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Photoactive Monofunctional Platinum(II) Anticancer Complexes of Multidentate Phenanthridine- Containing Ligands: Photocytotoxicity and Evidence for Interaction with DNA

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ABSTRACT

Pt(II) complexes supported by chelating, multidentate ligands containing π -extended, planar phenanthridine (benzo[*c*]quinoline) donors ($^{\mathbf{R}}\text{LPtCl}$) exhibit a promising *in vitro* therapeutic index compared with phenanthriplatin, a leading preclinical anticancer complex containing a monodentate phenanthridine ligand. Here, we report evidence for non-specific interactions of $^{\text{CF}_3}\text{LPtCl}$ with DNA through intercalation-mediated turn-on luminescence in O_2 saturated aqueous buffer. Brief irradiation with visible light (490 nm) was also found to drastically increase the activity of $^{\text{CF}_3}\text{LPtCl}$, with photocytotoxicity increased up to 87% against a variety of human cancer cell lines. Mechanistic studies highlight significantly improved cellular uptake of $^{\text{CF}_3}\text{LPtCl}$ compared with cisplatin, with localization in the nucleus and mitochondria triggering effective apoptosis. Photosensitization experiments with 1,3-diphenylisobenzofuran demonstrate $^{\text{CF}_3}\text{LPtCl}$ efficiently mediates the generation of singlet dioxygen ($^1\text{O}_2$), highlighting the potential of $^{\mathbf{R}}\text{LPtCl}$ in photodynamic therapy.

Introduction

A promising strategy for expanding the spectrum of activity of anticancer compounds and reducing side effects is to seek out complexes with novel structures that might operate via distinctive mechanisms of action.¹ Pt(II) complexes typically exert antineoplastic activity by disrupting a cancer cell's ability to replicate and repair DNA through direct interactions with nucleobases.^{2,3} Monofunctional platinum complexes (e.g., $[\text{cis-Pt}(\text{NH}_3)_2(\text{phenanthridine})\text{Cl}][\text{NO}_3]$ aka phenanthriplatin; Figure 1)⁴ are a class of drug candidates bearing only a single labile ligand, and thus are thought to operate differently than those such as cisplatin ($\text{cis-Pt}(\text{NH}_3)_2\text{Cl}_2$) and its analogues which contain two.⁵ For example, while cisplatin chelates DNA through formation of Pt-N bonds with adjacent purine nucleobases,⁶ single-molecule DNA-stretching experiments indicate a two-step DNA-binding process for phenanthriplatin in which intercalation of the phenanthridine unit triggers rapid unwinding of DNA that enables subsequent, efficient covalent binding.⁷ These modes of interaction with DNA complement wholly non-covalent groove binding, intercalation or insertion modes,⁸ such as are exhibited by cyclometallated Pt(II) complexes of 2,9-diphenyl-1,10-phenanthroline⁹ or π -extended ligands such as dipyridophenazine.¹⁰ As a result, bimodal chemotherapeutic approaches can be accessed, as can be seen in the example of mitochondrial-targeting monofunctional platinum complexes supported by tridentate, neutral,

quinoliny/pyridinyl imine ligands which induce tumor cell death through both autophagy and apoptosis.¹¹

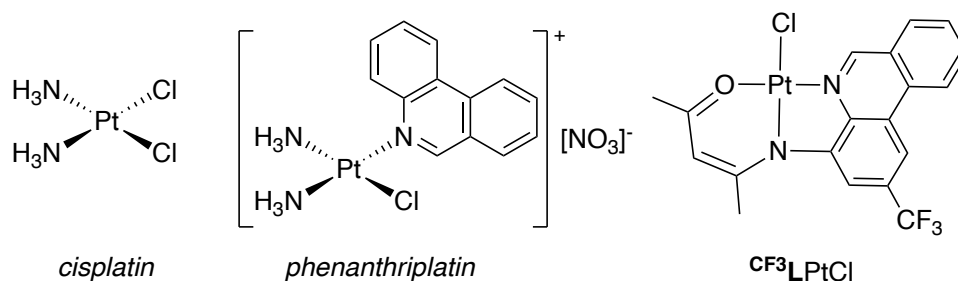


Figure 1. Structures of cisplatin,¹² phenanthriplatin⁴ and the multidentate phenanthridine-ligand supported Pt complex (CF_3LPtCl) discussed in this work.

We recently communicated the antineoplastic properties of CF_3LPtCl (Figure 1), a Pt(II) complex bearing a similar phenanthridinyl ligand moiety as in phenanthriplatin. In contrast to phenanthriplatin, our ligand design restricts the *N*-heterocycle to be both *cis* to the labile chloride and coplanar with the coordination plane of the metal by incorporating it into a chelating, multidentate ligand scaffold.¹³ CF_3LPtCl was found to exhibit a superior *in vitro* therapeutic index compared with both cisplatin and phenanthriplatin, with increased cytotoxicity and selectivity toward cancerous cell lines. In this contribution, we present evidence for non-covalent interactions with DNA through ‘turn-on’ luminescence experiments and enhanced cytotoxicity following brief irradiation with visible light (photocytotoxicity).

RESULTS AND DISCUSSION

Interaction of CF_3LPtCl with Random DNA Sequences and Other Biomacromolecules

“Light-switch” behaviour – enhancement or ‘turn-on’ of emission in the presence of nucleic acids¹⁴ – can enable application of coordination complexes, for example, as biomolecular or cellular probes.¹⁵ In addition, such complexes also bear promise in photodynamic therapy.^{16–18} DNA binding by luminescent intercalators can be probed using electronic absorption and emission spectroscopy.^{19–21} We examined the interactions between CF_3LPtCl and calf-thymus DNA (ct-DNA) using electronic absorption spectroscopy. The UV-Vis spectrum of the complex introduced to O_2 -saturated aqueous buffer in an aliquot of dimethylsulfoxide ($\text{DMSO} \leq 0.4\% \text{ v/v}$) contains a structureless absorption maxima between 465–480 nm that is broader relative to the spectra of the complex in otherwise unadulterated CH_2Cl_2 ($\lambda_{\text{max,abs}} = 475 \text{ nm}$; Figure 2a). The lowest energy absorption manifold of CF_3LPtCl is of mixed metal-to-ligand and intra-ligand charge-transfer (MLCT/ILCT) character, thanks to π -overlap between the ligand $\text{N}^{\wedge}\text{O}$ donor fragment and the chloride with filled Pt(II) $5d$ orbitals of appropriate energy and symmetry.²² R^nLPtCl complexes are moderately solvatochromic, as solvent-analyte dipole interactions and hydrogen bonding preferentially stabilize the ground state electronic structure of the complex. However, owing to the complex environment the analyte is in in these experiments, it is not obvious whether the broadening observed in the absorption spectrum is due to solvent-analyte or electrolyte-analyte interactions. Excimer emission at lower energy was not observed, similar to our previous studies in purely organic solvents.

In general, Pt(II) complexes can exhibit either bathochromism or hypsochromism in their absorption spectra upon intercalation. For example, the absorption band ascribed to the MLCT excitation of $[\text{Pt}(\text{tpy})\text{X}]^+$ ($\text{X} = ^-\text{OH}^{19}, ^-\text{SCH}_2\text{CH}_2\text{OH}^{23}$) shifts to significantly lower energy (< 1000

cm⁻¹) with increasing DNA concentration, while the intensity of higher energy intra-ligand π - π^* transitions attenuate compared to the free, solvated complex. In contrast to intercalation, binding of chromophores to the grooves of DNA has been shown to have minimal to no impact on the position of the absorption maxima, though changes in intensities can vary;²⁴ for example, as seen in a family of Pt(II) complexes supported by cyclometallating 2-(2-(1-benzyl-1*H*-1,2,3-triazol-4-yl)-6-phenylpyridine) ligands bearing alkyl tethers with terminal water-soluble functional groups.²⁵ In ^{CF₃}LPtCl, the peak positions of the MLCT/ILCT manifolds remain invariant with increasing ct-DNA concentration (Figure 2b), which could imply similar binding to double-stranded DNA grooves rather than intercalation.

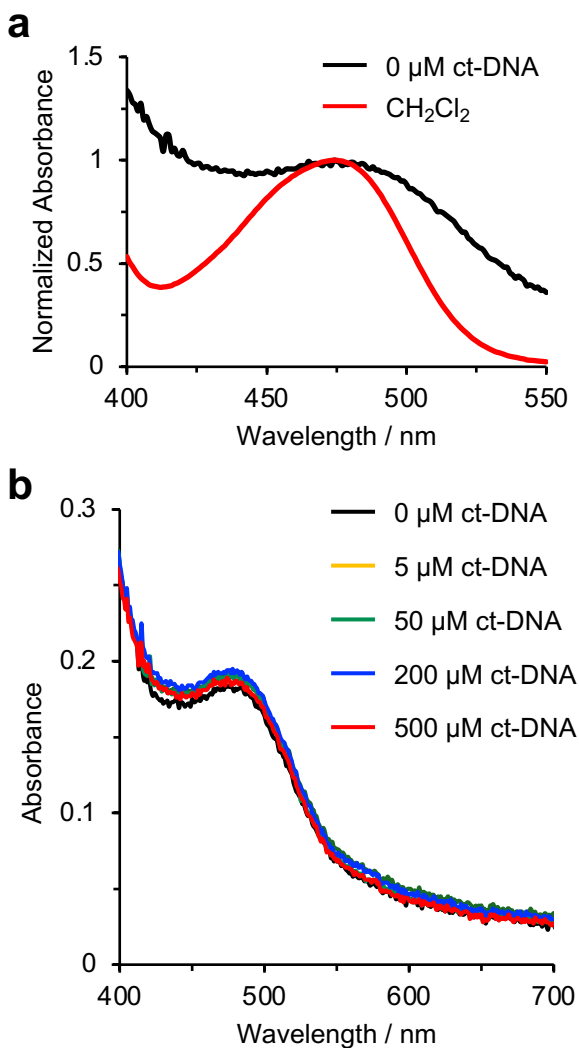


Figure 2. (a) Absorption spectrum of CF_3LPtCl in 500 μM aqueous solution of ct-DNA in 50 mM Na_3PO_4 buffer, pH 7.0; (b) effect of increasing ct-DNA concentration (50 mM Na_3PO_4 buffer, pH 7.0) to the absorption spectrum of CF_3LPtCl .

CF_3LPtCl emits strongly in deoxygenated organic solution ($\Phi_P = 16\%$ in CH_2Cl_2) with an emission maximum centered at 608 nm and a microsecond excited state lifetime ($\tau = 23 \mu\text{s}$).²² A significant quenching of the emission intensity and decrease in τ ($\tau = 0.48 \mu\text{s}$ in CH_2Cl_2) are observed in the presence of O_2 , consistent with radiative decay from a low-lying triplet excited state. Accordingly, emission from CF_3LPtCl is totally quenched in aerated aqueous buffer (blue

trace, Figure 3a). As no substantial differences are observed in the absorption spectrum under these conditions compared to in fluid organic solution, we do not attribute this behaviour to the formation of non-emissive aggregates.²¹ Interestingly, phosphorescence is observed in solutions of CF_3LPtCl containing ct-DNA (gray and red traces, Figure 3a) with an emission maximum centered at ~ 585 nm. The emission is shifted to higher energy by 647 cm^{-1} compared to in CH_2Cl_2 .²² This ‘light-switch’ behaviour in buffered aqueous solutions of ct-DNA is reminiscent of that of ethidium bromide (EthBr), a commonly used probe in displacement assays to estimate the binding affinity of intercalators with DNA.²⁶ Such behaviour has also been observed for Pt(II) complexes which function as DNA groove-binders.²⁵

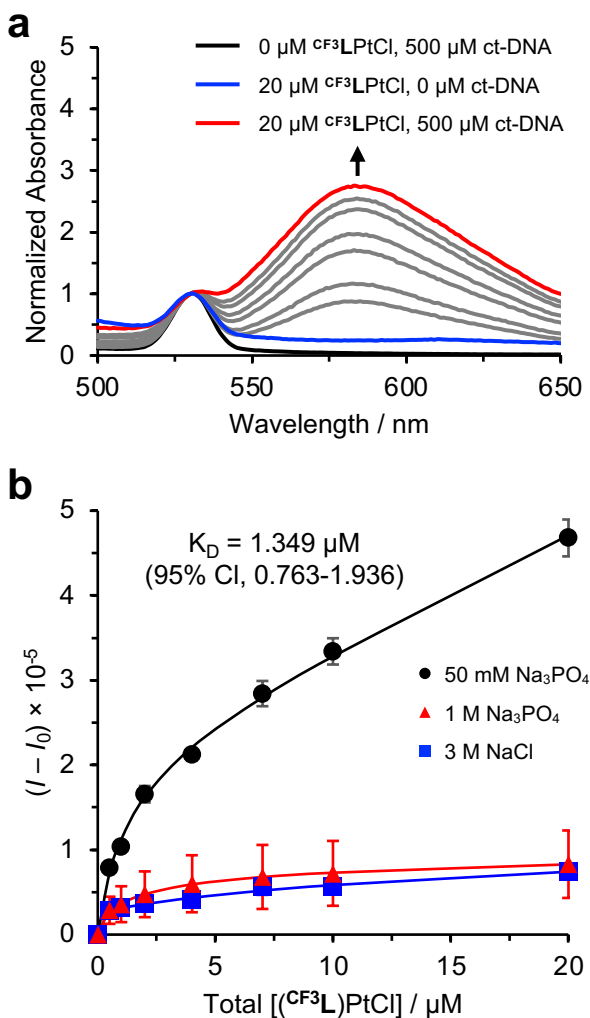


Figure 3. (a) Light switch behaviour in CF_3LPtCl with ct-DNA, showing increasing phosphorescence intensity with increasing ct-DNA concentration (50 mM Na_3PO_4 buffer, pH 7.0). (b) One site, total and nonspecific binding saturation model to estimate the degree of association of the complex with ct-DNA (500 μM) fitting intensity (I) at 583 nm ($\lambda_{\text{excitation}} = 450$ nm, 5 nm slit width).

While these observations cannot be used to conclusively differentiate intercalation from groove-binding, they do allow us to estimate the strength of association between CF_3LPtCl with DNA. Holding the concentration of ct-DNA constant, emission intensity increases with increasing

^{CF3}LPtCl concentration. This response can be fit to a one-site, total and nonspecific binding saturation model giving a binding dissociation constant (K_D) of 1.349 μ M (Figure 3b), comparable to that of EthBr (2.3 μ M).²⁶ In contrast, phenanthriplatin exhibits an order of magnitude weaker affinity for DNA ($K_D > 10 \mu$ M), presumably owing to the near-orthogonal orientation of phenanthridine with respect to the Pt coordination plane which may impact the extent of intercalation.⁷ Similar dissociation constants are observed with known groove binders such as netropsin (K_B/K_D , $2.4 \times 10^6 \text{ M}^{-1}/0.42 \mu\text{M}$)²⁷ and spermine (K_B/K_D , $2.1 \times 10^5 \text{ M}^{-1}/6.7 \mu\text{M}$).²⁸ An apparent decrease of K_D is observed in samples with increased ionic strength, independent of the charge of the anion (Figure 3b; see Figure S1 for emission spectra). This suggests that in buffered aqueous solutions, the complex may be forming a charged complex in the form of an aquo complex via labilization of the chloride. In contrast, in buffered aqueous solutions of ^{CF3}LPtCl and a RHAU-specific motif (RSM) that contains amino acids 53-105 of the protein RHAU and exhibits an α -helical helical structure similar to DNA,²⁹ no emission is observed (Figure S2). This suggests that the complex does not interact with proteins or that it may interact in a completely different manner that maintains or induces quenching of phosphorescence. We note that one mechanism in the development of cisplatin-resistance is due to quenching of the reactive cisplatin-aquo complex by interaction with proteins such as glutathione.³⁰

Interaction of ^{CF3}LPtCl with DNA Hairpin Oligonucleotides

The ct-DNA employed above was comprised of various sheared double-stranded DNA sequences and is therefore not indicative of any base-pair specificity for binding. To examine sequence specificity, we turned to a series of known double-stranded hairpin oligonucleotides.³¹ Each hairpin oligonucleotide contained the same sequence with the exception of the two nucleotides

preceding the hairpin and the complementary base-pairing nucleotides (Figure 4a). All 16 possible combinations of sequences were generated, and emission spectra were collected after incubation overnight with 20 μM $^{\text{CF}_3}\text{LPtCl}$ in 50 mM sodium phosphate at pH 7.0. As shown in Figure S3, all 16 oligonucleotides tested induced light-switch behaviour with $^{\text{CF}_3}\text{LPtCl}$. Relative intensities cannot be directly correlated with affinity due to factors such as quantum yield. As such, to measure affinity of $^{\text{CF}_3}\text{LPtCl}$ to the double stranded hairpin oligonucleotides, we repeated the titration of 0-20 μM $^{\text{CF}_3}\text{LPtCl}$ incubated overnight in 50 mM sodium phosphate, pH 7.0 with 5 μM of the oligonucleotides containing GG, TT, and TG (Figure 4b). Relative binding affinities for the GG, TT, and TG containing nucleotides were 0.682 μM , 1.512 μM and 6.345 μM , respectively.

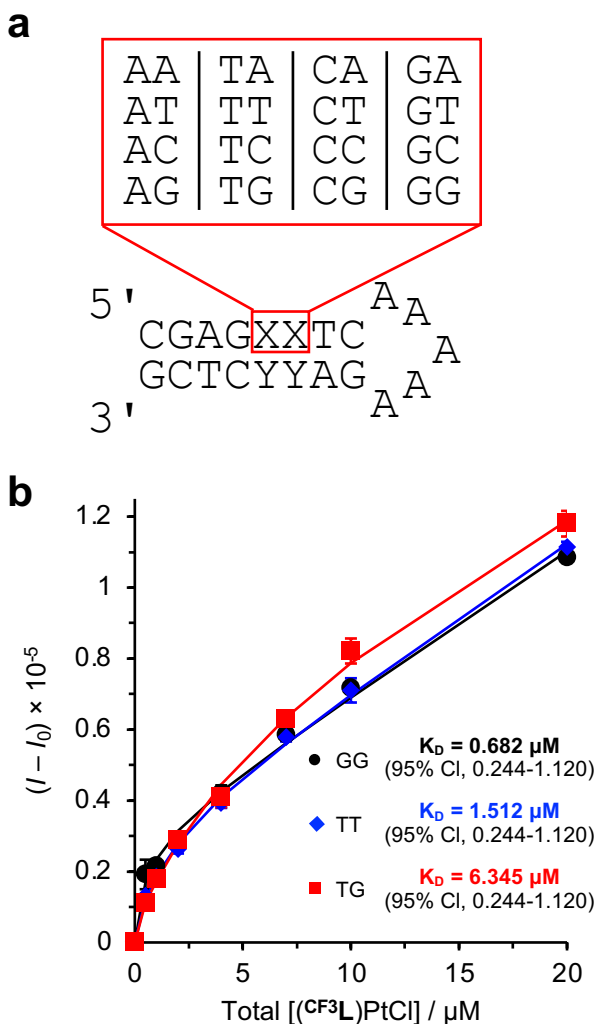


Figure 4. (a) Structure of hairpin oligonucleotides and (b) light switch behaviour and K_D of single hairpin oligonucleotides (50 mM Na₃PO₄ buffer, pH 7.0) fitting intensity (I) at 583 nm ($\lambda_{\text{excitation}} = 450$ nm, 5 nm slit width).

Altogether, this suggests ^{CF}₃L_{Pt}Cl binds with all double-stranded hairpin DNA oligonucleotides and that the relative binding affinities are indistinguishable. Although we have elucidated a clear binding capability between double stranded DNA and ^{CF}₃L_{Pt}Cl, it is not yet clear whether the interaction is occurring with melted single-stranded DNA (ss-DNA) or is specific to the double stranded DNA structure. To provide clarity, we collected emission spectra from the

titration of 0-20 μM $^{\text{CF}_3}\text{LPtCl}$ incubated in 50 mM sodium phosphate, pH 7.0, overnight with 5 μM of the DNA oligonucleotide with the sequence 5' – GTGGTGTGCAGCGAGAATAG. This oligonucleotide is single stranded with limited opportunity for intramolecular base pairing. Combining this oligonucleotide with increasing concentrations of $^{\text{CF}_3}\text{LPtCl}$ led to increasing emission intensities with an apparent K_D of 0.497 μM (Figure 5a-b). This indicates the $^{\text{CF}_3}\text{LPtCl}$ binds to both single-stranded and double-stranded DNA with similar efficacy.

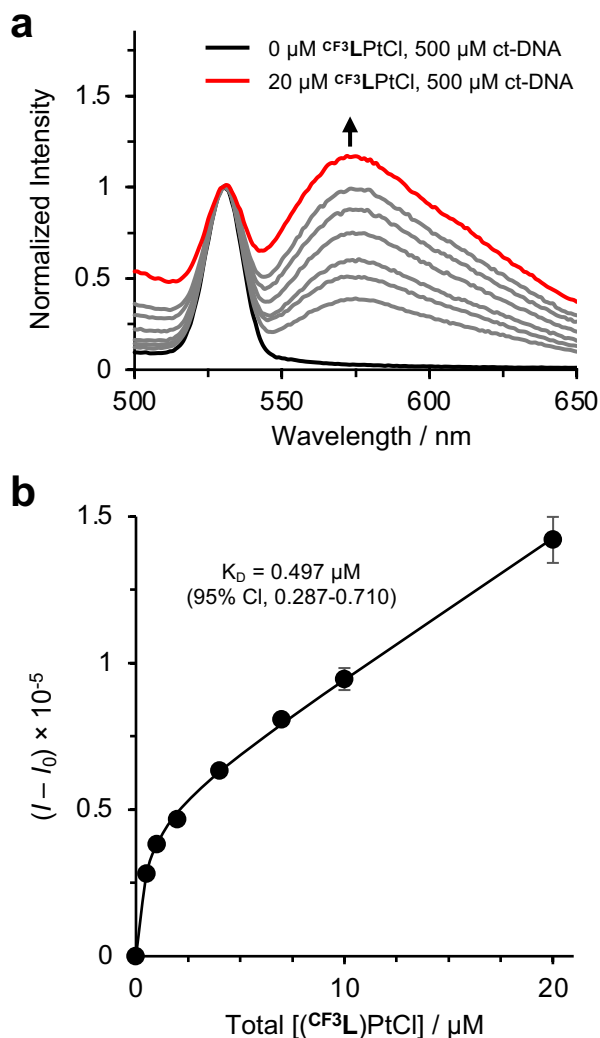


Figure 5. (a) Increasing emission intensity with increasing $^{\text{CF}_3}\text{LPtCl}$ concentration in buffered aqueous solutions of 5 μM DNA oligonucleotide (50 mM Na_3PO_4 buffer, pH 7.0) with the

sequence 5' – GTGGTGTGCAGCGAGAATAG; (b) apparent dissociation constant fitting intensity (I) at 583 nm ($\lambda_{\text{excitation}} = 450$ nm, 5 nm slit width).

To address the question of whether specific nucleotides are required or preferred for the binding of CF_3LPtCl with DNA, single-stranded pentamers of each DNA nucleotide were generated and samples containing 10 μM of each pentamer were exposed to 20 μM CF_3LPtCl in 50 mM sodium phosphate, pH 7.0. Surprisingly, only the guanine pentamer demonstrated any significant increase in emission intensity (Figure S4). Similar titration experiments under identical conditions with another pentamer with sequence 5' – AAGAA, guanosine nucleotides, and guanosine triphosphate (GTP), were also carried out. In all three cases, no light switch behaviour was observed. This implies guanine is necessary for the binding of CF_3LPtCl to DNA, but that a single guanine is not sufficient to encourage binding. The impact of guanine for the binding of CF_3LPtCl was validated using a ss-DNA oligonucleotide with a sequence of 5' – CTCCTCTCCACCCACAATAC. This ss-DNA oligonucleotide is identical to the one that was used in Figure 5, except with all guanines replaced with cytosines. Incubating a 5 μM solution of this ss-DNA oligonucleotide overnight with 20 μM CF_3LPtCl in 50 mM sodium phosphate at pH 7.0 did not lead to any appreciable emission enhancement (Figure S5). It also becomes apparent in the titration experiments with short ss-DNA and single-stranded pentamers that intercalation is the primary mode of interaction between DNA and CF_3LPtCl at least in these instances, as these strands do not have the opportunity to form grooves. Thus, the increased preference for guanine-rich DNA could be attributable to the increased dipole moment and number of hydrogen bonds in G-C base pair, compared with A-T pair, which leads to greater polarity and polarizability.³²

***In vitro* Photocytotoxicity**

Photodynamic therapy (PDT) is a promising research area, in which a light-absorbing molecule has its toxicity heightened following irradiation.³³ While PDT is traditionally understood in the context of the chemotherapeutic effect of singlet oxygen ($^1\text{O}_2$) / reactive oxygen species (ROS) generation,³³ photoactivatable platinum anticancer complexes, in particular, have been associated with diverse cell killing mechanisms.¹⁴ To evaluate the photocytotoxicity of CF_3LPtCl , we carried out MTT (3-(4, 5-dimethylthiazolyl)-2,5-diphenyltetrazolium bromide) cell viability assays against a panel of human cancer cell lines. The cells were seeded and incubated for 24 h, then treated with CF_3LPtCl . Cisplatin was engaged as a control. Following an additional 5 h of incubation, plates were either kept in the dark or irradiated with 490 nm light (2.36 mW/cm^2) for 15 minutes and cell viability was assessed. The results are shown in Table 1. CF_3LPtCl exhibits high potency in dark,¹³ but light treatment dramatically augmented its *in vitro* efficacy. For example, with platinum-resistant ovarian cancer cells (A2780cis) and cervical cancer cells (HeLa), 83.4% and 86.8% decreases, respectively, in IC_{50} are measured for CF_3LPtCl following irradiation with visible light. In comparison, the change in IC_{50} for cisplatin is <20% in each case. Similarly, increases in cytotoxicity are observed versus lung cancer (A549) and breast cancer (MDA-MB-231) cell lines, especially compared with cisplatin controls which in some cases show an *increase* in IC_{50} (e.g., vs. SKOV3; Table 1). A corresponding, though less statistically robust, effect is also observed versus ovarian cancer (SKOV3) cell lines. The phototoxic index ($\text{PI} = \text{IC}_{50}(\text{dark}) / \text{IC}_{50}(490 \text{ nm})$) of CF_3LPtCl in HeLa cells ($\text{PI} = 7.6$) thus approaches that reported for cyclometallated (2,2'-dipyridyl-4-methylbenzene)platinum chloride ($\text{PI} = 8$) which requires irradiation with higher energy light (405 nm).³⁴

Table 1. Photocytotoxicity profiles of cisplatin and ^{CF3}LPtCl against a panel of human cancer cell lines.

IC ₅₀ (μM)	A2780cis		SKOV3		A549		MDA MB 231		HeLa	
	Pt-resistant Ovarian Cancer		Ovarian Cancer		Lung Cancer		Breast Cancer		Cervical Cancer	
	Dark	490 nm	Dark	490 nm	Dark	490 nm	Dark	490 nm	Dark	490 nm
Cisplatin	125.0	102.3	141.5	179.0	246.5	231.1	201.4	198.2	166.3	133.2
	± 4.2	± 0.68	± 24.0	± 12.6	± 21	± 3.7	± 14.3	± 3.9	± 26.8	± 43.1
^{CF3} LPtCl	7.23	1.20	7.54	4.40	44.0	13.7	10.90	2.05	17.1	2.26
	± 2.3	± 0.94	± 4.7	± 0.1	± 16.1	± 1.6	± 0.52	± 1.90	± 2.8	± 0.99

The photocytotoxicity of $^{CF3}LPtCl$ was further studied by LIVE/DEAD cell assay using fluorescent microscopy (a combination of the ethidium homodimer (Erb) and a staining of acetomethoxycalcein, or calcein AM). Live cells were stained with calcein AM and yield a green fluorescence signal, whereas dead cells exhibit no fluorescence or a red signal due to the ethidium homodimer. As shown in Figure 6, HeLa cells treated with $^{CF3}LPtCl$ ($[Pt] = 10 \mu M$) for 24 h exhibited significant cell death in presence of irradiation while those without light irradiation under the same conditions all survived.

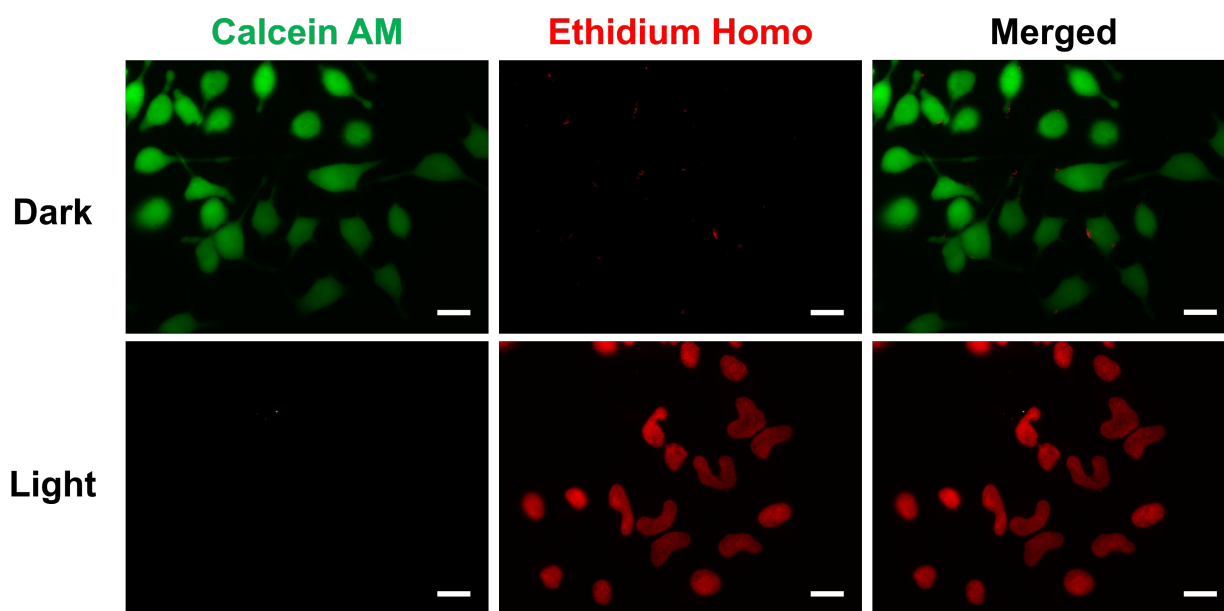


Figure 6. LIVE/DEAD cell assay of HeLa cells treated with $^{CF3}LPtCl$ ($[Pt] = 10 \mu M$) for 24 h followed by irradiation (15 min, 490 nm). Scale bar = 20 μm .

Mechanism of Action

To gain understanding about the mechanism of action, a series of cell-based studies were conducted. First, graphite furnace atomic absorption spectroscopy (GFAAS) was used to assess cellular uptake of the Pt compounds and subcellular distribution. In the experiment, one million A2780cis cells were treated with $^{CF3}LPtCl$ or cisplatin as a control ($[Pt] = 5 \mu M$). After 3 h, the

cellular Pt contents were determined using GFAAS. As shown in Figure 7a, the cellular uptake of $^{CF3}LPtCl$ was significantly higher than that of cisplatin. Upon isolating subcellular organelles, we determined the subcellular distribution of $^{CF3}LPtCl$: most of the Pt contents were found in nuclear and mitochondrial compartments (Figure 7b).

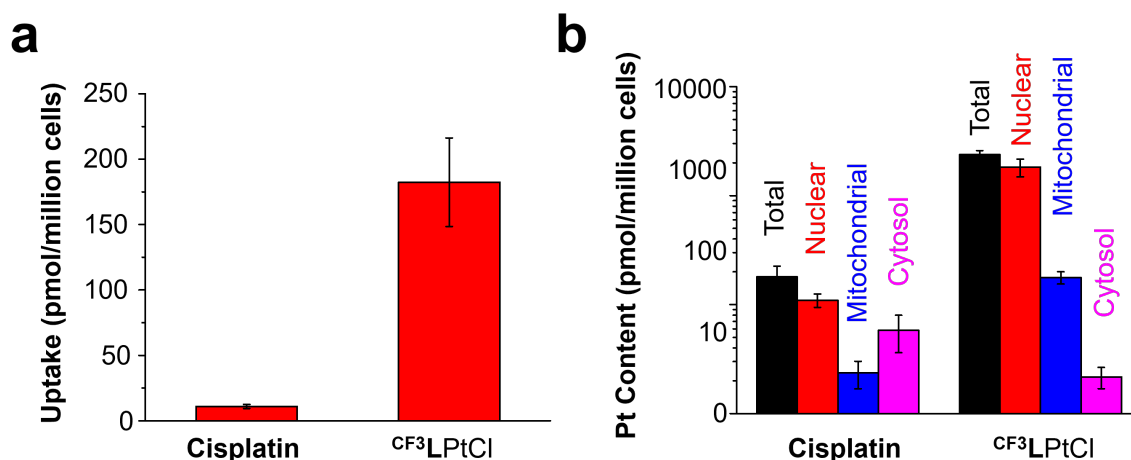


Figure 7. (a) Cellular uptake and (b) subcellular localization of cisplatin and $^{CF3}LPtCl$ in A2780cis cells ([Pt] = 5 μM for 3 h).

Next, we utilized flow cytometry to examine nuclear DNA and mitochondrial damage triggered by $^{CF3}LPtCl$ in presence or absence of 490 nm irradiation. Irradiation substantially increased $\gamma H2AX$ (Figure 8) and mitochondrial superoxide (Figure S7) in cells treated with $^{CF3}LPtCl$. This was not observed in the control sample treated with cisplatin. Likewise, in the flow cytometric analysis of apoptotic cells, we observed that the combination of $^{CF3}LPtCl$ and 490 nm irradiation effectively induced apoptosis in A2780cis cisplatin-resistant ovarian cancer cells (Figure S8). After incubating cells with $^{CF3}LPtCl$ ([Pt] = 10 μM) with subsequent 490 nm irradiation (2.36 mW/cm², 15 min), a large population of cells were in late (20.1%) and early (2.76%) apoptosis stages compared to the sample without irradiation, which only induced 4.43%

and 1.87% of cells to undergo late and early apoptosis respectively. Overall, we conclude that CF_3LPtCl readily enters cancer cells and is localized in nuclei and mitochondria. Upon irradiation, CF_3LPtCl triggers nuclear and mitochondrial damage in cancer cells, which then enter apoptosis.

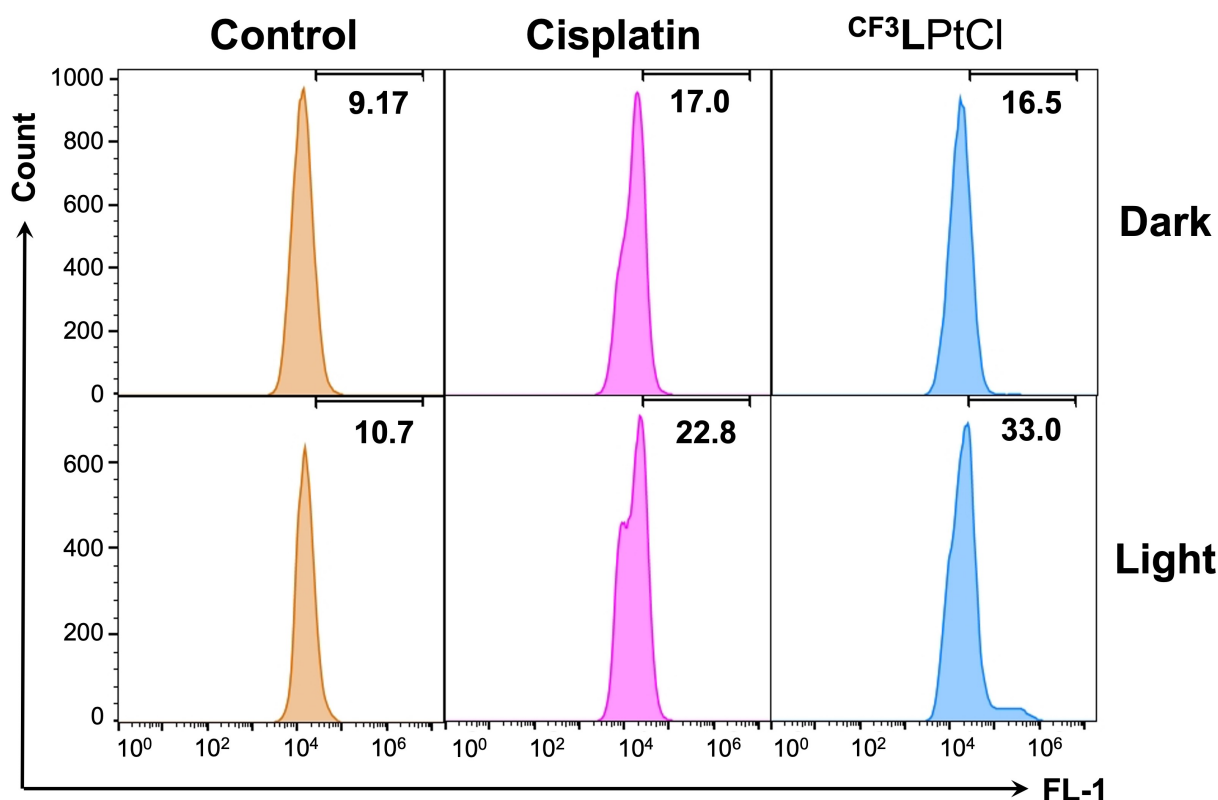


Figure 8. Flow cytometric analysis of DNA damage in A2780cis cells via γH2AX assays.

Singlet oxygen ($^1\text{O}_2$) is thought to be the primary cytotoxic species in PDT. Thus, it is critical to evaluate the ability of CF_3LPtCl to generate $^1\text{O}_2$. Dilute air-saturated, acetonitrile solutions of CF_3LPtCl and $[\text{Ru}(\text{bpy})_3][\text{PF}_6]_2$ ($\text{bpy} = 2,2'$ -bipyridine) as a control were prepared and combined with solutions of 1,3-diphenylisobenzofuran (DPBF; Scheme S1). The luminescence intensity at 475 nm ascribed to DPBF fluorescence (Figure S9) was monitored overtime upon continuous irradiation with light ($\lambda_{\text{excitation}} = 450 \text{ nm}$). An additional control of DPBF alone in air-

saturated CH_3CN was also prepared and so-monitored. As expected for the control, DPFB luminescence remained more or less constant within five minutes of constant irradiation indicating little to no $^1\text{O}_2$ present. In contrast, a rapid decrease in the emission intensity is evident in the presence of $[\text{Ru}(\text{bpy})_3][\text{PF}_6]_2$, as is expected for an effective $^1\text{O}_2$ sensitizer.³⁵ A comparably rapid decrease in luminescence intensity was also observed on addition of $^{\text{CF}_3}\text{LPtCl}$, indicating the complex is also capable of $^1\text{O}_2$ sensitization. Indeed, previous photophysical investigations of a series of $^{\text{R}}\text{LPtCl}$ showed a reduction of the excited state lifetimes of the phosphorescent excited state on exposure to air with bimolecular quenching constants of the order of $10^9 \text{ M}^{-1} \text{ s}^{-1}$ in CH_2Cl_2 at room temperature.²²

Conclusions

Combining planar moieties like phenanthridine with Pt(II) ions in monofunctional platinum drug candidates (e.g., in phenanthriplatin⁴) improves anti-cancer efficacy *in vitro* through an ability to access different mechanisms of action. ^{CF3}LPtCl bears a phenanthridine heterocycle forced co-planar with the coordination sphere of the Pt(II) ion thanks to a multidentate ligand design. Titration studies reveal a stronger binding affinity of ^{CF3}LPtCl for both double-stranded and single-stranded DNA compared with phenanthriplatin with a preference towards guanine-rich DNA. Owing to the co-planarization of the phenanthridinyl moiety with the Pt(II)-coordination plane, ^{CF3}LPtCl interacts with DNA primarily through intercalation (or groove-binding) with the whole-molecule planarity likely contributing to the enhanced affinity for DNA compared with phenanthriplatin. Irradiation of cell lines treated with ^{CF3}LPtCl exhibit enhanced *in vitro* efficacy compared with cell lines incubated in the dark. There is a greater uptake of the complex into the cell compared with cisplatin. Closer examination of subcellular distribution of ^{CF3}LPtCl reveal predominant uptake into the nucleus with significant concentrations in the mitochondria. Expression of mitochondrial superoxide and γ H2AX in irradiated ^{CF3}LPtCl-treated cell lines indicates the role of reactive oxygen species in the observed augmented efficacy. Consistent with this observation, ^{CF3}LPtCl is shown to be an efficient ¹O₂ photosensitizer. These results highlight the potential of ^{CF3}LPtCl as photodynamic therapy agent.

Experimental Section

General Materials and Methods: CF_3LPtCl was prepared as previously reported.¹³

DNA Binding Studies: For the titration studies, 10 mm × 10 mm, 0.7 mL nominal volume Spectrosil Quartz cuvettes equipped with PTFE stoppers (Starna Cells) were used. UV-Vis absorbance spectra were collected using a Helios Zeta UV-Vis spectrophotometer, while emission and excitation spectra were measured in a Horiba Fluorolog-3 spectrofluorometer ($\lambda_{\text{excitation}} = 450$ nm, 5 nm entrance/exit slit widths). All samples were present in 50 μM concentrations, in pH 7.0 Na_3PO_4 aqueous solution, unless stated otherwise, and incubated with 500 μM calf thymus DNA (ct-DNA) for 16 h at room temperature in the dark. The concentrations of ct-DNA samples (Thermo-Fisher Scientific, Ottawa, Canada) were determined by taking the absorbances at 260 nm with a NanoDrop 2000 spectrophotometer (Thermo-Fisher) and using $\varepsilon = 6600 \text{ M}^{-1}\text{cm}^{-1}$ as the molar absorption coefficient.³⁶ A 5 mM stock solution of CF_3LPtCl in DMSO was prepared prior to dilutions in aqueous solution. Emission spectra were collected for biological triplicates of 0 – 20 μM CF_3LPtCl in the presence of ct-DNA, with DMSO ultimately present in $\leq 0.4\%$ v/v. UV-Vis absorbance and excitation spectra were also collected for the 20 μM CF_3LPtCl with ct-DNA sample (0.4% V/V DMSO final concentration).

To investigate the effect of ionic strength, two sets of biological triplicates of 0-20 μM CF_3LPtCl were prepared in 1 M Na_3PO_4 and 3 M NaCl, pH 7.0 aqueous solutions. The emission spectrum of a 20 μM CF_3LPtCl sample without ct-DNA present was also collected to serve as control. The binding affinity of the CF_3LPtCl complex was explored using single-stranded DNA oligonucleotides known to form hairpins³¹ (Integrated DNA Technologies, Coralville, IA, USA) as well as single-stranded DNA oligonucleotides with the following sequences: : 5' – GTGGTGTGCAGCGAGAATAG, 5' – CTCCTCTCCACCCACAATAC, 5' – AAAAA, 5' –

GGGGG, 5' – CCCCC, 5' – TTTTT, and 5' – AAGAA. All samples were prepared in a similar manner as described for ct-DNA samples. Emission spectra were collected for samples containing 0–20 μM $^{\text{CF}_3}\text{LPtCl}$ in the presence of 5 μM hairpin DNA oligonucleotides. Samples of the shorter 5-mer oligonucleotides were prepared by preparing triplicate solutions of 10 μM 5' – AAAAA, 5' – GGGGG, 5' – CCCCC, 5' – TTTTT, or 5' – AAGAA containing 20 μM $^{\text{CF}_3}\text{LPtCl}$. The data were fitted to a one-site total and nonspecific binding saturation model, using equation [1] on a GraphPad Prism 9.

$$y = \frac{(B_{\text{max}})(x)}{x + K_D} + NS * x + BG \quad [1]$$

where B_{max} is the maximum specific binding ($B_{\text{max}} = 1$, for 1:1 DNA-Pt binding), K_D is the equilibrium dissociation constant, NS is the slope of the nonspecific binding, and BG is the amount of nonspecific binding.

Binding Studies with other Biomacromolecules. Additional emission spectra were also collected for 0 – 20 μM $^{\text{CF}_3}\text{LPtCl}$ in the presence of 20 μM of a truncation of the protein RHAU including amino acids 53-105 purified as described previously.³⁷

Singlet Oxygen Photosensitization Experiment. Solutions of 1,3-diphenylisobenzofuran (20 μM), $[\text{Ru}(\text{bpy})_3][\text{PF}_6]_2$ (200 μM) and $^{\text{CF}_3}\text{LPtCl}$ (200 μM) were freshly prepared in air-saturated CH_3CN at room temperature. To test the photosensitization potential of $^{\text{CF}_3}\text{LPtCl}$, A Horiba PTI QM-8075-11-C spectrofluorometer was used to perform timebased emission experiments with 1,3-diphenylisobenzofuran (DPBF). In a 1 cm pathlength cuvette, 2.5 mL of DPBF was mixed with 0.5 mL of the photosensitizer and mixed five times with a Pasteur pipet (see Scheme S1 for experimental principle). A control solution was also prepared by diluting a 2.5 mL DPBF solution

with 0.5 mL of air-saturated CH₃CN. The same cuvette was used for all experiments. [Ru(bpy)₂][PF₆]₂ is used as a standard photosensitizer.³⁵ The samples are then continuously irradiated at 450 nm, and the emission at 475 nm (see Figure S7 for the fluorescence spectrum of DPBF in MeCN) was monitored for five minutes using 1 nm slit widths.

MTT assays of cisplatin and ^{CF3}LPtCl against A2780cis, SKOV3, A549, MDA MB 231 and HeLa cells with and without 15-min irradiation by 490 nm LED: Cells were seeded in 96-well microplates (two plates from each cell line) in 100 µL cell suspensions (6×10^4 cells per mL) per well to begin and incubated for 24 h at 37 °C, 5% CO₂. Next, 50 µL volume of RPMI or DMEM with various concentrations of Pt compounds was added to each well of the microplates. The cells were then incubated for an additional 5 h at 37 °C, 5% CO₂. One plate was then kept in the dark and the other plate irradiated with 490 nm LED for 15 minutes. After the irradiation, medium in both plates were aspirated out and filled with 150 µL of fresh medium followed by one time washing with 200 µL fresh medium. The plates were then incubated for 24 h at 37 °C, 5% CO₂. Next, a volume of 30 µL MTT (Alfa Aesar) (5 mg mL^{-1} in PBS) was added to the cells and then the cells were incubated for an additional 2-4 h at 37 °C, 5% CO₂. The solutions were then aspirated, leaving behind insoluble purple formazan. A volume of 200 µL DMSO was added to the wells and the plates were shaken for 10 min. Next, the microplates were analyzed for absorbance at 562 nm with an ELx800 absorbance reader (BioTek, Winooski, VT, USA). Finally, the data were analyzed using Origin software to produce dose-response curves and to determine IC₅₀ values. All experiments were performed in triplicate.

Measurements of cellular Pt contents: A2780cis cells were seeded in a 6-well plate at a concentration of 2×10^5 cells/mL per well and incubated at 37 °C, 5% CO₂ overnight. These cells were treated with cisplatin or ^{CF3}LPtCl ([Pt] = 5 µM) for 3 h at 37 °C, 5% CO₂. The remaining living cells were harvested by trypsinization and counted. The cells were then digested in 200 µL 65% HNO₃ and shaken at 400 rpm on an Eppendorf ThermoMixer™ F1.5 at ambient temperature overnight. The Pt contents in the cells were analyzed by GFAAS (graphite furnace atomic absorption spectroscopy) after diluting the fractions 10x in water. All experiments were performed in triplicate.

Measurements of Cytosolic/Mitochondrial Pt contents: A2780cis cells were seeded on a 6-well microplate (2×10^5 cells/mL) and incubated at 37 °C 5% CO₂ overnight. The cells were then treated with either 5 µM cisplatin or ^{CF3}LPtCl and incubated for 3 h. The remaining living cells were harvested by trypsinization and counted. Cytosolic and mitochondrial fractions were isolated using the Thermo Scientific™ Mitochondria Isolation Kit for Mammalian Cells. Mitochondrial fractions were then dissolved in 100 µL 65% nitric acid and shaken at 400 rpm on an Eppendorf ThermoMixer™ F1.5 at ambient temperature overnight while the cytosolic fraction was maintained at -20 °C. Next, the fractions were diluted 10x in water and the platinum content was analysed using GFAAS. All experiments were performed in triplicate.

Measurements of Nuclear Pt contents: A2780cis cells were seeded on a 6-well microplate (2×10^5 cells/mL) and incubated at 37 °C 5% CO₂ overnight. The cells were then treated with cisplatin (5 µM) or ^{CF3}LPtCl (20 µM) and incubated for 3 h. The remaining living cells were harvested by trypsinization and counted. Next, the cells were suspended in 1 mL solution of PBS with 0.1% NP-40 followed by centrifugation to dissociate the cell membrane. This step was duplicated. The

isolated nuclei were digested in 200 μ L 65% HNO_3 on an Eppendorf ThermoMixer™ F1.5 at ambient temperature overnight. The platinum content was analyzed by GFAAS. All experiments were performed in triplicate.

Florescence imaging experiments for HeLa cells treated with CF_3LPtCl and Mitotracker Red:

HeLa cells were seeded in an imaging disk (MatTek) at a concentration of 5×10^4 cells with 2 mL of complete medium and incubated for 24 h at 37 °C. The cells were then treated with CF_3LPtCl (10 μ M) and incubated for 2 h at 37 °C, 5% CO_2 . Next, MitoTracker Red FM (Invitrogen) was added to the dish and incubated for another 30 minutes. The medium was aspirated out and washed 3 times with 1 mL of PBS. 1 mL PBS with 0.5% BSA was added into the dish and images were acquired using an Olympus IX70 inverted epifluorescence microscope equipped with a digital CCD camera (QImaging). Images were processed and intensities were quantified with ImageJ software (NIH).

LIVE/DEAD Cell Viability Assays: HeLa cells (5×10^4 cells per mL) were cultured on two 35 mm sterile glass bottom culture dishes (MATTEK Corporation) for 24 h at 37 °C. The cells were then treated with 10 μ M CF_3LPtCl for 5 h at 37 °C. Next, one dish was irradiated with 490 nm LED for 15 minutes while the other was kept in the dark. Both dishes were washed and replaced with regular DMEM medium and incubated at 37 °C overnight. Before the assay, the cells were washed with 1 mL PBS. A 100 μ L volume of LIVE/DEAD working solution (formed by mixing 1 mL of calcein AM and 5 μ L ethidium homodimer-1) was carefully added to the disks, which were then incubated at ambient temperature for 15 min. 1 mL PBS was added into each disk and images were acquired using an Olympus IX70 inverted epifluorescence microscope equipped with

a digital CCD camera (QImaging). Images were processed and intensities were quantified with ImageJ software (NIH).

MitoSOX™ (Mitochondrial ROS Production) Analysis: A2780Cis cells were seeded in two 6-well plates at a concentration of 2×10^5 cells per mL and incubated for 24 h at 37 °C under an atmosphere containing 5% CO₂. Next, the cells were treated with cisplatin (10 μM) or ^{CF3}LPtCl (10 μM) while one well was kept as a control and incubated 37 °C for 5h. One plate was irradiated with 490 nm light for 15 minutes while the other was kept in the dark. Both plates were washed with the medium and incubated overnight. MitoSOX Red Mitochondrial Superoxide Indicator (Thermo Scientific, Rochester, NY, USA) was then added to the cells to reach a concentration of 5 μM and incubated at 37 °C for 30 minutes. The cells were then collected, washed with PBS, and resuspended in PBS containing 0.5% BSA. The cell solutions were analyzed using the FL-2 channel on a BD Accuri C6™ flow cytometer.

Flow cytometric analysis of phosphorylation of H2AX (γH2AX): A2780Cis cells were seeded in two 6-well plates at a concentration of 2×10^5 cells per mL and incubated for 24 h at 37 °C under an atmosphere containing 5% CO₂. Next, the cells were treated with cisplatin (10 μM) or ^{CF3}LPtCl (10 μM) while one well was kept as a control and incubated 37 °C for 5h. One plate was irradiated with 490 nm for 15 minutes while the other was kept in the dark. Both plates were washed with the medium and incubated overnight. Cells were harvested from adherent cultures by trypsinization. Following centrifugation at 1500 rpm for 5 min, cells were washed with PBS. All samples were resuspended in BD fixation/Permeabilization solution (250 μL) for 20 minutes at 4°C. Then the supernatant was removed, the samples were resuspended with BD Perm/Wash

Buffer (1 mL) and centrifuged. About 50 μ L of buffer was left in the tube and 5 μ L of BD Alexa 488-anti γ H2AX antibody solution was added followed by 60 minutes incubation in the dark at room temperature. The cells were resuspended in PBS and analyzed using the FL-1 channel on a BD Accuri C6™ flow cytometer.

Flow Cytometric Apoptosis Assay: A2780cis cells were seeded in two 6-well plates at a concentration of 2×10^5 cells per well and incubated for 24 h at 37 °C. Next day, the cells were treated with CF3 LPtCl or cisplatin (10 μ M), while one well was kept as a control and incubated 37 °C for 5 h. One plate was irradiated with 490 nm for 15 min while the other was kept in the dark. Both plates were washed with the medium and incubated for 24 h at 37 °C. The medium was collected in clean 15 mL falcon tubes along with washed PBS solution. 1 mL trypsin was added to the wells. After 5 min, the cell suspensions were transferred to Falcon tubes that contained the media and PBS, and centrifuged at 1400 rpm at 4 °C for 5 min. The cell pellet was re-suspended in 1 mL PBS and the cells were counted. The cell pellet was collected again and an appropriate amount of $1 \times$ binding buffer was added to reach a concentration of 10^6 cells per mL. 100 μ L cell suspensions were added to new 2 mL Eppendorf tubes and 5 μ L Annexin V-FITC and 5 μ L PI solution was added. The cells were gently vortexed and incubated at ambient temperature for 15 min in the dark. 400 μ L binding buffer was added to each Eppendorf tube and the cell suspensions were transferred to flow cytometry tubes. Flow cytometry analysis was done using FL-1 and FL-3 channels on a BD Accuri C6™ flow cytometer at 10,000 events.

ASSOCIATED CONTENT

Supporting Information: Supporting figures (PDF)

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Conflict of Interest Statement

The authors declare no conflicts of interest.

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