Effects of light-activated diazido-Pt<sup>IV</sup> complexes on cancer cells in vitro

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Various Pt<sup>IV</sup> diazides have been investigated over the years as light-activatable prodrugs that interfere with cell proliferation, accumulate in cancer cells and cause cell death. The potencies of the complexes vary depending on the substituted amines (pyridine = piperidine > ammine) as well as the coordination geometry (trans diazide > cis). Light-activated Pt<sup>IV</sup> diazides tend to be less specific than cisplatin at inhibiting cancer cell growth, but cells resistant to cisplatin show little cross-resistance to Pt<sup>IV</sup> diazides. Platinum is accumulated in the cancer cells to a similar level as cisplatin, but only when activated by light, indicating that reactive Pt species form photolytically. Studies show that Pt also becomes attached to cellular DNA upon the light activation of various Pt<sup>IV</sup> diazides. Structures of some of the photolysis products were elucidated by LC–MS/MS; monoaqua- and diaqua-Pt<sup>II</sup> complexes form that are reactive towards biomolecules such as calf thymus DNA. Platination of calf thymus DNA can be blocked by the addition of nucleophiles such as glutathione and chloride, further evidence that aqua-Pt<sup>II</sup> species form upon irradiation. Evidence is presented that reactive oxygen species may be generated in the first hours following photoactivation. Cell death does not take the usual apoptotic pathways seen with cisplatin, but appears to involve autophagy. Thus, photoactivated diazido-Pt<sup>IV</sup> complexes represent an interesting class of potential anti-cancer agents that can be selectively activated by light and kill cells by a mechanism different to the anti-cancer drug cisplatin.
1. Introduction

In the quest for more selective acting anti-cancer therapies, various methods of localizing the cytotoxic effects of a chemotherapeutic agent to the tumour site have been investigated over the years, with varying measures of success; for example, targeting of tumour antigens with antibody–anti-cancer drug conjugates [1], antibody- and gene-directed enzyme prodrug therapy [2,3], folic-acid-based receptor targeting of cancer cells [4] and polymeric anti-cancer drugs with pH-controlled activation [5]. A more recent approach gaining attention is the photoactivation of a chemotherapeutic (PACT) in and around a tumour by use of visible light, preferentially longer wavelength red light that penetrates deeper into the tumour tissue. This treatment strategy differs from traditional photodynamic therapy, where light is used to activate a dye (the photosensitizer) that has accumulated in the tumour tissue to produce singlet oxygen from triplet oxygen, leading to oxidative stress and destruction of the cancer cell. In the case of PACT, an anti-cancer agent is generated in situ from a non-toxic prodrug through a photochemical process localized to the site of irradiation, ideally within the tumour itself, and without the need for oxygen (e.g. in hypoxic tissue).

Transition metal ion complexes are attractive for PACT owing to their rich and varied photochemistry, as discussed in depth in a recent review [6]. In particular, platinum(IV) complexes are well suited for this purpose and symbolize the first efforts of designing photoactivatable transition metal complexes for cancer chemotherapy [7]. Of the PtIV complexes investigated to date, the diazido-PtIV complexes (figure 1) developed in the research group of Prof. Peter Sadler appear most promising because of their superior dark stability and their ability to produce potent cytotoxic agents upon irradiation with visible light. This paper aims to review the literature of this remarkable class of compounds with a focus on photoactivation processes and effects on cellular systems.

2. Photoactivation

Compared with square-planar PtII complexes, octahedral PtIV complexes are relatively substitution inert and with the proper choice of ligands are more stable than their PtII counterparts under biological conditions. Thus, PtIV complexes are well suited as prodrugs for cytotoxic PtII drugs; for example, oxoplatin shows a similar spectrum of cytotoxic activity to cisplatin and is cross-resistant with cisplatin, consistent with it being in part a prodrug for cisplatin [8]. Activation of a PtIV prodrug generally takes place by chemical reduction to the corresponding PtII species by a biological reducing agent such as ascorbate, glutathione (GSH) or serum proteins. Irreversible
binding of aqua-Pt\textsuperscript{II} species to cellular DNA is now believed to be the event leading to cell death, usually by apoptosis as in the case of cisplatin [9]. However, because chemical reductions can take place throughout the body, Pt\textsuperscript{IV} prodrugs show little selectivity for tumour tissue; indeed, it was reported that treatment of cancer patients with the Pt\textsuperscript{IV} complex iproplatin gave even more severe side effects than the Pt\textsuperscript{II} anti-cancer agent carboplatin [10].

One approach to direct the activation process to the tumour itself is by use of light to induce a photolytic reductive elimination of a non-toxic but light-sensitive Pt\textsuperscript{IV} complex to a cytotoxic Pt\textsuperscript{II} species. This approach was initially tried with diiodido-Pt\textsuperscript{IV} diamines, which were activated by visible light to cytotoxic species that platinated DNA, but the dark stability of the complexes in the presence of biological reducing agents such as GSH was poor and the complexes showed relatively high dark activity [11,12]. The first light-activated Pt\textsuperscript{IV} complexes that were sufficiently stable in the presence of biological reducing agents were the diazido-Pt\textsuperscript{IV} diamines \textit{cis,trans,cis}-\textit{[Pt(N\textsubscript{3})\textsubscript{2}(OH)\textsubscript{2}(NH\textsubscript{3})\textsubscript{2}]} and \textit{cis,trans}-\textit{[Pt(en)(N\textsubscript{3})\textsubscript{2}(OH)\textsubscript{2}]} developed by Sadler and co-workers [13].

Various methods have been used to gain information on the photoactivation processes of diazido-Pt\textsuperscript{IV} complexes, including UV/vis, NMR, LC/MS/MS, as well as theoretical methods based on density functional theory (DFT).

### (a) Ultraviolet/visible studies

The first studies on the photochemistry of Pt azides were pioneered by Vogler, who followed the changes in the UV/vis spectra while irradiating with monochromatic light into the ligand-to-metal charge-transfer (LMCT) bands of both \textit{cis} and \textit{trans}-coordinated diazido complexes [14–16]. It was observed that both \textit{cis}- and \textit{trans}-coordinated diazido complexes underwent photoreductive eliminations to yield the reduced Pt\textsuperscript{II} species; for example, during the photolysis of trans-[Pt(CN)\textsubscript{4}(N\textsubscript{3})\textsubscript{2}]\textsuperscript{2–} to [Pt(CN)\textsubscript{4}]\textsuperscript{2–}, four isosbestic points were observed in the UV/vis spectra, wherein the LMCT band centred at $\lambda = 302$ nm disappeared completely paralleled by the rapid evolution of nitrogen [14]. The fate of the eliminated azide ligands differed depending on whether they were \textit{cis}- or \textit{trans}-coordinated. \textit{Cis}-coordinated diazido complexes cis-[Pt(N\textsubscript{3})\textsubscript{2}(PPh\textsubscript{3})\textsubscript{2}] and [Pt(N\textsubscript{3})\textsubscript{6}]\textsuperscript{4–} resulted in the formation of hexazabenzenes, which decomposed to gaseous nitrogen [15,16]. On the other hand, trans-diazido-Pt\textsuperscript{IV} complex trans-[Pt(CN)\textsubscript{4}(N\textsubscript{3})\textsubscript{2}]\textsuperscript{2–} underwent reductive photoelimination with the formation of azidyl radicals, which decomposed to N\textsubscript{2} and N atoms, and were identified by electron spin resonance (ESR) [14]. There was no evidence for azidyl radicals from the cis-complexes, however. These observations are important for later understanding of the biological differences between photoactivated \textit{cis}- and \textit{trans}-diazido-Pt\textsuperscript{IV} anti-cancer agents.

The first diazido-Pt\textsuperscript{IV} diamines to be investigated by Sadler and co-workers [17] for their photochemical reactivity were the \textit{cis}- and \textit{trans}-diammines 1 and 2. Azide-to-platinum(IV) LMCT bands are centred at $\lambda = 256$ and 285 nm for 1 and 2, respectively. Solutions of the complexes were stable in the dark, but irradiation with UVA light at $\lambda = 365$ nm ($p = 12$ mW cm$^{-2}$) caused a decrease in the LMCT bands; the decrease was more rapid for the \textit{trans}-compared with the \textit{cis}-isomer. The spectra of the \textit{cis}-isomer appeared ‘cleaner’, with an isosbestic point apparent, whereas the \textit{trans}-isomer showed more complicated spectra. Bubbles of a gas, presumed to be nitrogen, were observed in the irradiated solutions of both the \textit{cis}- and \textit{trans}-complexes [17]. The mixed-amine \textit{trans}-diazido-Pt\textsuperscript{IV} pyridine complex 4, which showed an LMCT band at $\lambda = 289$ nm, was also stable in the dark in solution but exhibited loss of the LMCT when irradiated with UVA light [18]. While Sadler and co-workers observed no isosbestic points during the photodecomposition of this complex, Westendorf et al. [19] reported an isosbestic point at approximately $\lambda = 260$ nm. Studies by high-performance liquid chromatography (HPLC; see §2c) of the reaction mixture showed, however, that a number of products simultaneously form during the photolysis of 4, so that the appearance of an isosbestic point is not always indicative of a one-step photochemical reaction. Moreover, UV/vis studies with Pt\textsuperscript{IV} diazides coordinated with various alkylamines (i.e. methylamine, propylamine, cyclohexylamine) and N-heterocyclics (i.e. thiazol, methylpyridines, quinoline) all show the loss of the (N\textsubscript{3} → Pt) LMCT
band without isosbestic points, further evidence that the irradiation with UVA yields a number of photoproducts with varying light absorbing properties [20].

(b) NMR studies

To better characterize the photoproducts formed from azido-PtIV complexes, various NMR methods have been used. It was observed by two-dimensional [1H,15N] heteronuclear single quantum correlation (HSQC) NMR that the first photoproducts of 1 and 2 that formed were still in the PtIV oxidation state, indicating isomerization or photoassisted exchange of one of the azides for water [17]. PtII species first appeared after a 1 h irradiation period, but even after 2 h, the majority of Pt species were still in the PtIV state, indicating sluggish activation to cytotoxic PtII complexes. Sadler and co-workers studied in detail the photochemistry of 1 [21], 2 [22] and 3 [13] by various multi-nuclear NMR methods. UVA light caused the formation of a number of PtII and PtIV species from all three compounds. Interestingly, at physiological pH and extracellular NaCl concentration (i.e. in phosphate-buffered saline solution), release of azide from 2 was observed, whereas under acidic conditions, N2 was the major product [21], evidence that the reaction conditions influence greatly the types of photoreactions diazido-PtIV complexes undergo. The release of an ammine ligand was observed for both isomers and increased with increasing pH. Unexpectedly, O2 was found to evolve in the photolysis of both isomers [21,22]. The formation of highly reactive PtIV-nitrene intermediates was postulated based on the photoproducts formed between 1 and dimethylsulfide [21], evidence that not only cytotoxic PtII species form photolytically, but other unexpected Pt species as well, which may contribute to the overall cytotoxicity of the light-activated complex.

During the irradiation with UVA light of the pyridine-containing complex 4, very little photoreduction to PtII was observed by one-dimensional 1H and two-dimensional [1H,15N] HSQC NMR spectroscopy in HClO4 solutions at pH 5 [18]. In addition, no evidence for the loss of the pyridine ligand was found, suggesting that pyridine remains firmly attached to Pt during the activation process. As in the case of 1 and 2, photolysis also resulted in the release of azide ion from 4, as detected by 14N{1H} NMR [23]. Azide is well known for cytotoxicity.

(c) High-performance liquid chromatography studies

HPLC coupled with UV/vis and MS detection offers possibilities to study both the rates of photolysis as well as the product composition at concentrations of Pt complexes closer to those used in cell culture experiments (i.e. less than 1 mM); because of low sensitivity of NMR methods, concentrations greater than 1 mM are usually required. Initially, Bednarski et al. [24] used HPLC with UV detection to study the stability of solutions of 1 and 3 with concentrations between 0.3 and 1.0 mM, irradiated with UVA light. HPLC coupled to MS detection has been more recently used to detect the traces of photoproducts formed in micromolar solutions of 4 and 5 [19,25]. Compared with the use of just UV/vis spectroscopy as described in §2a, reversed-phase HPLC coupled to UV/vis detection allows for a clean separation of substrate from products, providing concentration data for the determination of rate constants. In this way, Westendorf et al. [19] determined the first-order rate constants for the photodecomposition of 2 and 4 by UVA and white light; both complexes gave comparable rates of loss, which were approximately 40 per cent reduced when irradiated with white as compared with UVA light. With the help of HPLC separation of the photoproducts of 4 coupled to UV/vis and high-resolution MS/MS detection, the light-dependent formations of various PtIV and PtII species were characterized. NMR studies with 4 (discussed earlier in §2b) reported very little photoreduction of PtIV to PtII [18]. Consistent with this observation, the two PtII reduction products, [Pt(OH)2(py)NH3] and [Pt(OH)N3(py)NH3] detected by HPLC–MS/MS formed in low concentrations over 60 min (figure 2). Also consistent with earlier NMR results that indicated that the azide released was the MS detection of the PtIV complex [Pt(N3)3(OH)2NH3], which could only have formed in a photosubstitution reaction between 4 and free azide. There was also no evidence in
(a) Positive m/z 325.034
peak 2

Negative m/z 408.046
peak 1

Positive m/z 350.039
peak 3

Negative m/z 433.054
peak 4

(b) Positive m/z 331.444
peak 2

Negative m/z 414.095
peak 1

Figure 2. Photolysis reactions (UVA light) of compounds 4 (a) and 5 (b), studied by LC–MS/MS. Solutions contained 450 µM 4 or 900 µM 5, dissolved in 10 mM piperazine-N,N′-bis(2-ethanesulfonic acid) buffer (pH 7.4) at 37°C.

(d) Theoretical calculations

Recently, DFT and time-dependent DFT calculations have been used to help understand the photochemistry of cytotoxic diazido-PtIV complexes [26–29]. Salassa et al. [26] rationalized the photosubstitutions at PtIV of N−3 and NH3 to occur for 2 from either singlet and triplet excited states, but a mechanism for PtIV reduction could not be identified. Recently, Sokolv & Schaefer [29] proposed that the dissociation of two azido ligands from 2 might occur simultaneously with the formation of the azidyl radicals (N₃·) and reduction of the platinum centre from PtIV to PtII. This mechanism is consistent with the ESR spectrum observed with irradiated ethanolic solutions of trans-[Pt(N₃)₂(CN)₄]²⁻ that N atoms are detected, evidence for the decomposition of the chromato gram for the formation of free pyridine in the photolysis of 4. In the case of the piperidine analogue 5, only the dihydroxo-PtII reduction product [Pt(OH)₂(pip)NH₃] was detected by LC–MS/MS [25], indicating that the nature of the heterocyclic ring influences the photolytic pathways (figure 2).

Thus, NMR and HPLC with UV/vis and MS detection complement each other in studying the photochemistry of PtIV azides. The main advantage of LC–MS/MS is that the photochemistry can be studied at relatively low concentrations of Pt complexes compared with NMR. The main disadvantage is the analysis of very hydrophilic Pt complexes, such as 2, which are hardly retained on reverse-phase-packed columns, making their quantification, as well as the detection of very hydrophilic photolysis products, difficult [19].
of azidyl radicals [14]. Likewise, the analysis of atomic charges and electronic configuration of the pyridine complexes 4–6 showed that in the lowest-lying triplet geometry, the trans azides are both less negatively charged, whereas the Pt centre is less positively charged [23,27,28]. Such behaviour could account for a PtIV to PtII reduction mechanism and for the formation of N3 radical fragments. Nitrogen-based radicals could well contribute to the phototoxicity of the complexes.

These theoretical methods have been used to predict with good accuracy the UV/vis electronic absorption spectra of complexes 1 [26,29], 2 [29], 4 [23], 5 [28] and 6 [27]. Interestingly, for complex 6, the calculated low-intensity transitions in the blue region of the visible spectrum (\(\lambda = 414\) nm) might account for the photoactivity induced by blue light [27]. Hence, rational design of light-activated PtIV complexes could begin with DFT calculations to help choose the most promising compounds to synthesize and test.

3. Interactions with biomolecules

Reactive Pt species generated by light activation of PtIV diazides would be expected to react with biomolecules such as nucleotides, DNA and proteins. While little research has been reported to date on interactions with proteins, all of the PtIV diazides shown in figure 1 have been investigated for their reactivity towards nucleic acids because of the central role of DNA in the biological effects of cisplatin and other Pt anti-cancer drugs [9]. Furthermore, the effects of biological reducing agents such as ascorbate and GSH on the dark stability of PtIV diazides is key to anticipating selective activation by light.

(a) Reactions with nucleotides

Various heteronuclear NMR methods (e.g. two-dimensional [\(^1\)H,\(^{15}\)N] HSQC- and HSQC-TOCSY-NMR) have been used in the Sadler group [9] to study the reactions between light-activated diazido-PtIV complexes and mono- and dinucleotides, typically of guanine because of its importance in binding PtII anti-cancer drugs. Initial work showed by using these methods that irradiation (\(\lambda = 457.9\) nm) of a solution of diiodio-PtIV diamine trans, cis-[Pt(OAc)\(_2\)I\(_2\)(en)] in the presence of 5′-guanosine monophosphate (GMP) resulted in the same PtII-(5′-GMP)\(_2\) adduct formed directly when the corresponding PtII diamine was allowed to react with 5′-GMP [30]. In the case of diazido-PtIV complexes, irradiation of solutions of cis-complexes 1 and 3 with light from an Ar–Kr laser in the presence of either 5′-GMP or d(guanosinylphosphoguanosine) [d(GpG)] also resulted in the formation of PtII diamines bound in the third and fourth coordination positions with two guanines at N7 [13,24], again the same products found in the chemical reaction of cisplatin or [PtCl\(_2\)(en)] and 5′-GMP or d(GpG), respectively. Unlike with cisplatin and [PtCl\(_2\)(en)], however, no such products formed between 1 and 3 with 5′-GMP or d(GpG) in the dark.

Similar NMR studies with the trans-complex 2 showed that the photoactivation with 5′-GMP resulted in the formation of trans-[Pt(NH\(_3\))\(_2\)(5′-GMP-N7)2]\(^2+\), confirming photoreduction to the PtII species and the expected trans-coordination with N7 of guanine [17]. Interestingly, photoreactions proceeded faster in the presence than in the absence of 5′-GMP, suggesting that 5′-GMP traps reactive PtII species, preventing them from further photodecomposition reactions. Likewise, in the cases of the pyridine analogues 4 and 6, both the expected mono- and bis-GMP adducts of the PtII pyridine species were observed by two-dimensional [\(^1\)H,\(^{15}\)N] HSQC NMR, confirming photoreduction of PtIV to PtII [18,27]. The efficiencies for the photo conversion of 3 and 4 in the presence of 5′-GMP to mono- and bis-GMP adducts appeared to be around 50 per cent and 70 per cent, respectively [18,24]. As in the case of the other two complexes, no reactions between either 4 or 6 and 5′-GMP took place in the dark.

(b) Reactions with DNA

Early studies with cis-diiodio-PtIV diamines demonstrated irreversible platination of calf thymus DNA (ct-DNA) when photoactivation was carried out with visible light, as determined by
atomic absorption spectroscopy (AAS) [11,12]. Similarly, solutions of either cis-complexes 1 and 3 photolysed by UVA in the presence of ct-DNA resulted in irreversible binding of Pt to DNA, with little or no binding taking place in the dark [24]. While all-trans-complexes 2 and 4 showed similar rates of photolysis (see §2c), photolysed 2 bound much more rapidly to ct-DNA than did the photolysed pyridine analogue 4, evidence that the pyridine ring has a substantial influence on the rate of reaction with ct-DNA [19]. On the other hand, substituting the pyridine ligand in 4 for piperidine in 5 had no noticeable effect on the Pt binding kinetics to ct-DNA, further evidence that steric bulk is important in determining reaction rates with DNA [28]. The irreversible binding of photolysed 2 and 4 could be blocked by the presence of 100 mM chloride, supporting the idea that aqua-PtII species are the Pt species that react with ct-DNA, but that the less reactive chloride PtII species do not [19].

A considerable lowering in DNA platination levels was also observed when photolysed solutions of 4 were left to stand for 2h before being added to ct-DNA, suggesting that reactive PtII species trap each other over time and the product has reduced reactivity towards DNA [18]. GSH at physiological concentrations (between 1 and 10 mM) efficiently protected ct-DNA from platination by UVA-activated 2 and 4, further proof that reactive electrophilic Pt species form through photolysis and bind irreversibly to DNA [19].

The Brabec group has performed detailed studies on the structures of the DNA adducts resulting from platination by light-activated diazido-PtIV complexes. Initial transcription mapping studies with 3, activated by Ar–Kr laser light while incubated with pSP73KB plasmid DNA, indicated that the same guanosine–guanosine intrastrand cross-links are formed as with cisplatin, support for the idea that 3 serves as a light-activated prodrug for [PtCl2(en)] [31]. Similar transcription mapping studies with the all-trans pyridine complex 4 indicated that the binding sites on DNA were similar to those used by transplatin [18]. Relatively few interstrand cross-links were formed compared with other DNA adducts (i.e. intrastrand and DNA–protein cross-links), and mono-functional cross-links closed much faster for light-activated 4 than for transplatin. Results from ethidium fluorescence studies suggested that the distortions in DNA caused by adducts of 4 spanned more base pairs around the platination site than either cisplatin or transplatin. Furthermore, DNA adducts in a pUC19 plasmid from light-activated 4 were less efficiently removed than cisplatin and transplatin adducts by repair-proficient HeLa cell-free extract, further evidence for the uniqueness of the DNA-Pt adducts caused by 4 [18]. The DNA unwinding angle $\Phi$ caused by the binding of light-activated 4 to negatively supercoiled pUC19 plasmid was calculated by agarose gel electrophoresis to be $24 \pm 1^\circ$, considerably greater than the $13^\circ$ reported for cisplatin [28]. The piperidine analogue 5 gave comparable results to 4 in all of these studies, indicating that the steric bulk of the coordinating heterocyclic is influential for the binding behaviour to DNA [28]. In the case of the bis-pyridine analogue 6, the DNA unwinding angle was slightly larger ($28 \pm 3^\circ$) [32].

Kasparkova and co-workers [32] recently published data on the interactions of 6 with DNA that showed irreversible binding only when irradiated (with either UVA or visible light). Furthermore, by addition of thiourea to solutions of Pt-DNA adducts, the authors observed that about half of these adducts were still mono-functional after 24h, the rest having presumably closed to form inert bifunctional Pt adducts. Moreover, DNA adducts formed with light-activated 6 were much more efficient than cisplatin at stalling transcription by RNA pol II; inhibition of RNA polymerase would be expected to trigger a number of downstream cellular effects leading to cell death. The authors concluded that interactions of photoactivated 6 with cellular DNA could well play an important roll in the activity of the complex, but did not rule out participation of other mechanisms.

(c) Reactions with ascorbate and glutathione

A key requirement of a light-activatable PtIV prodrug is that the complex will not be reduced by bioreducing agents such as ascorbate and GSH before being irradiated at the site of the tumour. In fact, cis-diiodio-PtIV diamines failed in further development to anti-cancer agents because
the complexes were too rapidly reduced to reactive PtII species by GSH [33]. Thus, the ability for these agents to reduce various PtIV diazides has been the focus of considerable attention. Based on NMR methods, complexes 1 and 4 were found to be relatively stable in the presence of GSH for several weeks (e.g. 95% of 4 after 21 days in the presence of 6 mM GSH) [13,18].

Likewise, 5 mM ascorbate brought about no noticeable platination of ct-DNA by either 2 or 4 in the dark after 24 h at 37°C [19]. On the other hand, ascorbate did increase the level of DNA platination by UVA-activated 4, but not for light-activated 2, suggesting that light activation of 4 results in the formation of PtIV species that can be reduced by ascorbate, whereas the parent complex cannot.

4. Effects on cancer cells

The previously discussed studies show that photoactivated diazido-PtIV complexes generate DNA-binding Pt species as well as reactive nitrogen species (RNS). It is highly likely that these reactive photoproducts are cytotoxic on cancer cells and could have anti-cancer activity. Early work with cis-diiodio-PtIV diamines showed that irradiation with visible light produced cytotoxic photolysis products [11,12]. Thus, it is of central importance to characterize the cellular effects of the photoactivated diazido-PtIV complexes 1–6.

(a) Cytotoxicity and structure–activity relationships

Previous studies with diam(m)ine complexes 1–3 showed a stark increase in the antiproliferative activity against a human bladder cancer cell line (5637) [24], as well as cytotoxic activity against a transformed human keratinocyte line (HaCaT) [17] when irradiated with UVA light. The 50 per cent inhibitory concentrations (IC50) for light-activated 1 and 3 against the 5637 line were nearly 100-fold higher compared with cisplatin, but there was no cross-resistance in a 5637 cell line made fivefold resistant to cisplatin [24]. Interestingly, although cisplatin is threefold more potent than [PtCl2(en)] at inhibiting the growth of 5637 cells, 1 and 3 were of comparable potency. Also unexpected was the observation that the cis- and trans-diammine complexes 1 and 2 showed similar potency against the HaCaT line [17]; based on the IC50 values of cisplatin and transplatin, a much weaker effect for the trans-isomer 2 would have been expected had the compound simply served as a prodrug for transplatin. Thus, these data are not consistent with the idea that PtII photoproducts are alone responsible for cytotoxicity.

Light activation of 4 can also take place outside of cells. When 4 was irradiated with UVA in culture medium for 30 min without cells and then added to cultures of either 5637 or RT-4 human bladder cancer cells for 6 h followed by a 90 h incubation in fresh medium, no significant changes in the IC50 values (53.5 ± 15.2 and 72.6 ± 7.5 µM for 5637 and RT-4, respectively) were observed compared with when the compound was irradiated in the presence of cells (30.7 ± 5.0 and 136.9 ± 55.0 for 5637 and RT-4, respectively) [23].

The observation that cis- and trans-diazido-PtII diammines 1 and 2 have similar phototoxic activity on the HaCaT cell line spurred the Sadler group to develop further trans-diazido analogues [18,27,34]. In studies that directly compared the potency of cis- versus trans-diazido PtIV isomers, the trans were always the most phototoxic [34]. However, unlike in the case of the cisplatin resistant cell line 5637-CDDP, photoactivated complexes 1, 2 and 4 showed some cross-resistance to cisplatin in the A2780CIS human ovarian cancer cell line, which is approximately twice less sensitive to cisplatin than the native A2780 line [18,34]. These same studies also established that the substitution of an ammine (NH3) ligand for pyridine strongly increased the potency of the phototoxin; for example, IC50 values for 2 and 4 in the A2780 cell line were 99.2 and 1.9 µM, respectively. This remarkable structure–activity relationship between 2 and 4 was confirmed with two human oesophagus cancer cell lines [19] and the 5637 human bladder line [28]. In a panel of 13 human cancer cells lines, an average IC50 value of 55 ± 28 µM (range 29–139 µM) after 30 min irradiation (0.12 mW cm⁻²) was found for 4 when photoactivated with UVA, although visible light was also effective [23]. The selectivity towards this panel of cell lines was
far less than for cisplatin, which averages an IC$_{50}$ of 1.36 ± 1.28 μM (range 0.24–4.09 μM) for the same panel of lines [35]. Thus, not only is the potency of light-activated 4 less than cisplatin, the cytotoxicity is also less specific. Nevertheless, both 4 and 5 showed potent anticlonogenic activity on the SISO human cervix cancer cell line [28]; the clonogenic assay determines the ability of a single cell to grow into a colony and represents a more predictive test for anti-tumour activity than simply measuring cell growth inhibition.

With 2-methyl pyridine as the ligand, a 25-fold decrease in potency in the A2780 line was noted compared with 4, but the 3- and 4-methyl pyridines only minimally attenuated potency [34]. Interestingly, both of these later compounds were non-cross-resistant to cisplatin in the A2780CIS cell line. Substitution of the pyridine of 4 for the less hydrophilic but more basic piperidine (5) resulted in no great change in potency in six human cancer cell lines [28]. However, this compound was more rapidly photoactivated by both UVA and visible light than 4, which could be an advantage for a therapeutic [28].

The syntheses of trans-configured mixed-amino Pt$^{IV}$ complexes such as 4 and 5 are more laborious and the yields are lower than for simpler analogues with coordinating bis-heterocyclics. This is an important economic factor when the high price of platinum is considered. Accordingly, it was a significant finding that the more efficiently synthesized bis-pyridine Pt$^{IV}$ diazide 6 possessed comparable phototoxic potency to the mixed-amine analogue 4 in four cell lines [27]. Both UVA and blue light were capable of activating the compound while no cytotoxicity was seen in the dark. Thus, 6 would appear to be a suitable candidate for further preclinical studies, for example, testing for in vivo anti-tumour activity.

(b) Accumulation of platinum by cells and DNA damage

Uptake of Pt by cancer cells would seem a requirement for the activity of a light-activated Pt$^{IV}$ prodrug. Accumulation rates of the light-activated 1, 3–5 in the 5637 human bladder cancer cell line have been measured over 8 h by AAS and compared with the rates without light activation as well as with those for cisplatin [24,28]. In the cases of 1 and 3, accumulation of Pt in the cells was somewhat greater for the light-activated species compared with the dark controls, but the differences were not significant [24]. However, Pt accumulation was only 20 per cent compared with that found with cisplatin, which might partially explain the diminished cytotoxicity relative to cisplatin. On the other hand, for the mixed-amine Pt$^{IV}$ complexes 4 and 5, the cellular accumulation rate of Pt in 5637 cells was markedly greater in the light-activated experiments compared with the dark controls, which barely took up Pt at all [28]. Furthermore, levels of Pt accumulated in cells for the light-activated 4 and 5 were comparable to cisplatin over the first 6 h before plateauing off, which helps explain the greater activity of these compounds compared with 1 and 3. Thus, reactive Pt photoproducts would appear to enter cells and bind irreversibly to cellular components.

By using a highly sensitive inductively coupled plasma mass spectrometry method, platinum was in fact found bound to cellular DNA of HaCaT cells (0.35 ng Pt µg$^{-1}$ DNA) treated with 24 µM of UVA-activated 5, evidence that some kind of Pt-DNA adducts form in living cells [18]. In the case of 6, the level of platinum bound to cellular DNA of A2780 cancer cells was quantified by furnace atomic absorption spectrometry and found to be 16-fold greater (136.5–8.4 ng Pt µg$^{-1}$ DNA) than in cells exposed to cisplatin at the same concentration (24 µM) and duration of exposure (1 h) [32]. Moreover, the binding to cellular DNA took place only when cells were irradiated with UVA, while cisplatin platination of cellular DNA took place in the dark.

Damage to cellular DNA was hinted at by a single-cell gel electrophoresis (comet tail assay) for HaCaT cells exposed to UVA-activated 5. In contrast to cisplatin, which cross-links DNA and inhibits DNA migration after H$_2$O$_2$ treatment in a dose-dependent manner, a significant decrease in the DNA migration was only noted at concentrations of 5 at least twice the IC$_{50}$ values of the compound. This suggests that DNA cross-linking may not be associated with the observed cytotoxicity in this cell line [18]. However, this does not rule out the possibility that
Figure 3. Histographic representation of representative results from the flow cytometric analysis for oxidative stress by the DCFH-DA method in HL60 cells, exposed to 100 µM 4 and irradiated for 30 min with UVA. Fluorescence in cells was measured 0 (black), 2 (red) and 4 h (blue) following irradiation. The green curve is from irradiated negative control lacking 4 and the magenta curve is from the positive control exposed to approximately 200 µM t-butyl hydroperoxide only. The arrow symbolizes the direction towards more oxidative stress.

Mono-functional Pt-DNA adducts, which would not be observed in the comet tail assay, are somehow involved in the cytotoxicity of activated 5.

(c) Oxidative stress in cells

Aside from the possibility that cytotoxicity is elicited by irreversible binding of Pt to cellular DNA, as discussed in §2a, light-activated diazido-PtIV complexes could also produce RNS (e.g. azidyl radicals and N atoms) within cells that result in cellular damage. Such nitrogen-based radicals could ignite oxidative process in cells, which should be detectable by methods generally used to detect reactive oxygen species (ROS). For these studies, we have used a flow cytometric assay based on the detection of ROS with the lipophilic dichlorofluorescin diacetate (DCFH-DA), which diffuses into the cells and is hydrolysed by cellular esterases to the hydrophilic 2′,7′-dichlorofluorescin (DCFH), trapping the compound within the cells [36]. Oxidation of DCFH by ROS yields a highly fluorescent compound, 2′, 7′-dichlorofluorescein, which is detected by an increase in the fluorescents of the cells by the flow cytometer.

Figure 3 shows representative results from flow cytometry experiments where HL60 cells were exposed for 30 min to 2.0 µM DCFH-DA just after a 30 min UVA activation with 4 (100 µM). Cellular fluorescence was then measured 0, 2 and 4 h later. Immediately following photoactivation, cells showed the same intensity of fluorescence that cells displayed that were only exposed to UVA light for 30 min. However, by 2 h, a significant shift in the population of cells to higher fluorescent intensity had occurred. After 4 h, the intensity of fluorescence had already decreased and by 6 h, cellular fluorescence had returned to the starting levels. These results provide evidence that significant levels of ROS are generated within hours of photoactivation of 4 in cells and return to normal levels by 6 h. The nature of the signal transduction pathways that become activated remains to be determined.
**Figure 4.** Fluorescence microscopy (400× magnification) showing nuclei of MCF-7 cells, stained with 4',6-diamidino-2-phenylindole 48 h after treatment with 73 µM 4, either with UVA activated for 30 min (a) or dark control (b). Note that the number of cells in (a) are dramatically reduced, but the size and density of the nuclei are relatively unchanged. (Online version in colour.)

### (d) Mechanisms of cell death

Changes in the appearances of cancer cells after treatment with an anti-cancer agent can reveal information about the mechanism of cell death. Cells undergoing programmed cell death by apoptosis classically display shrinkage and condensation of the cell, packing of the nuclear material, budding on the cell membrane (blebbing), cellular fragmentation forming apoptotic bodies and nuclear break-up [37]. In the cases of UVA-activated cis-diazido-PtIV complexes 1 and 3, no such effects were observed by light microscopy in the 5637 cell line treated with IC90 concentrations of the complexes [24]. Rather, cells initially ballooned-up just after light activation, then shrank with loss of contact to neighbouring cells as well as the plastic bottoms of the culture vessels. Dramatic packing of nuclear DNA took place 17 h after irradiation, but unlike with apoptosis, no blebbing and cellular fragmentation was observed. Rather, destruction of nuclear material continued until many cells were left with no nuclei at all, leaving behind cell ghosts. By contrast, the morphological effects caused by cisplatin, although also not typical for apoptosis, were completely different in the 5637 line [24], evidence that 1 and 3 kill cells by a very different cellular mechanism than cisplatin.

In the case of the trans-diazido-PtIV complexes 4, no cell ballooning was observed immediately preceding light activation, and no dramatic changes in the nuclear material were observed either in the 5637 human bladder cancer or MCF-7 human breast cancer cell lines (figure 4). Only a slight swelling of the HL60 cells compared with untreated controls was noted; by contrast, cisplatin treatment set off apoptosis in the HL60 cell line [23]. Furthermore, no changes in the distribution of HL60 cells in the cell cycle relative to untreated controls could be found by flow cytometry 48 h after treatment with the IC90 concentration of photoactivated 4 [23]. On the other hand, cisplatin caused a typical S/G2 arrest in this cell line.

A hallmark of early apoptosis is the redistribution of phosphotidylserine (PS) from the cytosolic surface of the plasma membrane to the outer layer of the bilayer, which is typically detected with flow cytometry by marking PS on the outside surface of the cell with the PS-binding protein annexin V, which is marked with a fluorescent reporter molecule (fluorescein isothiocyanate-labelled annexin V) [38]. With HL60 cells treated at either the IC50 or the IC90 concentration of UVA-activated 4, very little translocation of PS from the inner to the outer leaflet of the plasma membrane was observed even after 48 h, while cisplatin gave a strong positive effect [23]. These results are inconsistent with apoptosis being a mechanism of cell death by photoactivated 4.

Further evidence that photoactivated 4 does not cause cells to enter into apoptosis was gained with the A2780 cell line, where no activation of caspase 3 and 7, classic markers of apoptosis, could
be detected after treatment with photoactivated 4 [18]. Moreover, the cleavage of poly adenosine diphosphate ribose polymerase, a known substrate of caspase 3, was also not observed by western blotting in cells treated with photoactivated 4, but was observed in cisplatin treated cells. Finally, microscopic analysis of Hoechst 33342-stained nuclei from A2780 cells treated with either cisplatin or UVA-activated 4 support the idea that cells do not die by apoptosis [23].

In search of alternative mechanisms of cell death, the possibility that UVA-activated 4 might initiate autophagic pathways was investigated. Autophagy is a cellular ‘self-eating’ process activated in cells to avoid starvation and diverse noxious effects [39,40]. Unlike apoptosis, where dramatic morphological changes in the cell can be viewed with light microscopy, autophagy is accompanied only by subtle changes in external cellular appearance. Changes in the cellular levels of key autophagic proteins have typically been used to monitor for autophagy, either by fluorescence microscopy or western blotting [41]. Two of these proteins are microtubule-associated protein light chain 3 (LC3), a ubiquitin-like protein associated with autophagosome, and sequestosome 1 (p62), a protein that delivers cargo to the autophagosome by binding to LC3 and is degraded by the autolysosome during autophagy [42]. HL60 cells exposed to IC90 concentration of photoactivated 4 for 6 h showed in western blotting a 2.5-fold increase in the levels of LC3B-II [23]. The presence of the lysosomal protease inhibitors E64d and pepstatin A increased the levels of LC3B-I still further, consistent with blocking the degradation of LC3B-II [42]. In parallel experiments, an IC90 dose of cisplatin caused no increase in the expression of the LC3B protein, probably because it induced apoptosis rather than autophagy in the HL60 cell line. The cellular levels of p62 consistently dropped on average by 40 per cent in HL60 cells treated with light-activated 4. These results are consistent with activated autophagic pathways during the early phases (6 h) of cell death. Whether the cells actually die by autophagy remains to be proven.

5. Conclusions and outlook

Light activation of non-toxic PtIV complexes appears to be a promising approach to increase the selectivity of Pt-base anti-cancer agents. Studies with all-tested diazido-PtIV complexes (i.e. 1–6) show photolysis bringing about irreversible binding of Pt to DNA that only minimally takes place in the dark, even in the presence of biological reducing agents. Binding of Pt to cellular DNA has been shown to take place in cells exposed to photoactivated PtIV diazides (i.e. 4 and 6), and this interaction with DNA could well be responsible for some of the cytotoxic effects. However, a lack of apoptosis in these cells suggests that other cell death pathways are activated, which could well be a result of different Pt-DNA adducts (both mono- and bifunctional) compared with cisplatin. Alternatively, RNS may also form (e.g. azidyl radicals, N atoms) upon light activation, bringing about oxidative stress in cancer cells. Our evidence from flow cytometry studies with 4 already supports this hypothesis.

There appears to be a difference between the mechanisms of toxicity depending on whether the two azide ligands are cis- or trans-configured. Nevertheless, both cis- and trans-diazido-PtIV complexes initiate cell death by (a) mechanism(s) different to that of cisplatin. Autophagy pathways appear activated and might play a role in the cytotoxic effects of some of the compounds. Furthermore, little cross-resistance is observed between cisplatin and photoactivated diazido-PtIV complexes, regardless of whether they are cis- or trans-coordinated. Complex 6 would appear to be an ideal candidate for further preclinical development, in particular, for testing in animal tumour models.

The main challenge in the development of better diazido-PtIV complexes for PACT will be to increase the wavelength of light capable of photoactivation. This would allow deeper penetration of light into the tumour and a more complete activation of the drug throughout the tumour mass. However, it will be difficult to extend the LMCT bands of diazidio-PtIV complexes much further into the visible region of the electromagnetic spectrum (i.e. into the red region) through synthesis of new complexes alone. Alternatively, it might be worthwhile to tether such light-activatable complexes to up-converting nanoparticles that specifically home in on tumour cells [43,44]. Such
nanoparticles could then up-convert near-infrared radiation, directed at the tumour with fibre optic devices, to emit visible light of the proper wavelength and sufficient intensity to achieve efficient photoactivation deeper within tumours.

We thank the members of COST Action CM1105 for many useful discussions.

References


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