obviously a potential target in this particular case, and thus chosen for more detailed study.

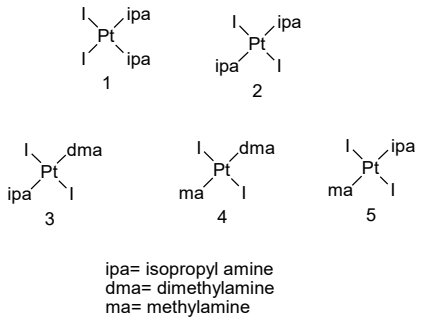

Figure 1. Platinum complexes studied in this work

3.1. DNA binding performed without UV irradiation

We first investigated in detail the interactions of the compounds with double stranded (ds) DNA by using various biochemical and biophysical methods. The nature of the interaction of these complexes with DNA was studied, followed by the evaluation of their binding kinetics to ds DNA by Atomic Absorption Spectroscopy (AAS). Finally, conformational distortions of DNA induced by binding to the Pt complexes were measured. The nature of this interaction has also been investigated in cell-free media and compared with previous results.

3.1.1. Transcription mapping of DNA adducts

In order to identify the preferential binding sites of the compounds, RNA synthesis on a DNA template modified by the platinum complexes **1**-**5** was performed. It has been demonstrated that in vitro RNA synthesis on DNA templates containing several types of adducts of platinum complexes can be prematurely terminated at the site of adduct formation or in close proximity to the Pt adducts.[16, 17] The experiments were carried out by using linear DNA fragment containing a T7 RNA polymerase promoter, modified at an r_b=0.015 by platinum compounds **1-5** and for comparative purposes also by cisplatin and transplatin. Fig. 2A shows that RNA synthesis on the template modified by the platinum complexes yielded fragments of defined sizes, which indicate that RNA synthesis on these templates was prematurely terminated. The sequence analysis revealed that the major bands resulting from termination of RNA synthesis by the adducts of the four *trans* compounds are similar to those produced by transplatin. Conversely, the bands generated by **1** are similar to those of cisplatin adducts (Fig. 2B). The results also indicate that the preferred binding sites for compounds **1**-**5** on DNA are guanine residues.

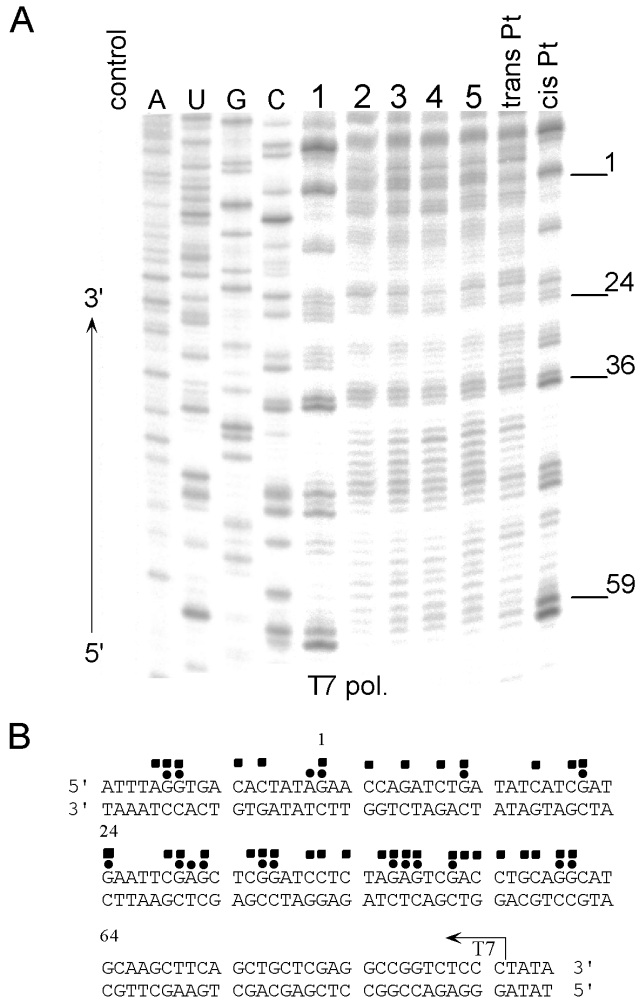

Figure 2. 3-RNA synthesis by T7 RNA polymerase on the NdeI/HpaI fragment of pSP73KB plasmid modified by cisplatin, transplatin, **1, 2, 3, 4** and **5**. **A**. Autoradiogram of 8% polyacrylamide/8 M urea sequencing gel. Lanes: control, non-platinated template: A, U, C, and G, chain terminated marker RNAs; **1-5**, transplatin, cisplatin, template modified by **1, 2, 3, 4, 5**, transplatin or cisplatin, respectively at r_b= 0.015. **B**. Sequence of the NdeI/ HpaI fragment of the pSP73KB plasmid. The arrow indicates the start of the T7 RNA polymerase, which used the upper strand as template. Numbers correspond to nucleotide numbering in the sequence of the pSP73KB plasmid. Circles and squares indicate stop signals from panel A, lanes cisplatin and transplatin, respectively.

3.1.2. Interstrand cross-linking

The transcription mapping experiments (Figure 3) suggest that the complexes **1-5** could form bidentate adducts on polymeric DNA, but whether these adducts were intrastrand or interstrand cross-links cannot be distinguished. Therefore, we quantify the interstrand cross-linking efficiency of **1-5** in linearized pSP73KB plasmid (2455 bp), which was modified by Pt compounds after it had been linearized by PvuII, an enzyme that cuts only once within pSP73KB plasmid. The samples were analyzed for the interstrand cross-links by agarose gel electrophoresis under denaturing conditions (Figure SM1). Under these conditions P^{32}-

5‘-end-labeled strands of linearized pSP73KB plasmid containing no interstrand cross-links migrated as a 2455-nucleotide single strand, whereas the interstrand cross-linked strands migrated more slowly as a higher molecular mass species. The intensity of the more slowly migrating band increased with the growing level of modification. The radioactivity associated with the individual bands in each lane was measured to obtain estimates of the fraction of non-cross-linked and cross-linked DNA. The frequency of interstrand cross-links was calculated by the Poisson distribution in combination with the r_b values and the fragment size (see summarized data in Table 1). The *cis* complexes compound 1 and cisplatin showed very similar cross-linking efficiency (% ICL per one molecule of the Pt complex) 5 and 6 %, respectively. The DNA interstrand cross-linking efficiency of *trans* platinum compounds **2**-**5** was 11-14 % which is similar or slightly higher than that of transplatin (12 %). [16]

Table 1. Percent Interstrand-CLs/adduct of CT-DNA modified by Pt complexes.

Compound	% interstrand CLs/adduct	Compound	% interstrand CLs/adduct
cisplatin	6	***3***	12
1	5	***4***	14
2	11	***5***	14

3.1.3 DNA unwinding

Electrophoresis in native agarose gel was performed to quantify the unwinding induced in pSP73 plasmid DNA by the platinum complexes by monitoring the degree of supercoiling (Figure SM1). The unwinding produced in the DNA duplex by the adducts of Pt complexes reduced the number of supercoils in closed, negatively supercoiled DNA and therefore the rate of migration through agarose gel, which made it possible to observe and quantify the degree of unwinding.[18]. The unwinding angle has been calculated using $\Phi = -18\sigma / r_b(c)$, whereby σ is the superhelical density and $r_b(c)$ is the value of r_b at which the supercoiled and relaxed forms co-migrate.[18] Under the present experimental conditions, σ was calculated to be -0.044 based on the data of cisplatin for which the $r_b(c)$ was determined in this study and $\Phi = 13°$ was assumed. By using this approach, we determined a DNA unwinding angle of 14 ± 3°, 22 ± 3°, 23 ± 3°, 23 ± 3°, and 24 ± 3° for complexes **1-5**, respectively. The unwinding angle value for compound **1** is very similar to the value of 13° determined for the adducts of cisplatin[18]. On the other hand, the unwinding angles produced by the adducts of *trans* complexes are significantly higher than the unwinding angle of 9° determined for the DNA globally modified by transplatin [18](Fig. SM2).

3.1.4 Quantification of monofunctional platinum adducts by thiourea treatment

Cisplatin and analogous bifunctional platinum compounds coordinate to DNA, first forming monofunctional adducts that afterwards close to form bifunctional adducts.[19, 20] To quantify the number of monofunctional adducts, thiourea (TU) is used to labilize and remove monofunctional DNA adducts [20, 21], The nucleobase nitrogen-platinum bond is weakened because of the strong *trans* effect of the coordinating sulfur of thiourea , thus making Pt susceptible to further substitution reactions. Thus, monofunctional Pt-DNA adducts are effectively removed from DNA, whereas bifunctional adducts are not.[20]

The treatment of the *trans* iodido complexes, **2, 3, 4** and **5** with TU at r_b = 0.05 was performed, including transplatin as a control for comparison purposes (Figure SM3). The results show that while transplatin formed ~60% of bifunctional adducts after 24 h of incubation, the four *trans* iodide complexes already formed ~80% bifunctional adducts, indicating that these iodide Pt complexes form bifunctional lesions more readily than transplatin.

3.1.5 DNA melting

Calf thymus (CT) DNA was modified by complexes **1-5** to various r_b values (0.02, 0.05, and 0.1). The melting curves of CT DNA were then recorded by measuring the absorbance at λ = 260 nm as a function of temperature. The plots in Figure 3 show that the melting temperature (t_m) of ct DNA modified by *trans* complexes **2-5** increase with the increasing level of platination while the modification of CT DNA by *cis* compound **1** resulted in a decrease in t_m value. It has been previously demonstrated that the thermal stability of DNA modified by metal complexes is affected by three factors: the stabilizing effects of both the positive charge of the metal moiety and DNA interstrand cross-links, and on the other hand a destabilizing effect of conformational distortions induced by intrastrand cross-links. [21] The positive impact of the *trans* compounds **2-5** on the thermal stability of CT DNA can be explained by increased formation of interstrand cross-links that prevent DNA strand dissociation. Interestingly, complex **1**, forms a much lower amount of interstrand cross-links and preferentially forms intrastrand cross-links that destabilize the helix.

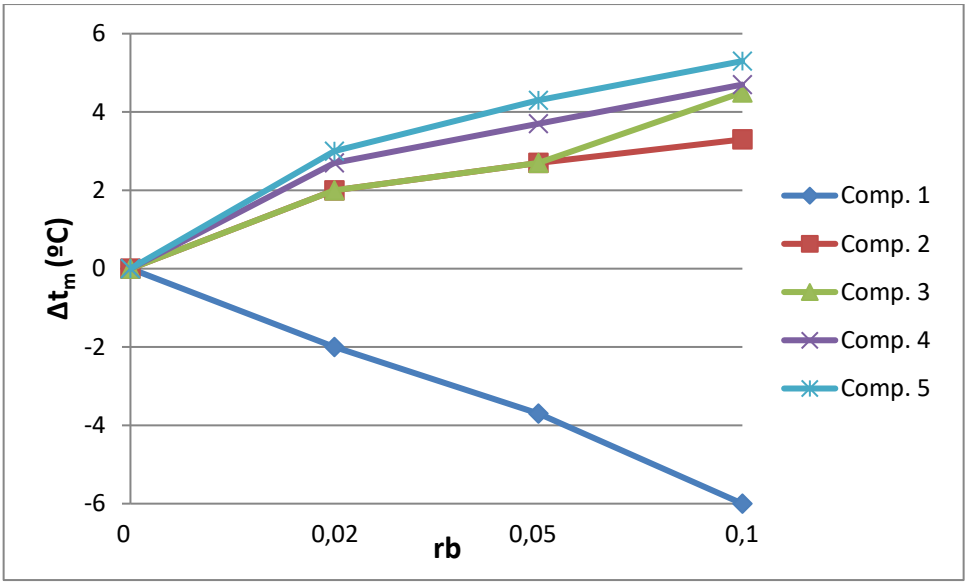

Figure 3. Plots of Δt_m of CT DNA modified by **1**, **2, 3, 4** and **5**. Δt_m is defined as the difference between the t_m values of platinated and unmodified DNA samples.

3.2. DNA binding performed under irradiation with UVA

3.2.1. Kinetics of binding to ct DNA

We selected compound **2** and **5** (as **3**, **4** and **5** showed a similar reactivity and DNA binding pattern) to study their interaction with DNA combined with UVA irradiation. This experiment was aimed at quantifing

the amount of platinum bound to DNA of complex **2** and **5**. Two set of samples of CT DNA were incubated with **2** at r_b = 0.1 in 10 mM $NaClO_4$ solution at 37 °C. The first set was irradiated during 4 h with UVA (λ_{max} =365 nm) immediately after addition of platinum complex and the second set was kept in the dark. Aliquots of these samples were withdrawn at 0; 2; 4 and 24 hours and assayed for Pt bound to DNA by FAAS [6].

The amount of Pt bound to DNA in samples irradiated with UVA increased over time more rapidly than if the samples were kept in the dark. Under continuous UVA irradiation, compounds **2** and **5** showed more than 70% and 80%, respectively, of Pt bound to DNA after 4 h (Figure 4). On the other hand, the amount of Pt bound to DNA in samples without irradiation was only 36 and 65 % for compounds **2** and **5**, respectively. This difference in binding levels without UVA irradiation is consistent with the greater bulkiness of the second amine ligand in **2** (isopropylamine) compare to the second amine ligand in **5** (methylamine). In general, we conclude that irradiation activates both compounds **2** and **5**, resulting in more rapid binding of Pt to DNA.

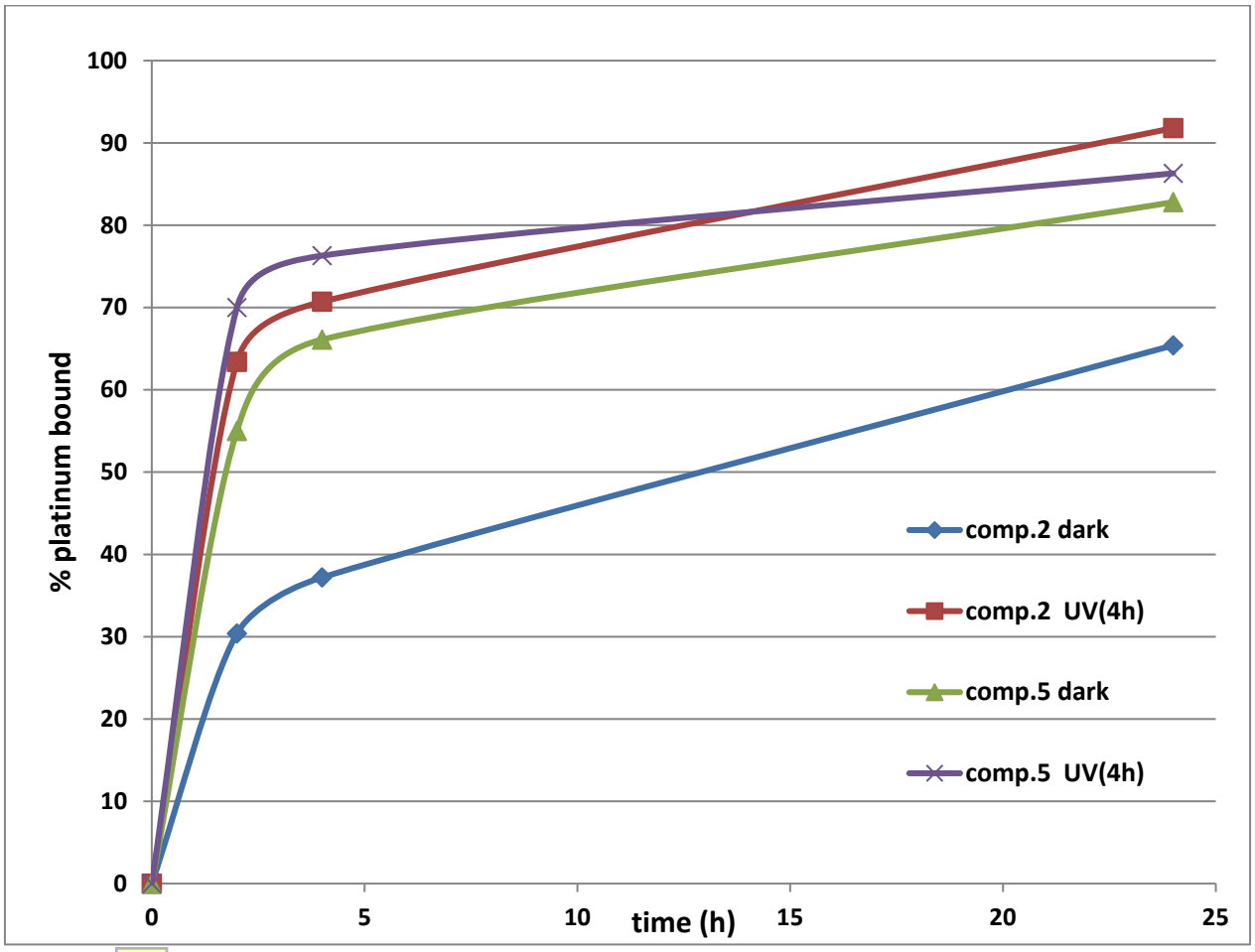

Figure 4. Kinetics of the reaction of complexes **2** and **5** with ct DNA, either photoactivated by UVA for up to 4 h. or kept in dark without irradiation.

3.2.2. Reactions of the complexes 2 and 5 with 5-GMP under UVA irradiation

To analyze the described effect with DNA in more detail, the reactivity of these compounds towards the nucleotide 5'-GMP was studied.

The interaction of compounds **2** - **5** with 9-ethyl guanine (9-EtG) and 5´-GMP in non-irradiating conditions has been evaluated previously.[8, 22] From those studies, it was known that the reactivity of these complexes followed the expected pattern for complexes with *cis* (complex **1**) and *trans* (complex **2** to **5**) geometries. The *trans* complexes, in particular, showed a similar trend in their reactivity through the experiments performed with the supercoiled pBR322, whereas the complex bearing the medium size aliphatic amines (complex **5**) showed only a moderate reactivity in those experiments.

With these results in mind, we studied the interaction of the *trans* compounds **2** and **5** with 5'-GMP; these were chosen because of their improved cytotoxicity (see below) and known reactivity with 9-EtG. The complexes were reacted under similar experimental conditions and with the same 4:1 ratio of Pt-complexes:GMP under irradiating conditions.

By monitoring the reaction of **2** with 5'-GMP by ^{1}H NMR, we clearly observed (Figure 5) formation of the monoadduct after only 1 h of UVA irradiation. However, after longer irradiation times (~ 2.5 h) this species disappeared. The sample solution became slightly murky and after centrifugation a colorless solid was observed. Compound **5** showed similar behavior towards 5´-GMP under irradiation. Its reactivity (Figure SM4) was triggered in the early stages of the reaction to produce precipitation at longer exposure times (2 h).

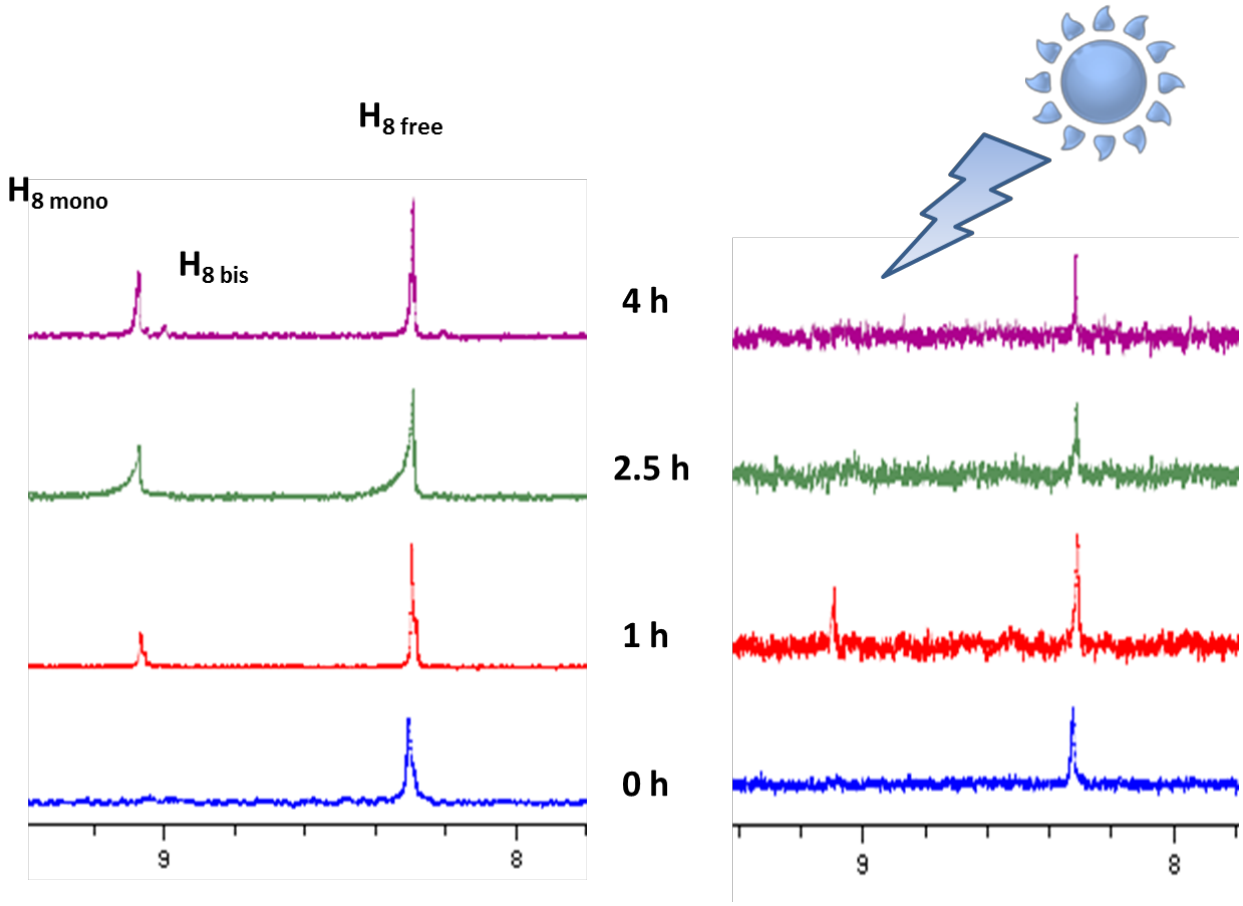

Figure 5. Comparison of the ^{1}H NMR spectra in the H8 area of reactions of complex **2** and 5'-GMP in the dark (left) and under irradiation with UVA (right) .

Attempts to fully characterize the solids isolated for compounds **2** and **5** were hindered due to their insolubility in common NMR solvents (except DMSO). The ^{1}H NMR from the final solid in DMSO was not of the best resolution, but it allowed us to establish that the amine ligands were not released in the final solid and that 5-GMP was also present (Figure SM5). The ESI mass spectra in both cases showed multiple fragmentations also indicative of GMP coordination.

To follow up to these experiments, we next studied the influence of irradiation on the cytotoxicity of the complexes on human cancer cell lines in culture.

3.3. Antiproliferative activity on cancer cells in vitro

The dark cytotoxicity of the complexes was evaluated previous [23]. Here, we investigated the effect of UVA radiation (λ_{max} = 350 nm) on the antiproliferative activity of the diiodido Pt(II)- complexes against two adherent human cancer cell lines: the non-small cell lung carcinoma, LCLC-103H and the uterine-cervical adenocarcinoma, SISO. Based on the results observed with the DNA and GMP irradiation, where we saw effects in the first hours, the irradiation duration of the cells preincubated with the Pt(II) complexes was set at 90 min. For these cytotoxicity studies, dark controls were always performed parallel to the irradiated samples, with and without platinum complex. Comparing the growth of cells not treated with compound to untreated dark controls, irradiation with UVA light for 90 min had no significant effect on growth: for the SISO and LCLC-103H cell lines, cell growth measured in four independent experiments with 90 min UVA-irradiation was 113 +/- 12% and 92 -/+ 24%, respectively, compared to dark controls. We then explored the possibility of selective activation of the diiodido complexes **1**, **2**, **3** and **5** by UVA-radiation in parallel incubations. Cells were treated with compound for 1 h followed by irradiation with UVA for 90 min. Then after 6 h the medium was changed and the cells allowed to grow an additional 88.5 h before cell growth was measured by the crystal violet method [23].

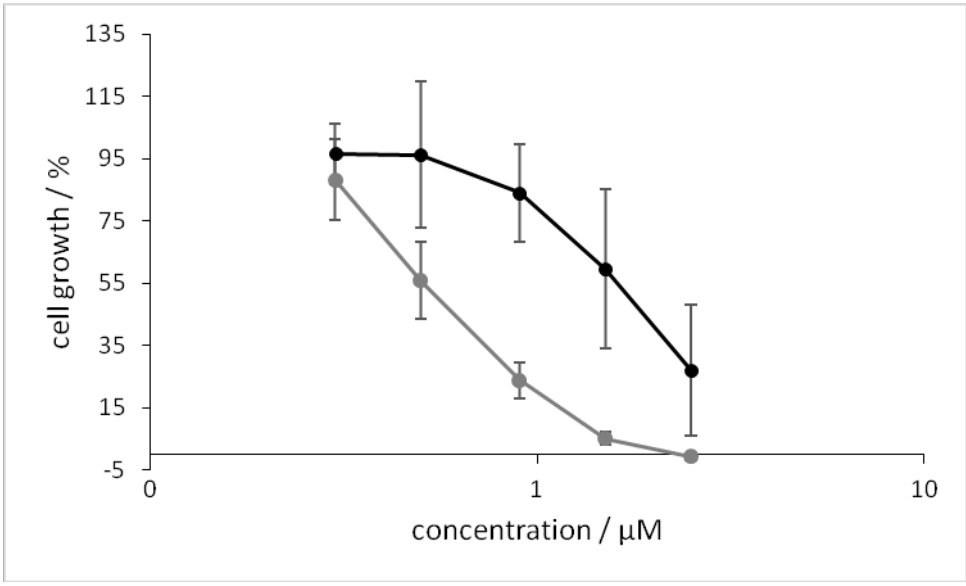

Figure 6. Growth inhibition of LCLC-103H cells after incubation with *trans*-[$PtI_2(ipa)_2$] (complex **2**), with 90 min irradiation with λ_{max} = 350 nm (grey curve) and in the dark (black curve). Averaged data from three independent, representative irradiation experiment in LCLC. Error bars represent ± SD.

The complexes were initially screened at 5 µM to determine whether further testing for IC_{50} values was warranted. Testing of the *cis* complex **1** at this concentration resulted in no changes in antiproliferative activity after irradiation of the cell lines SISO and LCLC-103H compared to non-irradiated controls. Thus, complex **1** was not evaluated further. On the other hand, the *trans* compounds **2**, **3** and **5** all showed noticeable improvement in antiproliferative potency when treatment was combined with irradiation.Representative dose-response curves are shown in Figure 6 for **2**, where it can be seen that there are noticeable increases in antiproliferative activity caused by UVA. In fact, the IC_{50} values of **2**, **3** and **5** all decreased in both cell lines after irradiation (Table 2); IC_{50} values for SISO and LCLC-103H lines dropped approximately 1.8- and 2.5-fold , respectively, when irradiated compared to the non-irradiated incubations. Nevertheless, the compounds tested appear to be quite active even in the dark, validating the cytotoxicity previously reported [8]. As a control, we compared the phototoxicity of the diiodido-Pt(II) complexes with *trans*-[$PtCl_2$(ipa)(ma)]; as expected, the photoactivation of the dichlorido complex was weaker than with the idodidos (Table 2).

Based on the DNA and 5'-GMP binding data in the previous two sections, the results of cytotoxicity testing would appear to support the idea that UVA light facilitated an increased activation of the diiodio Pt(II) complexes in cell culture. Alternatively, combinations of UVA light and the diiodio Pt(II) complexes could act synergistically to slow cell growth; e.g. a sub-cytotoxic exposure to UVA light for 90 min could enhance the DNA damage caused by the Pt(II) complexes. Further studies will be required to differentiate these two possibilities.

Table 2. Calculated IC_{50} values for **2**, **3**, **5** and the dichlorido complex in two human cancer cell lines, without and with irradiation with UVA ((λ_{max} = 350 nm, I = 0.12 mW / cm^2) for 90 min at 37 °CResults are averages of three independent experiments.

SISO	**IC_{50} / µM**		**Fold-activity increase**
compound	dark	UVA	
2 t-[$PtI_2(ipa)_2$]	2.46 ± 0.67	1.51 ± 0.55	1.63
3 t-[PtI_2(ipa)(dma)]	2.97 ± 0.29	1.65 ± 0.47	1.80
5 t-[PtI_2(ipa)(ma)]	4.98 ± 1.96	2.70 ± 1.02	1.84
t-[$PtCl_2$(ipa)(ma)]	2.64 ± 0.37	2.22 ± 0.66	1.19
LCLC-103H	**IC_{50} / µM**		**Fold-activity increase**
compound	dark	UVA	
2 t-[$PtI_2(ipa)_2$]	1.61 ± 0.55	0.63 ± 0.11	2.55
3 t-[PtI_2(ipa)(dma)]	2.83 ± 1.20	1.05 ± 0.27	2.69
5 t-[PtI_2(ipa)(ma)]	3.52 ± 1.46	1.44 ± 0.31	2.45
t-[$PtCl_2$(ipa)(ma)]	2.93 ± 0.60	1.77 ± 0.46	1.65

3.4 DFT calculations

In order to gain more insight into the photochemistry of the diiodido Pt(II) complexes, we ran a set of Density Functional Theory (DFT) and time-dependent DFT (TD-DFT) calculations on **1**, **2**, **5** and their 9-EtG adducts. The DNA basis mimic, 9-EtG, was employed instead of 5´-GMP to ease the computational work. After geometry optimization, we evaluated the single-singlet transitions on the complexes and gauged their excited-state chemistry. A summary of the most relevant bond distances for **1**, **2**, **5** and their 9Et-G derivatives are reported in Table SM1 and SM2.

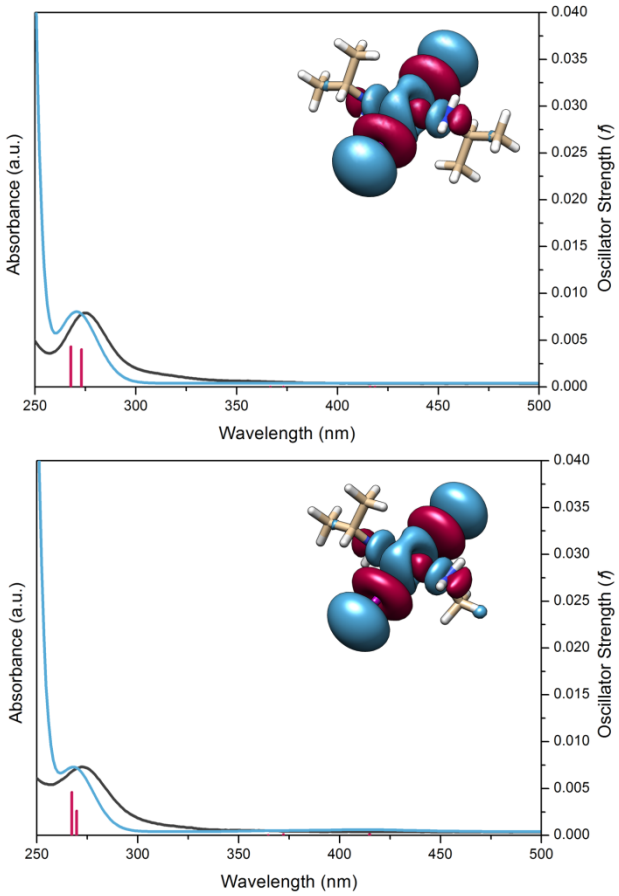

Figure 7. Experimental (black) and theoretical (light blue) absorption spectra for **2** (top), and **5** (bottom) in water at the CAM-B3LYP/LANL08/6-31G** level. Singlet-singlet transitions are shown as vertical bars with heights equal to their oscillator strengths. Inset: LUMO orbitals for **2** and **5**.

Singlet-singlet transition calculations and theoretical UV-vis spectra for **1**, **2**, **5** were in good agreement with experimental data (Figure 7 for **2** and **5** and Table SM3 for **1**). They display an absorption maximum in the 275–300 nm regions and an absorption tail up to around λ = 350 nm for **2** and **5** and λ = 400 nm for **1**. Analysis of the transitions composing the spectra (Table SM4) showed that they all have dissociative character (d–d/LMCT) due to dominant contributions of the LUMO (lowest unoccupied molecular orbital). This orbital is σ-antibonding with respect to the coordinated ligands in **1**, and towards the iodido ligands in **2** and **5** (inset of Figure 8 and Table SM5). Hence, light irradiation at λ = 365 nm should promote ligand dissociation, which in the case of **2** and **5** should lead to loss of the two halogens. Such a result is consistent with the increased DNA-binding properties of the two complexes compared to dark conditions.

A number of other platinum species can be generated upon dissolution of the complexes as well as during light irradiation. Therefore, we also optimized and calculated singlet excited states for 9-EtG derivatives of

2 and **5**. In particular, we studied 9-EtG mono-adducts bearing in the *trans* position either a iodido or an aqua ligand (**2-9EtG**, **2-9EtG-OH$_2$**, **5-9EtG** and **5-9EtG-OH$_2$**) and the *trans bis*-adducts **2-9EtG$_2$** and **5-9EtG$_2$** (Table SM6–8) Interestingly, all mono-adducts, as do the parent compounds, have dissociative transitions, indicating that light irradiation is capable of inducing further photoreactions. On the contrary, **2-9EtG$_2$** and **5-9EtG$_2$** have forbidden transitions at wavelength above 300 nm, and their LUMOs are a π-π* orbitals centered on the two 9-EtG.

4. MATERIALS AND METHODS

4.1. Materials and reagents

The stock solutions of the platinum complexes were prepared in N,N-dimethylformamide (DMF) and kept in the dark. The concentrations of platinum in the stock solutions and after dilution by water were determined by flameless atomic absorption spectrometry (FAAS). Calf thymus (CT) DNA (42% G + C, mean molecular mass approximately 20,000 kDa) and DMF were from Sigma (Prague, Czech Republic). Plasmids pUC19 [2686 base pairs (bp)] and pSP73KB (2455 bp) were isolated according to standard procedures. Restriction endonucleases and T4 polynucleotide kinase were purchased from New England Biolabs, T7 RNA polymerase and RNasin ribonuclease inhibitor from Promega (Mannheim, Germany). Ribonucleotide and deoxyribonucleotide triphosphates were from Roche Diagnostics, GmbH (Mannheim, Germany) and thiourea from Merck (Darmstadt, Germany). Agarose was from FMC BioProducts (Rockland, ME, USA) and radioactive reagents were from MP Biomedicals (Irvine, CA, USA).

4.1.1. Synthesis of the Complexes and their reactivity versus 5-GMP reactivity under irradiation

Complexes **1, 2, 3, 4** and **5** were synthesized and characterized according to previous publications from our laboratories [8].

4.1.2. Sample preparations for monitoring the reactivity of the complexes under irradiation by NMR

Samples of complex **2** and **5** were prepared by mixing 3 mM of complex **3** and 5 with 5'-GMP (3 mg, 22.5 x10^{-3} mmol) in D_2O:acetone-d_6 (2:2) to a final volume of 0.5 mL in an NMR tube. The tube was maintained at 37 °C under slight stirring during the entire experiment (24 h) in a thermoshaker for the series of samples assayed in dark (samples non irradiated) and in carrousel device inside a photoactivator (Luzchem Research, Inc. Option 1) for the samples assayed using UVA treatment (UV lamps of λ_{max} = 350 nm to achieve an irradiation of 270 nm). ^{1}H spectra monitoring was acquired from t = 0 to 24h. (With the first spectra recorded (t=0), the preparation of the sample had not been taken into consideration.) It is important to point out that the addition of the 5´-GMP solution to the compounds was very slow to avoid precipitation. In these preparations, we were required to use a larger volume of water than previously used for these kinds of complexes[8]. This new procedure required at minimum 10 to 15 minutes to mix both solutions (compound and 5´-GMP) which had been previously warmed up to 37 °C. The final white solid (WS), in both cases, was isolated by centrifugation and characterized by ESI mass spectrometry. The ^{1}H NMR was only recorded for WS-compound 2 in DMSO (figure SM5).

ESI+ for WS-Compound **2:** $[Pt(ipa)(ipa)(GMP)I]^+$+Na^+ at 592.9 m/z

ESI+ for WS-Compound **5**: $[Pt(ipa)(ma)(GMP)_2NaCl]^{2+}$ at 563.1 m/z , $[Pt_2(ipa)(ma)IGMP]^+$ at 951.9 m/z and $[Pt_2(ipa)_2(dma)I_2(GMP)]^+$ at 1102.,9 m/z; this last being the most abundant.

- **DNA melting**

The stock solution of the already described platinum complexes was prepared in dimethylformamide (DMF) and its concentration was determined by flameless absorption spectroscopy (FAAS). The melting curves of unplatinated CT DNA or that modified by platinum complex were recorded by measuring the absorbance at 260 nm (1 nm bandwidth, averaging time 10 s, heating rate 0.4 °C/min) as a function of temperature. The experiment was run simultaneously on six masked 1 cm pathlength cuvettes of 1.2 mL volume using a Peltier-controlled 6-sample cell-changer in a Varian Cary 4000 UV/vis spectrophotometer. The melting curves were recorded in a medium containing $NaClO_4$ (10 mM) buffered with Tris-HCl (1 mM) + EDTA (0.1 mM, pH 7.4). The value of the t_m was determined as the temperature corresponding to a maximum of the first derivate profile of the melting curves. The t_m values could be determined with an accuracy of ± 0.3 °C [24].

- **Transcription mapping of DNA adducts**

Transcription of the (NdeI/HpaI) restriction fragment of pSP73KB DNA treated with platinum complexes and T7 RNA polymerase and electrophoretic analysis of the transcripts were performed according to the protocols recommended by Promega [Promega Protocols and Applications, 43-46 (1989/90)] [16, 17]. The DNA concentration used in this assay was 4.0×10^{-5} M (relative to the monomeric nucleotide content).

- **Interstrand cross-linking assay**

The platinum complexes were incubated for 24 h at 37 °C with 0.8 µg of pSP73KB plasmid (2455 bp) after it had been linearized by PvuII and 5'-end labeled [γ-^{32}P]ATP using T4 polynucleotide kinase. The number of interstrand cross-links was analyzed by electrophoresis under denaturing conditions on an alkaline agarose gel (1%) [25]. After the electrophoresis had been completed, the intensities of the bands corresponding to single strands of DNA and interstrand cross-linked duplex were quantified. The frequency of interstrand cross-links was calculated as % ICL/Pt = XL / 5372 × r_b (pSP73KB plasmid contained 4910 nucleotides), where % ICL/Pt is the number of interstrand cross-links per adduct multiplied by 100, and XL is the number of interstrand cross-links per molecule of the linearized DNA duplex calculated assuming a Poisson distribution of the interstrand cross-links as XL = −ln A, where A is the fraction of molecules running as a band corresponding to the non-cross-linked DNA.

- **Characterization of platinum adducts by thiourea (TU)**

Double-stranded CT DNA (1×10^{-4} M) was incubated with transplatin and the *trans* platinum complexes **2**-**5** at $r_i = 0.05$ in $NaClO_4$ (10 mM) at 37 °C for 24 h. The reaction was stopped by dialysing the samples overnight (4 °C). In parallel experiments, the reactions were stopped by addition of TU solutions (0.5 M). These samples with TU were incubated 1 h at room temperature and then dialysed overnight at 4 °C. The platinum content of DNA with and without TU treatment was determined by FAAS [24].

- **Unwinding of negatively supercoiled plasmid DNA**

Unwinding of closed circular supercoiled pUC19 plasmid DNA was assayed by an agarose gel mobility shift assay. The unwinding angle Φ, induced per one DNA adduct of platinum complex, was calculated upon the determination of the r_b value at which the complete transformation of the supercoiled plasmid to the relaxed form was attained. Samples of plasmid DNA at a concentration of 2.2×10^{-4} M (related to the monomeric nucleotide content) were incubated with platinum complexes at 37 °C in the dark for 24 hours. Samples were precipitated by ethanol and redissolved in TAE (Tris-acetate/EDTA) buffer. One aliquot of the precipitated sample was subjected to electrophoresis on 1% agarose gels running at 25 °C in the dark with TAE buffer and the voltage set at 40 V. The gels were then post-stained with Ethidium Bromide,

followed by photography with a transilluminator. The remaining aliquot was used for the determination of r_b values by FAAS [5].

- **DNA binding of photoactivated platinum complex: kinetics of binding to CT DNA**

This experiment was aimed at quantifying the binding of platinum complex **2** and **5** (we did the experiment only with three compounds because all platinum complexes showed similar properties of binding to DNA). Two sets of samples of CT DNA were incubated with this Pt complex at r_b = 0.1 in $NaClO_4$ (10 mM) at 37 °C. The first set was irradiated for 4 h with UVA (λ_{max} = 365 nm) light immediately after addition of the platinum complex and the second set was kept in the dark. Aliquots of these samples were withdrawn at 0, 2, 4 and 24 h and assayed for Pt bound to DNA by FAAS as described previously [6]. The light source used in DNA photocleavage experiments was a Photoreactor LZC-ICH2 from Luzchem (Canada) fitted with UVA lamps (4.3 mW/cm^2, λ_{max} = 365 nm). The temperature in the light chamber during irradiation was approximately 37 °C.

- **Phototoxicity studies**

Human cancer cell lines were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ) (Braunschweig, Germany). RPMI cell culture medium 1640 from PAN Biotech (Aidenbach, Germany) was supplemented with 10 % fetal calf serum and antibiotics (penicillin and streptomycin).. The UVA lamps used for the cell experiments were Hitachi FL 8BL-B 8W lamps, which emit between λ 300-400 nm with λ_{max} = 350 nm [see Fig S3 in ref. 26]. Five lamps were fitted into a Luzchem Expo panel (Luzchem Research Inc., Ontario, Canada), mounted to the roof of the incubator 25 cm above the microtiter plates that were maintained at 37 °C [27]. A 3 mm thick acrylic glass filter between the lamps and the plates cut-off any light λ < 300 nm.

Cells were seeded at a density of 1000 cells per well (SiSo) and 250 cells per well (LCLC-103H) into microtiter plates and incubated for 24 h at 37 °C, in an atmosphere of 5 % CO_2. Afterwards, the cells were treated with dilutions of complexes in 0.1% DMF in medium for 1 h in the dark, followed by illumination with UVA ((λ_{max} = 350 nm, I = 0.12 mW / cm^2) for up to 90 min. Subsequently the cells were kept in the dark for 6 h before the medium was replaced by fresh culture medium (200 µL each well). The cells were then incubated an additional 88.5 h before the medium was discarded from the plates. Cells were fixed with 10 % glutaraldehyde. An antiproliferation assay based on the staining of cells with crystal violet was performed as previously described [23]. Estimated IC_{50} values are averages of at least three independent experiments.

- **Computational studies**

DFT and TD-DFT [28, 29] calculations on **1**, **2**, **5** and their 9-EtG adducts were performed with the Gaussian 09 [30] (G09) program package at the PBE1PBE:LANL08/6-31G**[31-33]level of theory for geometry optimization and at the CAM-B3LYP/LANL08/6-31G**[31, 32, 34] level to evaluate single-singlet transitions. The solvent effect was included by using the polarizable continuum model (PCM method) [35], with water as solvent. GaussSum 2.2.5 [36] was used to simulate the electronic spectra and to visualize the excited-state transitions as electron density difference maps [36, 37]. Molecular graphics images were produced using the UCSF Chimera package from the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco (supported by NIH P41 RR001081) [38].

5. CONCLUSIONS

The results presented here show that *cis* diiodido complex **1** binds to DNA in a similar way to cisplatin, in agreement with previous studies [14]. The interaction of the *trans* compounds **2**-**5** with DNA, on the other hand, differs considerably from that of transplatin. Measurement of cross-links showed approximately 11-14% of ICLs formed by these complexes, which is similar to the interstrand cross-linking efficiency of transplatin (12%). In contrast, the results demonstrate that *trans* compounds **2**-**5** form much lower levels of monofunctional adducts than transplatin after 24 h, evidence that *trans* compounds **2**-**5** form higher amounts of interstrand crosslinks than either cisplatin or **1**. Because of the stereochemistry of the complexes, it has been demonstrated that intrastrand cross-links between neighboring nucleotides in the same strand of DNA cannot form but rather 1,3-cross-links occur, in which two platinated bases are separated by a third base. These adducts are highly distortive for the DNA double helix.[39] The presence of greater amounts of 1,3-intrastrand cross-links formed by compounds **2-5** is further supported by two facts: a) a more efficient DNA unwinding induced by *trans* compounds **2-5** (22-24 °) in comparison with transplatin (9 °); and b) the t_m values of CT-DNA globally modified by compounds 2-5 only showed an increase of 4-6 °C. It is in contrast to transplatin, which increases the t_m of CT-DNA by 12.5 °C [24] while forming similar amount of interstrand cross-links compounds **2-5**

Interestingly, irradiation of the *trans* diiodido Pt(II) complexes **2 – 5** with UVA light led to a more rapid binding of Pt to CT DNA than when the complexes were incubated with CT DNA in the dark. Representative complexes **2** and **5** also reacted faster with 5'-GMP under irradiation with UVA compared to dark controls. The irradiation of the cells treated with the *cis*-diiodido compound did not lead to an increase in cytotoxicity, however the *trans* diiodido Pt(II) complexes **2**, **3** and **5** all showed noticeable improvements in antiproliferative potency when treatment was combined with UVA irradiation. Computational studies have showed the presence of dissociative electronic transitions, indicating that UVA irradiation is capable of inducing further photoreactions on the monoadducts and parent complexes. On the contrary, **2-9EtG$_2$** and **5-9EtG$_2$** have forbidden transitions at wavelength above 300 nm, and their LUMOs are a π-π* orbitals centered on the two 9-EtG, which seems to correlate with the reactivity profile observed by NMR.
All together, these results support the hypothesis that diiodido-plaitnum complexes possess interesting photobiological properties and are worthy of further, more detailed investigations.

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