Platinum Anticancer Drugs

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A Photoactivatable Platinum(IV) Complex Targeting Genomic DNA and Histone Deacetylases

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Abstract: *We report toxic effects of a photoactivatable platinum(IV) complex conjugated with suberoyl-bis-hydroxamic acid in tumor cells. The conjugate exerts, after photoactivation, two functions: activity as both a platinum(II) anticancer drug and histone deacetylase (HDAC) inhibitor in cancer cells. This approach relies on the use of a Pt^{IV} pro-drug, acting by two independent mechanisms of biological action in a cooperative manner, which can be selectively photoactivated to a cytotoxic species in and around a tumor, thereby increasing selectivity towards cancer cells. These results suggest that this strategy is a valuable route to design new platinum agents with higher efficacy for photodynamic anticancer chemotherapy.*

It has been previously shown that photoactivation of nontoxic platinum(IV)-azido complexes can generate potent, cytotoxic species capable of causing cell death in a number of cells.[1] After irradiation with ultraviolet (UV) or visible (Vis) light, they easily undergo photoreduction to form bioactive Pt^{II} species that are able to irreversibly platinate DNA. The photoactivation in tumor cells brings about destruction of the nuclear DNA,[2] causing cell death.

In the nucleus, DNA is noncovalently associated with histones to form the nucleosomes which make up chromatin subunits. Agents, such as histone deacetylase (HDAC) inhibitors, that induce hyperacetylation of histone proteins could increase the accessibility of DNA within chromatin[3] and consequently potentiate the anticancer activities of platinum and other DNA-damaging drugs.[4] These facts suggest that cell death triggered by simultaneous HDAC inhibition and DNA damage could be a viable alternative approach in cancer therapy.

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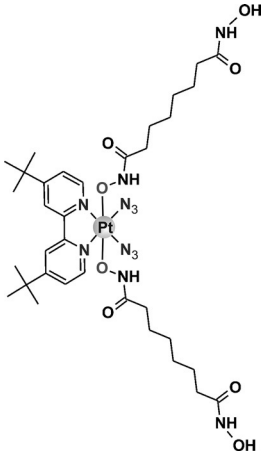

Figure 1. Structural formula of of *cis,trans*-$[Pt(N_3)_2(Sub)_2(tBu_2bpy)]$ (**1**).

Herein, we designed a photoactivatable platinum(IV)-diazido complex, *cis,trans*-$[Pt(N_3)_2(Sub)_2(tBu_2bpy)]$ (**1**), with suberoyl-bis-hydroxamic acid (SubH) as axial ligands, where tBu_2bpy stands for 4,4′-di-*tert*-butyl-2,2′-bipyridine (Figure 1). SubH is a HDAC inhibitor, which inhibits the activity of HDACs at submicromolar concentrations and exhibits a profound dose-dependent inhibition of tumor cell proliferation at micromolar concentrations.[5] Interestingly, SubH also shows potent synergistic interaction with antitumor Pt^{II} complexes at equimolar concentrations.[5c] Thus, **1** was designed to achieve a dual functionality of the new prodrug, simultaneously targeting genomic DNA and HDACs both induced by low energy UVA or Vis irradiation. Our strategy has been to develop a new photoactivatable Pt^{IV} system which is kinetically inert (in the dark) to suppress the substitution reactions with biomolecules, but should be capable of controlled release by irradiation of clinically effective levels of Pt^{II} species binding nuclear DNA as well as bioactive ligands (HDAC inhibitor) that may potentiate toxic effects of the Pt^{II} drugs by an independent pathway. Notably, the substitutional inertness of the new system in the dark, as well as the relative stability of Pt^{IV} complexes to hydrolysis,[6] would prevent the premature release of biologically active Pt^{II} and HDAC inhibitor species in vitro or in vivo. This approach relies on the use of a photoactivatable Pt^{IV} pro-drug with a dual mechanism of action for localized tumors accessible by fiber-optic devices, thereby also decreasing the damage to normal, noncancerous cells.

Thus, we specifically designed the complex *cis,trans*-$[Pt(N_3)_2(Sub)_2(tBu_2bpy)]$ (**1**) (Figure 1) which exists as an

inert platinum(IV)-diazido prodrug form when kept in the dark, but is rapidly activated to efficiently release simultaneously the cytotoxic Pt^{II} species and HDAC inhibitor SubH upon UVA irradiation. Complex **1** was prepared by a synthetic procedure described in the Supporting Information (section Results and Scheme S1). The final compound was characterized by elemental analysis, infrared, ^{1}H, and ^{195}Pt NMR spectroscopy (for details, see the Experimental section in the Supporting Information). Photodecomposition of **1** was studied by means of ^{195}Pt NMR spectroscopy (Supporting Information, section Results and Figure S1).

The first experiments were aimed at quantifying the binding of **1** to mammalian DNA in cell-free media. Two samples of double-helical calf thymus (CT) DNA were incubated with **1** at the r_i value of 0.1 (r_i is defined as the molar ratio of free platinum complex to nucleotide phosphates at the onset of incubation with DNA) in $NaClO_4$ at 37 °C as described in detail in the Experimental section of Supporting information. One sample was irradiated with UVA (365 nm, 4.3 mW cm^{-2}) or visible (458 nm, 65 mW cm^{-2}) light immediately after the addition of **1**; the other sample was kept in the dark. Aliquots of both samples were withdrawn at various time intervals; free, unbound platinum was removed by gel filtration through a Sephadex G-50 coarse column, and DNA was assayed for platinum content by flameless atomic absorption spectrometry (FAAS). Only a very small amount (ca. 5%) of platinum from **1** bound to DNA was found in the sample which was kept in the dark, even after a long time of incubation (24 h; Figure 2).

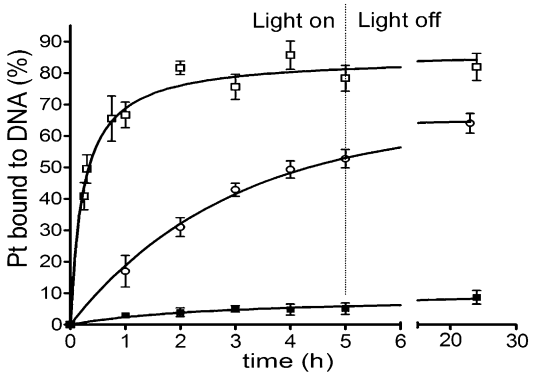

Figure 2. Kinetics of the reaction of **1** photoactivated by UVA (□), visible light (○) or incubated in the dark (■) with double-helical CT DNA in 10 mM $NaClO_4$ at 37 °C. Each data point represents the mean ± SD of at least three independent experiments.

In contrast, the amount of platinum from **1** bound to DNA in irradiated samples increased with time. Under continuous UVA irradiation, platinum from **1** was able to bind DNA so fast that 50% of Pt was bound after 24 min ($t_{50\%}$); the plateau of the platination was reached after 2 h of continuous irradiation when nearly 80% of platinum present in the sample was bound to DNA. Similarly, 50% of Pt from **1** was bound after 4 h of continuous irradiation by visible (blue) light (Figure 2). Thus, these DNA binding experiments clearly indicated that **1** was almost inactive and unable to bind DNA in the dark, but under irradiation by UVA or visible light, it was activated and platinum from **1** strongly bound to DNA. Notably, the results of these DNA binding studies in cell-free media indicate that the rates of binding of platinum from **1** to natural double-helical DNA, when the reaction mixture was irradiated by UVA or visible light were relatively high compared to the binding of nonirradiated conventional cisplatin ($t_{50\%} = 120$ min[7]). Moreover, the results of transcription mapping experiments (Supporting Information, Figure S9) suggest that the Pt^{II} species formed after photoactivation target similar DNA regions as conventional cisplatin. Interestingly, in view of the deeper tissue penetration of red light, irradiation of a solution containing **1** and DNA with red light ($\lambda \geq 630$ nm) also resulted in a significant increase of platinum bound to DNA by about 14%, if compared with the sample kept in the dark.

We also tested whether **1** can be activated in the dark by biological reductants so that platinum from **1** would bind DNA. The ability of platinum from **1** to bind DNA in the presence of glutathione and ascorbic acid was examined (see the Supporting Information). Compound **1** was stable in the dark even in the presence of the biological reductants, so the binding of Pt from **1** to DNA was not activated in the presence of intracellular reducing agents in the dark (Supporting Information, Figures S2–S4).

The photoactivated dose-dependent inhibition of cell viability (IC_{50} values) for **1** towards human ovarian cell lines

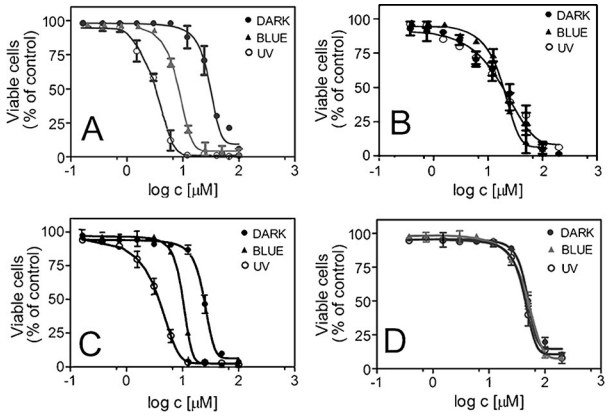

Figure 3. Phototoxicity of **1** (left panels A, C) and cisplatin (right panels B, D) in human ovarian A2780 cells (panels A, B) and A2780cisR (panels C, D). The recovery period after irradiation was 24 h. Each data point represents the mean ± SD of at least two independent experiments each performed in triplicate.

Table 1: IC_{50}[a] values (μM) obtained by neutral red assay for **1** and cisplatin.[b]

	A2780			A2780cisR		
	Dark	Blue	UVA	Dark	Blue	UVA
Cisplatin	21 ± 3	19 ± 2	18 ± 1	50 ± 1	50 ± 3	45 ± 2
1	31 ± 2	8.4 ± 0.4	3.3 ± 0.3	24.2 ± 0.7	10.2 ± 0.2	3.9 ± 0.5

[a] The concentration of the complex that inhibited dye uptake by 50%. The lower value indicates the higher toxicity to cells. [b] For other details, see Figure 3. Each value is the mean of three independent experiments performed in triplicate.

was evaluated using neutral red uptake assay after 30 min of irradiation with UVA, blue, or sham irradiation followed by 24 h of recovery (Figure 3, Table 1).

The corresponding data for cisplatin were also determined for comparative purposes. The cytotoxic effect of cisplatin was not significantly affected by light (UVA or blue) neither in cisplatin-sensitive A2780 nor resistant A2780cisR ovarian adenocarcinoma cells (Figure 3B and D, Table 1). In contrast, the cytotoxicity of **1** markedly increased (6–9-fold) upon irradiation with UVA. Therefore the cytotoxicity in both cell lines tested was significantly greater than that of cisplatin (6–11-fold) under the same experimental conditions (Figure 3A and C, Table 1). Visible blue light also caused elevated cell death in the presence of **1**. Qualitatively identical results were also obtained after longer (72 h) recovery time after irradiation (Supporting Information, Figure S5, Table S1). Furthermore, the photoactivated dose-dependent inhibition of cell viability towards human ovarian cell lines was also evaluated for Pt^{IV} analogues of **1**, containing biologically inactive hydroxido or acetato axial ligands instead of SubH groups, namely *cis*,*trans*-$[Pt(en)(N_3)_2(OH)_2]$ and *trans*,*cis*-$[Pt(bpy)(OAc)_2(N_3)_2]$ (Supporting Information, Table S1). Notably, the cytotoxicity of these derivatives of **1** was also markedly increased upon irradiation with UVA, but significantly less than that of **1**. This difference in cytotoxicities of **1** and its Pt^{IV} derivatives after photoactivation was markedly more pronounced in A2780cisR cells resistant to cisplatin, indicating that the mechanism of action of **1** also involves, on top of the process(es) associated with platination of DNA, other processes not affected by the mechanisms of resistance developed by cells towards DNA damaging cisplatin.

Complex **1** was designed such that the Pt^{II} product of its photoreduction should bind nuclear DNA similarly to cisplatin or its derivatives. Besides, the extra functionality of this compound lies in the HDAC inhibitory activity of its axial Sub ligands released upon light-induced reduction in cells. HDACs inhibition induces hyperacetylation of histone proteins complexed with DNA, thereby increasing the accessibility of DNA within chromatin to DNA-binding agents[3a,b] with consequent enhancement of the extent of DNA damage by platinum adducts. In this way, the anticancer activity of this drug can be significantly potentiated. Therefore, further studies were aimed at determining the effect of **1** on HDAC activity in living cells.

Total activity of HDACs determined directly in A2780 cells decreased compared to the basal (control) level, when A2780 cells were treated with free SubH (Figure 4A). This observation is in good agreement with the well-known HDACs inhibitory effect of SubH (particularly to HDAC1 and HDAC3).[5a,c,8] The treatment of A2780 cells with **1** in the dark resulted in a decrease of HDACs activity below the basal level in the untreated A2780 cells. Importantly, the effect of **1** on the activity of HDACs was further potentiated when the cells were irradiated with either blue or UVA light. Conversely, cisplatin (applied in the dark or under irradiation; Figure 4A) or Pt^{IV} analogues of **1** containing biologically inactive axial ligands (Supporting Information, Figure S6) had no significant effect on HDACs activity in A2780 cells under the experimental condition used.

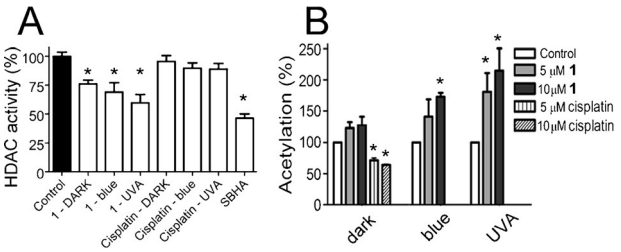

Figure 4. Effect of irradiated **1** on HDACs in A2780 cells. A. The HDAC activity in cells treated with **1**, cisplatin, or SubH at their 18 μM concentrations was assessed as the ability to remove an acetyl group from an acetylated substrate. HDAC activity in control, untreated cells was taken as 100%. It was verified that in the presence of 1 μM trichostatin A (a potent and specific inhibitor of HDAC), the deacetylase activity was inhibited to 4 ± 2% of the control. The data represent mean ± SD of three independent experiments. An asterisk denotes a significant difference ($p < 0.01$) from the untreated control. B. Quantification of acetylated histone H3 in cells treated with **1** (10 μM). The level of acetylated histone H3 measured for the control was taken as 100%. The results are means of three independent experiments, and standard deviations are shown by error bars.

The results obtained by direct analysis of HDAC activity were complemented by the quantification of total levels of genome-wide histone H3 acetylation in A2780 cells (Figure 4B). The results of this analysis indicated that the levels of histone H3 acetylation in A2780 cells were increased compared to the basal (control) level, if A2780 cells treated with **1** were photoactivated. Thus, the results demonstrating the increased level of histone H3 acetylation owing to the irradiation of the cells treated with **1** are consistent with the reduced HDAC activity compared with that found for control (irradiated but nontreated cells).

There is an important implication of our findings that photoactivatable Pt^{IV} complexes induce cytotoxicity and HDAC inhibitory activity. There must be another factor, in conjunction with the DNA binding capability of Pt(II) species formed upon photoactivation of **1,** responsible for its enhanced cytotoxicity in contrast to analogous photoactivatable Pt^{IV} complexes containing biologically inactive axial ligands.

One of the factors playing a key role in the platinum drug-mediated cytotoxicity is the arrest of RNA synthesis by Pt–DNA adducts.[9] It has been shown that the ability of platinum drugs to block RNA polymerase is enhanced by the presence of sterically encumbering DNA adducts of Pt^{II} species.[10] After photoreduction, the tBu_2bpy ligand remains coordinated to the platinum moiety and, after platinum is bound to DNA forming DNA adducts, they can create a severe steric block for RNA polymerases.

The results shown in Figure S7 (Supporting Information) show that the DNA adduct of photoactivated **1** can represent a serious steric obstacle for RNA polymerization. This may significantly contribute to the considerable cytotoxic activity of **1** when photoactivated.

Bifunctional platinum compounds coordinating base residues in DNA form various types of interstrand and intrastrand cross-links (CLs). Such cross-links in the target DNA are important factors involved in the DNA damaging

action of genotoxic agents, although their relative efficacy remains unknown. Nonetheless, interstrand CLs are considered to be more inhibitory to DNA replication and transcription because the damage involves both complementary strands and precludes the correct function of RNA and DNA polymerases.[11] The repair of interstrand CLs is also not easily achievable as it has to employ both nucleotide excision and recombinational repair pathways. Thus, interstrand CLs might be significantly responsible for anticancer activity of platinum drugs.[12] Therefore, we quantitated the interstrand cross-linking efficiency of photoactivated **1** in 212 bp fragment of natural pUC19 DNA (Supporting Information, Figure S8). The intensity of the interstrand CL-containing DNA fraction increased with growing levels of platination. The frequency of interstrand CLs of photoactivated **1** was calculated as described earlier,[13] and was found to be $34\pm4\%$ and $31\pm2\%$ (mean and standard deviation, $n=3$) for DNA modified by **1** photoactivated by UVA and visible light, respectively. Thus, the efficiency of irradiated **1** to form interstrand CLs in DNA is markedly enhanced (ca. 5-fold) compared to that of cisplatin (6%).[14]

In conclusion, the platinum(IV) complex **1** has been prepared and characterized. It is remarkably stable in the dark, even in the presence of cellular reducing agents such as glutathione and ascorbic acid, but readily undergoes photoinduced ligand substitution and photoreduction reactions. When **1** is photoactivated in human ovarian carcinoma A2780 cells, it is highly toxic: pronouncedly more cytotoxic than its photoactivatable Pt^{IV} derivatives containing biologically inactive axial ligands or the Pt^{II} anticancer drug cisplatin (Table 1; Supporting Information, Table S1). Notably, photoactivated **1** was more toxic than its Pt^{IV} derivatives containing inactive axial ligands, or cisplatin in the dark in cisplatin-resistant A2780cisR cells. The resistance factor (defined as the ratio of IC_{50} values in resistant (A2780cisR) and cisplatin-sensitive parent cells (A2780)) was 1.2 for photoactivated **1**, whereas it was markedly higher for its Pt^{IV} derivatives containing inactive axial ligands or cisplatin (ca. 2-fold). This suggests that the mechanism underlying the biological action of photoactivated **1** is different from that of its Pt^{IV} derivatives containing inactive axial ligands or cisplatin and its clinically used derivatives, allowing photoactivated **1** to successfully overcome the resistance mechanisms operating in the case of cisplatin. Thus, photoactivated **1** has an unprecedented in vitro cytotoxicity, better than that of cisplatin or its antitumor derivatives used in the clinic. The results also show that the mechanism underlying the biological action of photoactivated **1** also involves an inhibitory effect towards HDACs, resulting in elevated levels of histone acetylation. It may, consequently, increase the accessibility of DNA in chromatin to DNA damage caused by the platinum moiety. Furthermore, photoactivated **1** coordinating to DNA forms markedly more interstrand CLs. These CLs are highly deleterious lesions that block transcription and replication more efficiently than other types of lesions induced in DNA by antitumor platinum drugs. Therefore, it is not surprising that photoactivated **1** shows significantly higher potency to block RNA polymerization (the major effect mediating antitumor effects of conventional platinum drugs) than cisplatin and its clinically used analogues. Thus, the enhanced cytotoxicity of irradiated **1** in comparison with cisplatin (both nonirradiated and irradiated) could be a consequence of combination of: i) histone deacetylase inhibition that induces hyperacetylation of histone proteins complexed with DNA; ii) effective steric blockage of RNA polymerase II inhibiting the prolongation of RNA transcription; and iii) more extensive and/or distinct global perturbations of DNA owing to the DNA adducts (particularly interstrand CLs) favorable for the antitumor effect.

Our results lend strong support to the view that the strategy to conjugate the photoactivatable Pt^{IV} compounds with biologically active ligands (possessing their own biological effect(s)) might be also applicable in vivo and in the tumors resistant to cisplatin and its clinically used derivatives. The results of our work might have broad implications for photoactivated chemotherapy of cancer owing to extensive use of platinum-based drugs in cancer treatment, and they suggest that strategies based on simultaneous HDACs inhibition and DNA damage by platinum drugs induced by light may result in efficient antitumor activity, taking advantage of the selective and targeted activation in tumor cells.

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