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Light-Activated Destruction of Cancer Cell Nuclei by Platinum Diazide Complexes

Patrick J. Bednarski,[1,*] Renate Grünert,[1] Michael Zielzki,[1] Anja Wellner,[1] Fiona S. Mackay,[2] and Peter J. Sadler[2,*]

[1] Department of Pharmaceutical and Medicinal Chemistry
Institute of Pharmacy
University of Greifswald
17487 Greifswald
Germany

[2] School of Chemistry
University of Edinburgh
West Mains Road
Edinburgh EH9 3JJ
United Kingdom

Summary

A possible way to avoid dose-limiting side effects of platinum anticancer drugs is to employ light to cause photochemical changes in nontoxic platinum prodrugs that release active antitumor agents. This strategy could be used in the treatment of localized cancers accessible to irradiation (e.g., bladder, lung, esophagus, and skin). We report here that nontoxic photolabile diam(m)ino platinum(IV) diazido complexes inhibit the growth of human bladder cancer cells upon irradiation with light, and are non-crossresistant to cisplatin. Their rate of photolysis closely parallels that of DNA platination, indicating that the photolysis products interact directly, and rapidly, with DNA. Photoactivation results in a dramatic shrinking of the cancer cells, loss of adhesion, packing of nuclear material, and eventual disintegration of their nuclei, indicating a different mechanism of action from cisplatin.

Introduction

The platinum diammine complexes cisplatin and carboplatin are highly effective anticancer drugs, but their use is limited by dose-limiting side effects, by their restricted spectrum of anticancer activity, and by the development of resistance after repeated use in treatment [1]. As an approach to the avoidance of toxic side effects, we are exploring the use of light in causing photochemical changes in nontoxic platinum prodrugs that then release active antitumor agents [2–4].

In general, the d-shells of transition metal ions are a rich source of electronic transitions in the visible region of the spectrum. Particularly intense transitions can arise from ligand-to-metal or metal-to-ligand charge transfer (LMCT and MLCT). Examples of MLCT include Ru^{II} complexes, which are useful in studies of electron transfer pathways in proteins [5] and DNA [6]. Importantly, photoactivated excited-state metal complexes often possess enhanced chemical reactivity compared to the ground state. We have chosen octahedral $5d^6$ Pt^{IV} complexes, which possess LMCT bands, for photoactivation studies because they are kinetically inert under biological conditions and can undergo two-electron reduction to form potentially reactive square-planar Pt^{II} complexes [7]. The orally active Pt^{IV} drug JM216, for example (Figure 1A), undergoes chemical reduction in vivo to form active Pt^{II} analogs [8]. Pt^{IV} complexes containing iodo ligands (e.g., *trans*, *cis*-$[Pt(en)(OAc)_2I_2]$ [en = ethylenediamine]) possess intense LMCT bands and are photoactive, but biological thiols, particularly glutathione (GSH), readily attack the coordinated iodo ligand [9]. This leads to a premature reduction of Pt^{IV} to Pt^{II}, and makes these iodo complexes unsuitable for in vivo use.

Recently, we synthesized the photolabile diazido Pt^{IV} complexes *cis*, *trans*, *cis*-$[Pt(N_3)_2(OH)_2X_2]$ **4** (X = NH_3) and **5** (X_2 = en), potentially prodrugs of the Pt^{II} anticancer complex **1** (cisplatin) and its ethylenediamine analog **2**, respectively (Figure 1A) [10]. These Pt^{IV} complexes possess intense azide-to-Pt^{IV} charge-transfer bands, and the presence of axial hydroxo ligands decreases the reduction potential [7], and therefore increases stabilization of Pt^{IV} relative to Pt^{II} (in the dark). Indeed, we found [10] that the presence of 5 mM GSH (within the concentration range found in cancer cells [11]) had little effect on the stability of complex **4**. It seemed likely, therefore, that these complexes might reach the nucleus of cancer cells intact and exhibit phototoxicity.

Here we have studied the effects of complexes **4** and **5** on the growth and morphology of human bladder cancer cells, both in the dark and in the light, and the photolysis of the complexes in the absence and presence of a dinucleotide (d(GpG)) and DNA using irradiation conditions similar to those for the cell work. Intriguingly, G-G adducts were formed much more rapidly via photoactivation of the diazide complexes than they were with cisplatin, and no crossresistance to cisplatin in cancer cells was observed.

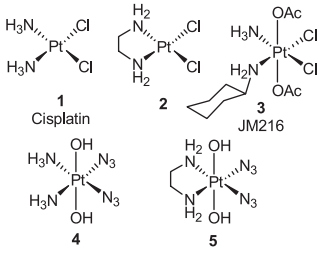
Results and Discussion

Cytotoxicity Studies

First, we investigated the effect of the photoactivation of complexes **4** and **5** on the growth of 5637 human bladder cancer cells. Control experiments showed no phototoxicity in these cells on irradiation at λ = 366 nm, and azide itself was relatively nontoxic (50% growth inhibitory concentration (IC_{50}) = 3.1 mM in the dark, 2.8 mM with light). When the cancer cells were treated with complexes **4** and **5** for 6 hr at 37ºC in the dark, the complexes exhibited very low inhibition of growth (Figure 1B). When they were irradiated with light (λ = 366 nm; I = 5.2×10^{-8} einsteins min^{-1}) during a 6 hr treatment, a selective inhibition of cell growth was observed, and the IC_{50} values decreased significantly from >300 μM to 49 and 63 μM for complexes **4** and **5**, respectively (Figure 1C). In contrast, the growth-inhibitory activities of both cisplatin (**1**) and the ethylenediamine complex **2** were unaffected by light. Cisplatin and **2** are more potent than their

*Correspondence: bednarsk@uni-greifswald.de (P.J.B.); p.j.sadler@ed.ac.uk (P.J.S.)

A



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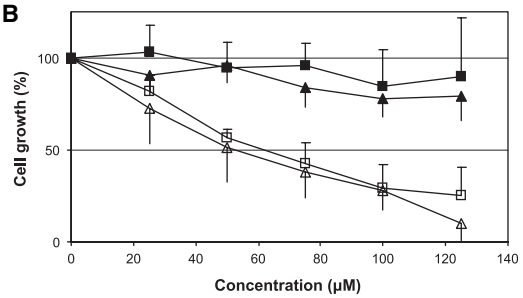


C

	IC$_{50}$ (μM)			
	5637 (N = 4)		5637-CDDP (N = 3)	
Pt-Complex	**with light**	**without light**	**with light**	**without light**
4	49.3 ± 28.1*	357 ± 81*	66.8 ± 17.5	> 200
Cisplatin	0.76 ± 0.18	0.78 ± 0.09	3.63 ± 0.93	3.03 ± 0.38
5	63.0 ± 20.2**	440 ± 143**	79.8 ± 16.6	>200
[Pt(en)Cl$_2$]	2.28 ± 0.13	2.47 ± 0.78	14.5 ± 4.1	9.67 ± 1.66

N= no. of independent experiments, *) $p < 0.01$, ** $p < 0.02$ (two-sided, paired Students t-test)

Figure 1. Effect of Light on the Activity of Platinum Diazido Complexes 4 and 5 toward Human Bladder Cancer Cells

(A) Structures of complexes 4 and 5, and of cisplatin 1, its chelated ethylenediamine (en) analog 2, and the Pt(IV) complex 3 (JM216), which has been in clinical trials and is activated by chemical reduction.

(B) 5637 Human urinary bladder cancer cells (German Collection of Microorganisms and Cell Culture, No. ACC 35) were seeded into 96-well microtiter plates and grown for 24 hr before treatment. Cells were exposed to 5 concentrations of 4 (triangles) or 5 (squares), respectively, for 6 hr at 37ºC, either in the dark (closed symbols) or with concurrent irradiation (open symbols). After the 6 hr drug exposure, the culture medium was replaced with fresh medium without HEPES, and the cells were then allowed to grow for an additional 90 hr. Data points represent the averages of four independent experiments, and error bars represent standard deviations. With the exception of the lowest concentration, the differences between the paired results from light and dark experiments are statistically significant ($p < 0.01$, two-sided, paired Student's t test). Error bars represent ±1 SD.

(C) IC$_{50}$ values for the inhibition of cell growth by complexes 4, 5, cisplatin, and [Pt(en)Cl$_2$] in the 5637 cell line and cisplatin-resistant 5637 cell line (5637-CDDP), with and without a concurrent irradiation with light for 6 hr at 37ºC. A two-sided, paired Student's t test was used to establish statistical significance.

photoactivated analogs 4 and 5, respectively, but this is not an unexpected finding, because the antitumor drugs carboplatin and oxoplatin, prodrugs of cisplatin that are chemically less reactive than the parent drug, are approximately 10- to 15-fold less active in the 5637 cell line compared to cisplatin.

Importantly, when irradiated, the platinum diazide complexes were equally toxic to 5637 and cisplatin-resistant 5637 cells (5637-CDDP). The 5637-CDDP cells exhibited a resistance factor (RF = IC$_{50}$[5637CDDP]/IC$_{50}$[5637]), of approximately 5 (both in the presence and absence of light), whereas RF was approximately 1 for the diazide complexes 4 and 5 in the presence of light (Figure 1C). This suggests that the mechanisms of cytotoxicity are different for cisplatin and the cytotoxic products of photolysis.

Fluorescence Microscopy

Changes in the morphologies of 5637 cells after exposure to either cisplatin, complexes 4 (*cis*, *trans*, *cis*-[Pt(N$_3$)$_2$(OH)$_2$(NH$_3$)$_2$]) or 5 (*cis*, *trans*-[Pt(en)(N$_3$)$_2$(OH)$_2$]), both in the dark and in the light, were investigated by fluorescence microscopy. Cells treated for 6 hr with either 1.25 or 2.5 μM cisplatin showed no changes in their morphologies 17 hr following treatment (data not shown). At 90 hr after exposure to 2.5 μM cisplatin (approximately the IC$_{30}$ concentration), the cells had grown larger than the controls, and possessed a multitude of white vacuoles (Figure 2A). The fluorescing nuclei also appeared larger and were more diffuse than in the control cells, and fluorescence could be seen in the cytoplasm. Nevertheless, the cells maintained contact with each other. Interestingly, the classic hallmarks of apoptosis (i.e., shrinkage and condensation of the cell, packing of the nuclear material, budding and cellular fragmentation, and nuclear breakup [karyolysis]) were not observed [12].

The effects of 4 on cellular and nuclear morphologies were documented 17 and 90 hr after a 6 hr treatment with and without irradiation with light (λ = 366 nm). Representative results for compound 4 are shown in Figure 2. In contrast to cisplatin, complex 4 had already caused rounding ("ballooning") of the cells by the end of the 6 hr irradiation period (Figure 2B). At 17 hr, the changes were characterized by cellular shrinkage and loss of contact with neighboring cells, as well as the plastic bottoms of the culture vessel (Figure 2C). Nuclear packing was dramatic in the fluorescence images, especially for cells treated with 100 μM complex. The cytoplasm also showed some fluorescence. At 90 hr, some of the cells had survived in the presence of the complex at doses of 25 and 50 μM, but they appeared larger than the control cells, with enlarged nuclei (Figure 2D). With 100 μM of the complex present (approximately the IC$_{30}$ concentration), however, the only cells that remained were shrunken, and most lacked a nucleus. Those nuclei still remaining were small and weakly stained, suggesting nuclear breakup. However, budding and cellular fragmentation were not observed, as would be expected with apoptosis [12].

These dramatic changes in the morphology of the cells were observed only when treatment with complex 4 was accompanied by irradiation. Without light, no changes in the cells were seen up to a concentration of 100 μM of 4 (Figure 2E). At 500 μM, the cells appeared to take on an appearance similar to that of cells treated with cisplatin. Comparable effects on the appearance of 5637 cells were found with complex 5 (see Figure S1 in the Supplemental Data available with this article online). These results indicate that photoactivatable complexes 4 and 5 cause a very different cytotoxicity compared with cisplatin. Further work will be required to elucidate the exact mechanism of cell death caused by the light-activated Pt^{IV} complexes; however, these initial results

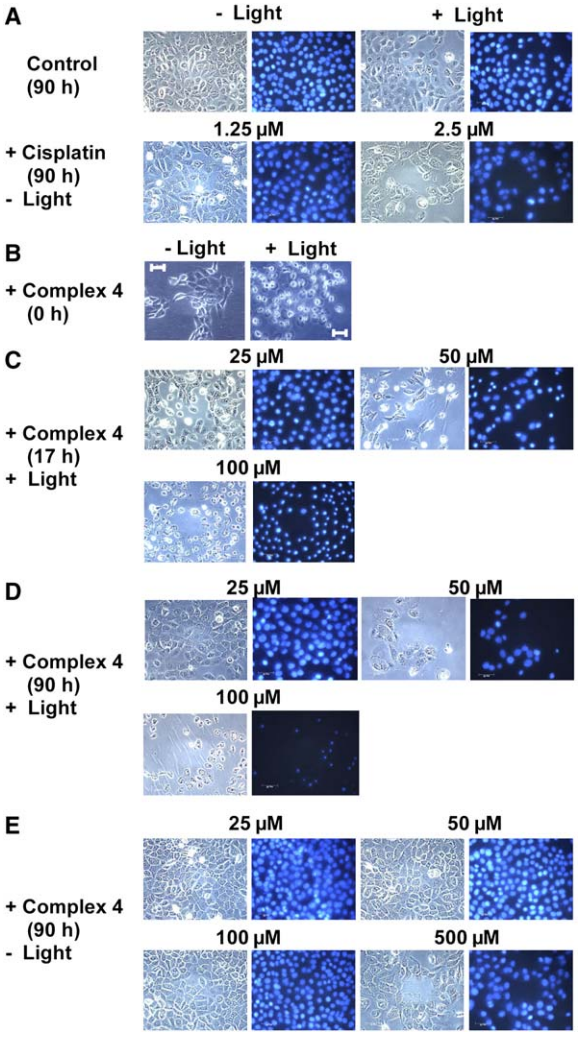


Figure 2. Phase-Contrast and Fluorescence Microscopy Studies at 400× Magnification of the Effect of Platinum Complexes and of Light on the Morphology of 5637 Cells and Their DNA Distribution

Cells were seeded into NUNC SonicSeal slide wells and allowed to grow for 24 hr before treatment.

(A) Effect of light but no addition of platinum, and 90 hr after a 6 hr treatment with cisplatin (without irradiation).

(B) Immediate effects of 100 μM 4 on 5637 cells after a 6 hr treatment without (left image), and with (right image) irradiation with light. Scale bar = 50 μm.

(C) At 17 hr after a 6 hr treatment with complex 4 with a 6 hr concurrent irradiation with light (λ = 366 nm).

(D) At 90 hr after a 6 hr treatment with complex 4 with 6 hr concurrent irradiation with light.

(E) At 90 hr after a 6 hr treatment without a 6 hr concurrent irradiation with light.

are not fully consistent with apoptosis being the mechanism of cell death.

Cell Uptake

We then studied the effect of light on the time-dependent uptake of complexes **4** and **5** by 5637 bladder cancer cells by atomic absorption spectroscopy (AAS). It is evident that light has no significant effect on Pt uptake up to 8 hr (Figure S2). Over the same time period, cisplatin reached approximately 5-fold higher concentrations of intracellular platinum, explaining some of the reduced activity of **4** compared with **1**.

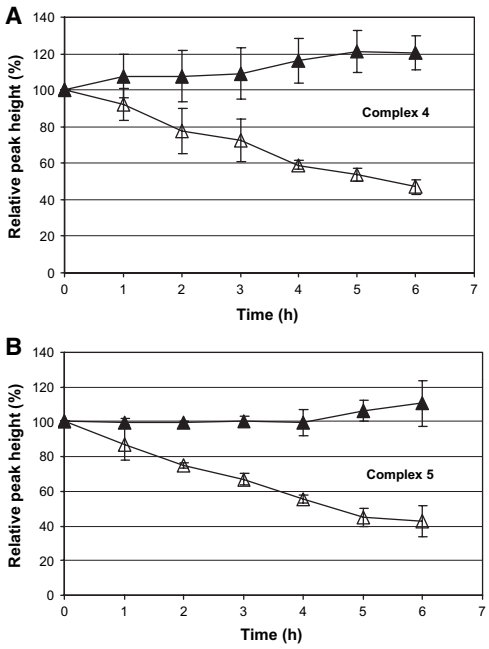


Figure 3. Effect of Light on the Stability of Platinum Diazido Complexes

(A) Complex **4**, and (B) complex **5**, in the presence (open symbols) and absence (closed symbols) of light. Solutions of the complexes (0.3–1.0 mM) in 50 mM phosphate buffer (pH 7.4) were incubated in 96-well plates (200 μl/well) at 37°C (water bath) and irradiated at a distance of 17 cm from a 40 W Lamag UV lamp (λ = 366 nm; intensity = $5.2 \pm 0.1 \times 10^{-8}$ einsteins min^{-1}). Error bars represent ±1 SD.

Stability and Photoactivation

Next, the effect of light, of the same wavelength and intensity, on the stability of the complexes alone was investigated. The complexes were stable in phosphate buffer pH 7.4 in the dark at 37°C, Figure 3, but decomposed in the presence of light. This was also confirmed by 2D [^{1}H, ^{15}N] NMR studies of ^{15}N-labeled complexes (Figure S3), which show the existence of photoisomerization and photoreduction pathways. ^{15}N NMR peaks with chemical shifts in the range −66 to −73 ppm characteristic of $^{15}NH_3$-Pt^{II} species were clearly visible after irradiation of an aqueous solution of the diammine complex **4** for 26 min. After 246 min, the major crosspeaks in the 2D [^{1}H, ^{15}N] HSQC NMR spectrum (Figure S3A) had ^{1}H, ^{15}N shifts of 4.02, −68.59 ppm (peak b), 3.87, −66.79 ppm (peak c), and 4.03, −72.60 ppm (peak d, minor). The $^{1}J(^{15}N\text{-}^{195}Pt)$ coupling constants associated with species b and c (286 Hz) suggest that they contain N ligands *trans* to Pt-NH_3 groups [13]; possible N ligands include NH_3 (which would require isomerization of the $Pt(NH_3)_2$ unit) or a breakdown product of azide. For the ethylenediamine complex **5**, a Pt^{II} product ($\delta(^{1}H)$, $\delta(^{15}N)$ 5.11, −33.43, Figure S3B) was detected after only 11 min. The $^{1}J(^{15}N\text{-}^{195}Pt)$ value of 377 Hz suggests that this is likely to be an aqua or hydroxo complex. It is notable that these products of photolysis are not the major products when nucleotides are present in irradiated solutions, suggesting that they or their precursors are

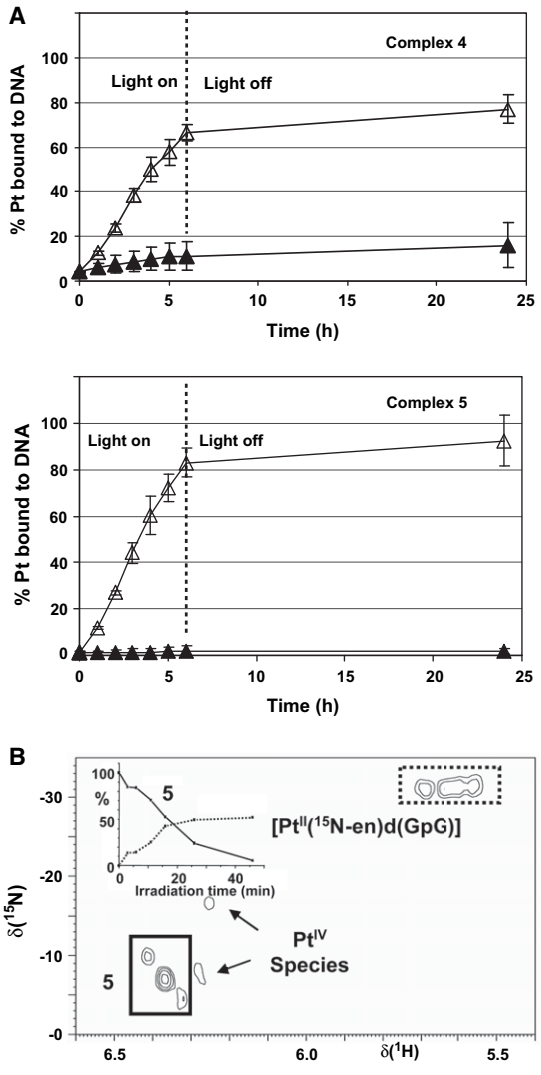


Figure 4. Binding of Photoactivated Platinum Diazido Complexes 4 and 5 to DNA

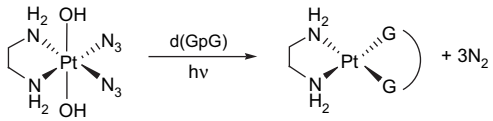
(A) Mixtures of Pt complexes (15 μM) and calf thymus DNA (0.25 mg/ml) in 10 mM PIPES buffer (pH 6.9) were incubated in 96-well microtiter plates (200 μl/well) at 37°C for various times in the dark (▲), or in the light (△; 17 cm from a 40 W Lamag UV lamp, λ = 366 nm; intensity = 5.2 ± 0.1 × 10^{-8} einsteins min^{-1}). The time-dependent changes in the amounts of Pt bound to DNA were determined using a flameless atomic absorption spectroscopy (AAS) method. Note that platination ceases when the light is switched off at 6 hr. Error bars represent ±1 SD.

(B) 2D [^{1}H, ^{15}N] HSQC NMR spectrum of a solution containing **5** *cis*, *trans*-[Pt(^{15}N-en)$(N_3)_2(OH)_2$] (0.75 mM) and d(GpG) (0.5 mM) after irradiation for 26 min (λ = 365 nm). The inset graph shows that formation of the crosslinked Pt^{II} adduct is complete within 30 min. Some photo-induced isomerization/substitution of the Pt^{IV} starting complex is also evident.

highly reactive with nucleobases (see below, Figures 4 and S4).

DNA Binding

Since the target for cisplatin and related anticancer drugs is DNA [14], we investigated the platination of calf thymus DNA by complexes **4** and **5** in the dark and with light of the same intensity and wavelength as in the cell experiments. The photolysis rates (Figure 3) closely parallel the rates of irreversible DNA platination by these two complexes (Figure 4A), indicating that the photolysis products react directly with DNA. The platination of DNA is directly dependent on light, and almost ceases when the light is switched off at 6 hr. Two-dimensional [^{1}H, ^{15}N] NMR studies of ^{15}N-labeled complexes provided further insight into the nature of the photochemical decomposition processes and their reactions with DNA bases. Irradiation of complex **5** in the presence of d(GpG) quickly gave rise to new crosspeaks (Figure 4B), assignable to Pt^{IV} isomers/substitution products and to the Pt^{II} species [Pt(^{15}N-en)d(GpG)-*N7,N7*], an intrastrand GG crosslink of the type formed by cisplatin on DNA. The ^{1}H NMR spectra in Figure S4 confirm that binding occurs to N7 of G. Overall, the photoreaction can be represented as:



It is notable that platination of DNA can occur more rapidly via this photoactivation pathway than by the usual chemical activation of cisplatin, which is determined by slow, rate-limiting hydrolysis steps [15, 16].

Significance

Stable *cis*-diam(m)ine *trans*-dihydroxo platinum(IV) diazido complexes bind to DNA rapidly upon activation by visible light, and can give rise to d(GpG) crosslinks of the type produced on DNA by the anticancer drug cisplatin. Intriguingly, the GG adducts are formed much more rapidly via photoactivation of diazide complexes than they are with cisplatin. The photoactivatable Pt^{IV} diazido complexes studied here platinate DNA by a different pathway to cisplatin, which involves the step-wise hydrolysis of the bound chloride ligands. The products from photolysis of 4 and 5 appear to react directly with DNA. Photoactivation may give rise to reactive products that do not have counterparts for Pt^{II} drugs. These Pt^{IV} diazido complexes are nontoxic to 5637 human bladder cancer cells in the dark, but are toxic to the cells upon irradiation. Photoactivation causes dramatic effects on the morphology of bladder cancer cells, including disintegration of their nuclei. The mechanism by which they kill cancer cells, therefore, appears to be different from that of cisplatin. Indeed, we found that these platinum diazido complexes were equally cytotoxic to 5637 cells and cisplatin-resistant 5637 cells. This new class of photoactivatable platinum complexes could, therefore, be useful in the treatment of cancers that are accessible to light, including bladder, lung, esophagus, and skin cancers. Since activation can be localized to the area of irradiation, this procedure has potential for avoiding the side effects that often accompany the use of cisplatin. Existing photodynamic agents rely on the conversion of ground state triplet oxygen to toxic singlet-state oxygen [17]. The activity of platinum diazide complexes does not rely on the presence of oxygen, which is a potential advantage, as some tumors are oxygen deficient.

Experimental Procedures

Materials

The human bladder cancer cell line 5637 (ACC 35) [18] was obtained from the German Collection of Microorganisms and Cell Culture (Brauschweig, Germany). The cisplatin-resistant cell line, 5637-CDDP, was raised from the 5637 line in our laboratories, as previously described [19]. Cell culture media, serum, antibiotics, trypsin/EDTA, DAPI, and spectral-grade DMF were from Sigma-Aldrich (Taufkirchen, Germany). Porcine pancreatin was from Fluka (Taufkirchen, Germany), and HEPES was from Merck (Darmstadt, Germany). All plastic culture supplies were from Starsted (Nümbrecht, Germany). Cisplatin was obtained from ChemPur (Karlsruhe, Germany). Complexes **4**, **5**, and [Pt(en)Cl_2] were synthesized in our laboratories following published procedures [10].

NMR

1D [^{1}H], 2D [^{1}H,^{15}N] HSQC NMR spectra were recorded on a Bruker DMX 500 NMR spectrometer (^{1}H: 500.13 MHz; ^{15}N: 50.7 MHz; Bruker UK Ltd., Coventry, UK) in 90% H_2O/10% D_2O, using dioxane (3.764 ppm) as the internal $\delta(^1H)$ standard. All $\delta(^{15}N)$ values were referenced externally to $^{15}NH_4^+$ at δ = 0 ppm. The pH value of all samples was adjusted to 5 with $HClO_4$ before recording spectra so as to ensure slow exchange (on the NMR time scale) of NH protons of the Pt^{IV} species present. Spectra were acquired at 25ºC, and processed using Xwinnmr (version 2.0, Bruker UK Ltd.) software. The light source was a 365 nm UV lamp (2 × 15 W tubes, model VL-215L; Merck Eurolab, Poole, UK).

Cytotoxicity Studies

The cell lines were maintained in RPMI medium supplemented with 10% fetal calf serum (FCS) and antibiotics (benzylpenicillin and streptomycin). Cells were kept at 37ºC in an atmosphere of 5% CO_2 air. Under these conditions, the 5637 line achieved a maximum doubling time of 26 hr.

The 5637 and 5637-CDDP cells were seeded into 96-well microtiter plates in 100 μl medium at a density of 1000 cells/well. The plates were returned to the incubator for 24 hr. The next day, complexes **4** and **5** were dissolved directly in culture medium (supplemented with 10 g/l HEPES) to achieve a stock solution of 400 μM. Medium was then sterilized by filtration through a 0.22 μm Millex GV filter unit (Millipore, Bedford, MA). The stock solution was serially diluted 2-fold. The cells in the microtiter plates were treated with 100 μl of either stock solution or the serial dilutions, respectively. The 5637 cells were exposed to final concentrations of 125, 100, 75, 50, and 25 μM of complexes **4** or **5** with light and 500, 400, 300, 200, and 100 μM without light, while the 5637-CDDP cells were exposed with and without light to 200, 100, 50, 25, and 12.5 μM of the Pt^{IV} complexes. One plate was returned to the incubator (dark control), while the second was placed in the irradiation apparatus. The apparatus consisted of a black aluminum block held in a water bath at 37ºC and placed 17 cm below a Lamag 40 W UV-lamp (λ = 366 nm). Studies with a ferrioxalate actinometer [20] estimated the intensity of UV_{366}-light to be 5.22×10^{-8} einsteins/min (standard deviation, $\pm$ 0.12×10^{-8} einsteins/min) in the wells of the microtiter plates with this apparatus. The plate was irradiated for 6 hr. The culture medium was then removed by aspiration from both the irradiated plate and dark control and replaced with 200 μl fresh medium before being returned to the incubator for 90 hr. The medium was discarded, and the cells were fixed with a 1% glutaraldehyde solution. Staining of the cells with crystal violet, measurement of cell-bound dye (λ = 570 nm), and the calculation of the IC_{50} values have been described in detail elsewhere [21].

Fluorescence Microscopy

For the phase-contrast and fluorescence microscopy studies on the effects of platinum complexes and light on the morphology of 5637 cells and their DNA distribution, cells were first seeded into NUNC SonicSeal slide wells and allowed to grow for 24 hr before treatment. Cells were treated with specified concentrations of complexes **4** and **5** in a manner analogous to that described above. A 6 hr treatment with and without concurrent irradiation with light (λ = 366 nm) at 37ºC was performed with the same apparatus as that used for irradiation in the cytotoxicity experiments. Treatment was ended by replacing the medium with fresh medium. The plates were then returned to the incubator for either 17 or 90 hr. After these time periods, the medium was removed, and the cells were washed with phosphate buffer, fixed with a 3:1 mixture of methanol:acetic acid for 10 min, and then stained with 1 μg/ml DAPI in a 1:1 solution of methanol:PBS for 15 min. Inverse microscopy was carried out on an Axiovert 200 (Carl Zeiss, Göttingen, Germany) at a magnification of 400×; cells were irradiated with light at λ_{ex} = 365 nm (BP-filter) and observed at λ_{em} > 397 nm (LP-filter).

Table 1. Temperature Program Employed in the Atomic Absorption Spectroscopic Analysis of Cell Lysates

Phase	T (ºC)	Length (s)	Heating Rate (ºC/s)	Argon Flow (l/min)
Drying	90	55	0	0.1
Pyrolysis	1400	15	100	0.1
Atomization	2500	3	0	0.0
Burn out	2900	3	0	0.2
Cooling	20	10	0	0.2

The standard curves were linear, with a correlation coefficient of approximately 0.9979. All standards and samples were measure in triplicate; the sample-to-sample relative SD was <5%. The day-to-day reproducibility was between 9% and 22%.

Cell Uptake

A single-cell suspension of 5637 cells growing as a monolayer was prepared by trypsinization, and cells were seeded into 25 cm^2 culture vessels at a density of 100,000–180,000 cells per vessel. Cells were allowed to grow for 3–5 d, at which time they reached a density of 1–2 × 10^6 cells per vessel. On the day of treatment, complexes **4** and **5** were dissolved directly in culture medium (supplemented with 10 g/l HEPES) as described above. A portion of the filtrate was diluted with culture medium to give a final Pt concentration of 100 μM. The medium from the cells in culture was replaced with 7.5 ml of medium containing either **4** or **5**, at a concentration of either 100 or 150 μM. In the case of cisplatin, a 10 mM solution in DMF was diluted by the culture medium to give a final concentration of 50 μM. The flasks were either returned to the incubator (dark control) or irradiated at λ = 366 nm, as described above.

At time t = 0, and at specified time intervals thereafter, the medium was aspirated out of the flasks, from both a "dark" and "light" flask. The cells were washed five times with 1 ml PBS, and then 1 ml of a trypsin/EDTA solution was added for 1 min at 37ºC. Following removal of the trypsin/EDTA solution, the cells were returned to the incubator for 15 min. A cell suspension was made by continuous rinsing of the flask bottom with 3 ml Dulbecco's buffer containing 5% FCS by means of a 5 ml pipette. From the resulting cell suspension, 200 μl were removed and added to 10 ml Isoton and the number of cells counted with a Coulter Counter Z2 instrument (Beckman-Coulter, Fullerton, CA). The remaining cell suspension was added to Eppendorf vials and centrifuged for 5 min at 5000 × g. The supernatant was discarded, and the cell pellet was resuspended in 250 μl Dulbecco's buffer and centrifuged again. The resulting cell pellet was stored at −20ºC until further use.

On the day of the AAS analysis, the cell pellets (approximately 10^6 cells) were thawed and 150 μl of a 1% pancreatin/phosphate buffer solution was added. The contents were mixed and the vials incubated at 37ºC for 5 min. Samples were cooled to RT before analysis.

For the flameless AAS analysis, a 989 QZ AA spectrometer (Unicam, Cambridge, UK) equipped with a GF90 oven and autosampler was used. ELC graphite cuvettes were employed in all measurements. Pt was measured at 265.9 nm with a lamp current of 80% and a slit opening of 0.2 nm. Deuterium lamp compensation was used as the method for background correction.

For the creation of the standard curve, 5 standards between 12 and 150 ppb Pt were used: 5 μl of standard Pt solutions (in 5% HNO_3) were diluted into 15 μl 1% pancreatin/phosphate buffer solution in the graphite cuvettes. In the case of the samples, 15 μl aliquots of the cell lysates were added to the cuvettes together with 5 μl of 5% HNO_3 solution (see Table 1 for details of the temperature program employed in the analysis).

Table 2. Temperature Program Employed in the Atomic Absorption Spectroscopic Analysis of DNA

Temp (ºC)	Length (s)	Heating Rate (ºC/s)	Argon Flow (l/min)
95	30	0	0.1
1400	10	100	0.1
2500	3	0	0.0
2700	3	0	0.2
20	10	0	0.2

Stability and Photoactivation

Solutions of complexes **4** and **5** were prepared at concentrations between 0.30 and 1.00 mM in 50 mM phosphate buffer (pH 7.4). Aliquots of 50 μl were added to each well of a 96-well microtiter plate, which was placed in the irradiation apparatus described above and illuminated with light (λ = 366 nm) at 37ºC. Parallel to this study, a second plate, filled with the same solution of either **4** or **5**, was wrapped in aluminum foil to avoid exposure to light and held at 37ºC in the same water bath. Alternating between the light and dark solutions, the solutions from one row of 8 wells were removed, mixed together, and a 20 μl aliquot injected into a Merck-Hitachi HPLC system, consisting of an L-7100 pump, an L-450 diode array detector, an L-7360 column oven (30ºC), and a Reodyne injector. The column used for the separation was a (strongly cationic) Nucleosil 100-10 SA 250 × 4.0 mm column preceded by a 11 × 4 mm precolumn of the same material. The eluent consisted of 1:9 acetonitrile:phosphate buffer (20 mM, pH 7.0) mixture, set at a flow rate of 0.7 ml/min. Detection was carried out at λ = 257 nm. Under these conditions, complexes **4** and **5** had retention times of 3.8 and 3.9 min, respectively. Peak areas were used for quantification.

Sampling was done at 1 hr intervals for 6 hr. The area of the peak at t = 0 was arbitrarily set at 100%; the percent area of the remaining peaks was set relative to that at t = 0. All experiments were repeated three times.

DNA Binding

Solutions containing 15 μM of complex **4** or **5**, 0.25 mg/ml calf thymus DNA, 10 mM $NaClO_4$ and 10 mM PIPES buffer (pH 6.9) were pipetted into two 96-well microtiter plates at 50 μl/well. One plate was wrapped in aluminum foil and placed in a water bath at 37ºC, while the second plate was placed in the irradiation apparatus and irradiated with light (λ = 366) nm at 37ºC. After defined time periods, the contents of 4 wells (200 μl) were removed, combined, and added with mixing to 100 μl ice-cold 0.9 M NaOAc solution. To this solution was added 900 μl ice-cold ethanol, and the 2 ml Eppendorf vials were closed and dropped into liquid nitrogen. The Eppendorf vials were removed from the liquid nitrogen and allowed to warm to −20ºC for 30 min. The vials were then centrifuged at 5400 × g for 10 min at −9ºC to pellet the precipitated DNA. The DNA pellet was resuspended in 300 μl 0.3 M NaOAc, precipitated again with 900 μl ice-cold ethanol, frozen in liquid nitrogen, and thawed to −20ºC before pelleting the precipitated DNA. This procedure was repeated once more before the DNA was taken up in a solution of 500 μl 0.5% HNO_3 and incubated for 2 d at 70ºC to hydrolyze the DNA.

The concentration of DNA bases was measured with a spectrophotometer at λ = 260 nm (ε = 8900 M^{-1} cm^{-1}). The amount of Pt was determined using a flameless 989QZ AAS (Unicam) set at λ = 265.9 nm using 10 μl of sample. The quantification range was 0.25–2.95 μM Pt. Each sample was measured twice and averaged (see Table 2 for the temperature-time program of the spectrophotometric analysis).

The R value was calculated as the quotient of platinum bound to DNA to the amount of DNA nucleotides (R = platinum [μmol]/DNA nucleotides [μmol]). The ratio of R/R_{max}, where R_{max} = 0.019 at 0.25 mg/ml DNA, is the fraction of total Pt bound to DNA. All experiments were repeated three times.

Supplemental Data

Figures showing phase-contrast and fluorescence microscopy of 5637 cells, effect of light on cellular uptake of platinum, effect of irradiation on complexes **4** and **5**, and NMR studies of GG adduct formation are available at http://www.chembiol.com/cgi/content/full/13/1/61/DC1/.

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