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

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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 diazido platinum(IV) diazide 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 37°C 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...
were exposed to 5 concentrations of 96-well microtiter plates and grown for 24 hr before treatment. Cells
Microorganisms and Cell Culture, No. ACC 35) were seeded into
(B) 5637 Human urinary bladder cancer cells (German Collection of
tination.
ethylenediamine (en) analog
2
(A) Structures of complexes 4 and 5, and of cisplatin, its chelated ethylenediamine (en) analog 2, and the Pt(V) 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 39) were seeded into 96-well microtiter plates and grown for 24 hr before treatment. Cells
were exposed to 5 concentrations of 4 (tris) or 5 (squares), res-
pectively, for 6 hr at 37°C, 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 ad-
tional 90 hr. Data points represent the averages of four indepen-dent experiments, and error bars represent standard deviations.

With the exception of the lowest concentration, the differences be-
tween the paired results from light and dark experiments are statisti-cally significant (p < 0.01, two-sided, paired Student’s t test). Error bars represent ± 1 SD.

(C) IC50 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 irradia-
tion with light for 6 hr at 37°C. A two-sided, paired Student’s t test was used to establish statistical significance.

Figure 1. Effect of Light on the Activity of Platinum Diazido Com-
plexes 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(V) complex 3 (JM216), which has been in clinical trials and is activated by chemical reduc-

B

A

\[
\text{IC}_{50} (\mu M) \quad \begin{array}{|c|c|c|c|c|c|}
\hline
\text{Complex} & \text{5637} & \text{5637-CDDP} \\
\hline
\text{with light} & \text{without light} & \text{with light} & \text{without light} \\
\text{4} & 44.3 ± 2.8^* & 357 ± 61 & 66.8 ± 17.5 & >200 \\
\text{cisplatin} & 0.76 ± 0.18 & 0.78 ± 0.09 & 3.63 ± 0.93 & 3.03 ± 0.38 \\
\text{5} & 163 ± 20.2** & 440 ± 145** & 79.8 ± 16.5 & >200 \\
[Pt(en)(Cl)2] & 2.28 ± 0.13 & 2.47 ± 0.78 & 14.5 ± 4.1 & 8.07 ± 1.66 \\
\hline
\end{array}
\]

**Note:** Error bars represent ± 1 SD.

exhibited a resistance factor (RF = IC50[5637/CDDP]/ IC50[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 cyto-
toxicity 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(N3)2(OH)2(NH3)2]) or 5 (cis, trans-[Pt(en)(N3)2(OH)2]), both in the dark and in the light, were investigated by flu-

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 IC30 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 Supple-

mental 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 PtIV complexes; however, these initial results
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.

**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 [1H, 15N] NMR studies of 15N-labeled complexes (Figure S3), which show the existence of photoisomerization and photoreduction pathways. 15N NMR peaks with chemical shifts in the range 266 to 273 ppm characteristic of 15NH3-PtII 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 [1H, 15N] HSQC NMR spectrum (Figure S3A) had 1H, 15N shifts of 4.02, 268.59 ppm (peak b), 3.87, 266.79 ppm (peak c), and 4.03, 272.60 ppm (peak d, minor). The 1J(15N-195Pt) coupling constants associated with species b and c (286 Hz) suggest that they contain N ligands trans to Pt-NH3 groups [13]; possible N ligands include NH3 (which would require isomerization of the Pt(NH3)2 unit) or a breakdown product of azide. For the ethylenediamine complex 5, a PtII product (d(1H), d(15N) 5.11, −33.43, Figure S3B) was detected after only 11 min. The 1J(15N-195Pt) 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
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:

$$\text{Pt}^{\text{IV}}\text{d(GpG)}\xrightarrow{\text{hv}}\text{Pt}^{\text{II}} + 3\text{N}_2$$

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 diazido 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 diazido 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 (Braunschweig, 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). All plastic culture supplies were from Starstedt (Nümbrecht, Germany). Cisplatin was obtained from ChemPur (Karlsruhe, Germany). Complexes 4, 5, and [Pt(en)Cl2] were synthesized in our laboratories following published procedures [10].

NMR

1D [1H], 2D [1H,15N] HSQC NMR spectra were recorded on a Bruker DMX 500 NMR spectrometer ([1H]: 500.13 MHz; [15N]: 50.7 MHz; Bruker UK Ltd., Coventry, UK) in 90% H2O/10% D2O, using dioxane (3.764 ppm) as the internal [3H] standard. All [1H,15N] values were referenced externally to 3.764 ppm. The pH value of all samples was adjusted to 5 with HClO4 before recording spectra so as to ensure slow exchange (on the NMR time scale) of NH protons of the PtIV species present. Spectra were acquired at 25ºC, and processed using Xwinmr (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% CO2 air. Under these conditions, the 5637 line achieved a maximum doubling time of 28 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) 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 256 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 prepared 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 lysis buffer containing the number of cells counted with a Coulter Counter ZF 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 G990 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% HNO3) 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% HNO3 solution (see Table 1 for details of the temperature program employed in the analysis).
Absorption Spectroscopic Analysis of DNA

Solutions containing 15 μM of complex 4 or 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 37°C. 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 37°C 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 (30°C), and a Reodney 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 pre-column 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.

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

<table>
<thead>
<tr>
<th>Temp (°C)</th>
<th>Length (s)</th>
<th>Heating Rate (°C/s)</th>
<th>Argon Flow (l/min)</th>
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Stability and Photocaustion

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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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double-stranded GG oligonucleotides with cisplatin and cis-