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Targeting Mitochondrial DNA with a Platinum-Based Anticancer Agent

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SUMMARY

An analog of the anticancer drug cisplatin (mtPt) was delivered to mitochondria of human cells using a peptide specifically targeting this organelle. mtPt induces apoptosis without damaging nuclear DNA, indicating that mtDNA damage is sufficient to mediate the activity of a platinum-based chemotherapy. This study demonstrates the specific delivery of a platinum drug to mitochondria and investigates the effects of directing this agent outside the nucleus.

INTRODUCTION

cis-diamminedichloroplatinum(II), or cisplatin, is an effective chemotherapeutic agent that is used in nearly 50% of all cancer patients (Galanski et al., 2005). The second generation platinum-based drugs, carboplatin and oxaliplatin, also play an important role in modern chemotherapy. These compounds bind to nuclear DNA, resulting primarily in intrastrand DNA crosslinks (Todd and Lippard, 2009). The lesions they cause inhibit transcription, ultimately triggering apoptosis and cell death (Todd and Lippard, 2009). It is important, however, to understand whether alternative cellular targets besides nuclear DNA can potentiate the activity of platinum-based drugs because they offer the opportunity to treat resistant tumors. Furthermore, a greater understanding of other platinum drug targets might allow treatment-limiting side effects to be mitigated.

Owing to their central role in facilitating apoptosis, mitochondria are being actively explored as potential anticancer drug targets (Fulda et al., 2010). Mitochondria contain their own circular DNA (mtDNA), the potential importance of which as a target during platinum-based chemotherapy has not been fully evaluated. Previous studies have proposed mitochondrial and not nuclear DNA as the critical target of cisplatin in potentiating its anticancer activity (Cullen et al., 2007), and under certain conditions higher levels of cisplatin adducts are observed in mitochondrial relative to nuclear DNA (Murata et al., 1990; Olivero et al., 1995). Mitochondria also appear to play a role in mediating cellular resistance to cisplatin. Cisplatin-resistant cell lines have elevated mitochondrial membrane potentials (Andrews and Albright, 1992; Isonishi et al., 2001), sustain less damage to mtDNA when treated with the drug (Hirama et al., 2006), and exhibit substantially less mitochondrial uptake of cisplatin (Groessl et al., 2011) compared to nonresistant parent lines.

To investigate more precisely the effects of mitochondrial targeting by a potential platinum chemotherapeutic, we designed a complex that would selectively localize to this organelle. A mitochondria-penetrating peptide (MPP) was appended to the cis-[Pt(NH3)2]2+ DNA-binding unit of cisplatin and carboplatin. MPPs are short, cell-permeable peptide sequences comprising alternating lipophilic and cationic residues that exhibit minimal toxicity toward human cells (Horton et al., 2008; Yousil et al., 2009). Here, we describe the synthesis and biological properties of a platinum(II) complex conjugated to the N terminus of an MPP to determine the effect of mitochondrial targeting on the activity of a platinum-based agent. This study probes the consequences of platinum directed specifically to mitochondria in a cancer cell.

Platinum-peptide conjugates reported previously use a variety of different linking strategies. Such conjugates have been prepared by attaching the peptide to the nonleaving group ligand (amino) of a platinum(II) complex (Robillard et al., 2000; Barragan et al., 2009; Damian et al., 2010), the leaving group ligand (carboxylate) of a platinum(II) complex (Ndinguri et al., 2009), or through axial ligands of a platinum(IV) prodrug (Mukhopadhyay et al., 2008; Graf et al., 2012). Here, we began with the platinum(II) complex [Pt(succac)(NH3)2][NO3], where succac equals succinylacetone, as detailed in the Supplemental Information (available online). Structural and spectroscopic characterization data for this complex are shown in Figure S1. The succac ligand contains (1) a β-diketone group for coordination to platinum as the leaving group ligand, and (2) a dangling carboxylic acid functionality for amide-bond formation. [Pt(succac)(NH3)2][NO3] was conjugated to the N terminus of the MPP r[Fx]x, where r and Fx are the unnatural amino acid residues ω-arginine and ω-cyclohexylalanine, respectively. This peptide was selected for conjugation because it exhibits no toxicity toward human cells (Horton et al., 2012), is composed of artificial amino acids, and is therefore not degraded by proteases (Fonseca et al., 2011). This peptide/platinum conjugate is referred to as mtPt (Figure 1A). A fluorophore-labeled analog, mtPt-TAMRA
was also prepared featuring attachment of carboxy-
tetramethylrhodamine (TAMRA) on the amino side chain of a
C-terminal lysine. For both compounds, the platinum unit was
attached while these peptides remained on the solid-phase
support. The peptides were then cleaved from the resin with
neat trifluoroacetic acid (TFA) and purified by reverse-phase
high-performance liquid chromatography (HPLC). The purified
Pt-peptide conjugates were characterized by ESI-MS and
195Pt nuclear magnetic resonance (NMR) spectroscopy (Notes
S1B and S1C; Figures S1 and S2). The ability to tether
cis-di-
aminoplatinum(II) groups to peptides on the solid-phase via
linkages in their leaving groups using \([\text{Pt(succac)(NH}_3)_2\text{]}^+\)
represents a general strategy for the design of platinum-peptide
conjugates.

In order to assess the intracellular localization of mtPt, fluores-
cence imaging studies were carried out with the TAMRA-labeled
target analog. Localization was first assessed in
HeLa cells, for they have a well-defined
mitochondrial network that can be readily
imaged. Representative images revealing
colocalization of the TAMRA conjugate
with MitoTracker Deep Red (Invitrogen)
are shown in Figure 1C. Imaging was
also performed in wild-type (WT) A2780
and cisplatin-resistant A2780 CP70
ovarian cancer cell lines. Pearson’s corre-
lation coefficients (PCCs) quantitatively
describing colocalization of the two dyes
in these cell lines are given in Table S2.
For both cell lines, mtPt(TAMRA) localizes
specifically to the mitochondria with PCCs
ranging from 0.36 to 0.53. These results
also clearly indicate that mtPt(TAMRA)
does not localize in the nucleus; Pearson’s
correlation coefficients for overlap with
the nuclear stain are all effectively zero.

The cytotoxicity of mtPt was then
studied using the MTT assay in A2780
WT and A2780CP70 ovarian cancer cell
lines. Cisplatin, \([\text{Pt(NH}_3)_2\text{]}^+\), and
\([\text{Pt(succac)(NH}_3)_2\text{]}^+\)
were included as
controls. The results are summarized in
Table S1and Figure S3, and representa-
tive dose-response curves for mtPt are depicted in Figure 1D.
Cisplatin is toxic to A2780 cells at submicromolar concentra-
tions, with an IC\(_{50}\) of 0.60 ± 0.08 \(\mu\)M. In the resistant CP70 line,
however, the IC\(_{50}\) value (5.2 ± 1.4 \(\mu\)M) increases by almost an
order of magnitude. The calculation of resistance factors (RFs),
defined as the ratios of IC\(_{50}\) values of resistant to wild-type cell
lines, provides an estimate of differential toxicity. The RF for
cisplatin is 8.7, indicating that it is susceptible to resistance
factors expressed in A2780CP70 cells. \([\text{Pt(acac)(NH}_3)_2\text{]}^+\)
and
\([\text{Pt(succac)(NH}_3)_2\text{]}^+\)
are both less toxic than cisplatin (IC\(_{50}\) values
of 13.9 ± 1.9 and 220 ± 40 \(\mu\)M, respectively), but exhibit similar
resistance factors. The lower toxicity for the latter two com-
pounds is expected, given that chelating \(\beta\)-diketonate ligands
decrease the rate of departure of the leaving group from the
platinum coordination sphere, which leads to lower biological
activity (Wilson and Lippard, 2012). The toxicity of mtPt is greater

![Figure 1](https://example.com/f1.png)

**Figure 1.** mtPt Localizes to Mitochondria and Is Active against a Cisplatin-Resistant Cell Line

(A) Structure of mtPt.

(B) Structures of cis-[Pt(NH\(_3\))\(_2\)Cl\(_2\)] (cisplatin) and
\([\text{Pt(NH}_3)_2\text{]}^+\).

(C) Intracellular localization of mtPt-TAMRA in
HeLa cells by fluorescence microscopy.

(D) Cytotoxicity of mtPt. Viability of A2780 WT and
cisplatin-resistant A2780CP70 cells treated for
72 hr with mtPt. Mean values plotted, \(n \geq 3\), error
bars represent SEM.

See also Note S1A, Table S1, and Figure S1 for
further information on the characterization of the
compounds tested.
than that of the precursor diketonate compounds, with an IC\textsubscript{50} of 7.5 ± 0.3 μM, but importantly, the activity is unaffected in the resistant cell line (Figure 1D). The mechanisms used by this cell line to evade the action of platinum drugs, including increased nucleotide excision repair and drug efflux (Parker et al., 1991), are presumably less able to interfere with the action of a drug that targets mitochondria, allowing mtPt to retain activity in both cell lines.

Further links between the activity of mtPt and events occurring within mitochondria were sought by assessing DNA damage, effects on cell cycle, and the generation of mitochondrial reactive oxygen species. Damage to nuclear and mitochondrial DNA was assessed by monitoring the efficiency of PCR amplification of long segments of isolated nuclear and mitochondrial DNA. A2780 cells were exposed to mtPt, \[\text{Pt(acac)(NH}_3\text{)}_2\text{(SO}_4\text{)}\] or cisplatin for 6 hr. PCR amplification of an 8.9 kb mitochondrial genomic segment and a 12.2 kb nuclear genomic segment was analyzed and compared to results for untreated controls. mtPt caused a statistically significant reduction in the amplification of the mitochondrial but not the nuclear DNA segment (Figure 2A). The reverse effect was observed for cisplatin (Figure 2A). These results indicate that mtPt damages mitochondrial DNA without causing a significant amount of lesions on nuclear DNA. This conclusion was corroborated by flow cytometry studies, which revealed that mtPt does not induce cell-cycle arrest in A2780 cells (Figures 2B and S4). The genomic DNA-targeting compounds cisplatin and \[\text{Pt(acac)(NH}_3\text{)}_2\text{(SO}_4\text{)}\] in contrast, both induced significant cell-cycle arrest. Although the mechanism of action for mtPt is clearly different from that of platinum drugs acting in the nucleus, apoptotic cell death still occurs, as judged by Annexin V/Sytox staining and flow cytometry (Figures 2D and S5).

Damage to mitochondrial DNA can produce reactive oxygen species (ROS) that lead to mitochondrial dysfunction and cell death (Santos et al., 2003). We therefore assessed whether mtPt treatment increases the levels of superoxide in mitochondria. mtPt induced significant increases in mitochondrial superoxide in A2780 cells following 24 hr of treatment, as judged by studies using a fluorogenic dye that is specific for \text{O}_2^– ROS. The \[\text{Pt(acac)(NH}_3\text{)}_2\text{(SO}_4\text{)}\] parent compound, in contrast, caused minimal superoxide production (Figure 2C). These results support the hypothesis that mtPt generates mitochondrial dysfunction.

To further probe a potential connection between damage to mitochondrial DNA and mtPt cytotoxicity, we assessed mtPt activity in mouse embryonic fibroblasts (MEFs) expressing a form of mitochondrial DNA polymerase gamma (PolG) lacking functional 3’- 5’-exonuclease activity. The PolG m/m line is

Figure 2. The Cellular Effects of mtPt Treatment

(A) DNA damage caused by platinum drugs. Relative amplification of an 8.9 kb mitochondrial specific gene segment is reduced by 6 hr of treatment with 15 μM mtPt but not by 30 μM treatment with \[\text{Pt(acac)(NH}_3\text{)}_2\text{(SO}_4\text{)}\] (acac-Pt) or 15 μM treatment with cisplatin. Mean values plotted, n = 4, all values normalized to nontreated control. Error bars represent SEM. *p < 0.05; **p < 0.01.

(B) Cell-cycle arrest caused by platinum drugs. In contrast to cisplatin and the parent compound, mtPt does not induce cell-cycle arrest. Quantitative analysis of distribution into G1, S, and G2 cell-cycle phases was assessed by propidium iodide staining and flow cytometry. Mean values plotted, n = 3. Error bars represent SEM.

(C) Assessment of mitochondrial ROS production by MitoSox staining of A2780 cells treated with the indicated concentrations of mtPt and \[\text{Pt(acac)(NH}_3\text{)}_2\text{(SO}_4\text{)}\] for 24 hr. Mean values plotted, n = 3, all values normalized to nontreated control. Error bars represent SEM.

(D) mtPt induces slow-onset apoptosis. Annexin V/Sytox Red staining of A2780 cells treated with 30 μM mtPt at indicated time points. See also Figure S2.
proofreading-deficient and accumulates mtDNA point mutations at a 3- to 5-fold higher rate than their WT counterparts (Kujoth et al., 2005). We therefore hypothesized that they might be highly sensitive to agents that damage mtDNA. Indeed, we observed that PolG m/m cells are more sensitive to mtPt than wild-type cells, with greater levels of apoptosis being generated at an equivalent dose (Figure 3). This potentiation of toxicity was not observed for cisplatin or \([\text{Pt(acac})(\text{NH}_3)_2](\text{SO}_4)_{0.5}\) (Figures 3 and S6). These data suggest that mtPt-induced apoptosis results from damage specifically to mtDNA.

**SIGNIFICANCE**

In summary, we have prepared and characterized a mitochondrially localized conjugate of cisplatin using chemistry that can be readily applied to access a wide range of platinum(II)-peptide conjugates targeted to subcellular locales. Although less cytotoxic than cisplatin, presumably owing to slower ligand exchange kinetics, mtPt is equally effective at killing wild-type and cisplatin-resistant ovarian cancer cells. We find that mtPt is delivered to mitochondria and damages mtDNA. Moreover, we present evidence that a cell line with reduced mitochondrial genomic integrity is highly sensitive to treatment with mtPt, linking its activity directly to mtDNA damage. Nuclear DNA damage and cell-cycle arrest, both well-characterized aspects of the cisplatin mechanism of action, were not observed for the mitochondrially-targeted compound. These findings provide direct evidence that damage to mtDNA by platinum chemotherapeutics is toxic to cancer cells and indicate that mitochondria are potential targets for anticancer therapy.

**EXPERIMENTAL PROCEDURES**

**General Materials and Methods**

Succinylacetone (Levenson, 1994) and \([\text{Pt(acac})(\text{NH}_3)_2](\text{SO}_4)_{0.5}\) (Wilson and Lippard, 2012) were synthesized as previously described. Peptides were also prepared as reported (Mourtada et al., 2013) and purified by reverse-phase HPLC. Other reagents were purchased from commercial vendors.

**Physical Measurements**

NMR spectra were acquired on a Bruker DPX-400 spectrometer in the Massachusetts Institute of Technology (MIT) Department of Chemistry Instrumentation Facility (DCIF). For Fourier transform infrared spectroscopy (FTIR) spectra, samples were prepared as KBr disks and data were recorded with a ThermoNicolet Avatar 360 spectrophotometer running the OMNIC software. Electrospray ionization mass spectrometry (ESI-MS) measurements were acquired on an Agilent Technologies 1100 series LC-MSD trap. Solutions used for biological studies were dissolved in MilliQ water (or PBS for cisplatin) and sterile-filtered. The platinum concentrations of the solutions were determined by graphite-furnace atomic absorption spectroscopy (GFAAS) using a Perkin-Elmer AAnalyst600 spectrometer.
Synthesis of [Pt(succac)(NH3)2]NO3
Cisplatin (500 mg, 1.67 mmol) and AgNO3 (552 mg, 3.25 mmol) were stirred in 10 ml H2O in the absence of light at room temperature for 16 hr. The resulting mixture was filtered to remove AgCl. To the filtrate, a solution of NaOH (67 mg, 1.68 mmol) and succinylacetone (269 mg, 1.70 mmol) in 5 ml of H2O was added dropwise. After stirring at room temperature (RT) for 5 hr, the resulting solution was concentrated to dryness at 60°C under reduced pressure to afford an orange oil. This oil was dissolved in 3 ml of H2O and acidified with three drops of 25% HNO3. Acetone (50 ml) was added, and the resulting turbid white suspension was stirred for ~3 min, resulting in the deposition of an oily orange-brown residue. The turbid supernatant was decanted and mixed with 50 ml of a 1:1 (v/v) mixture of acetone and diethyl ether. Upon stirring at RT for ~5 min, an orange-brown residue deposited again. The cloudy supernatant was decanted and poured directly into 150 ml of diethyl ether. The mixture was stored at ~40°C for 1.5 hr and filtered to collect a white solid. The white solid was washed with 2 × 10 ml diethyl ether and then dried in vacuo. The yield equals 203 mg (28%). Characterization data are given in Note S1A.

Synthesis of mtPt
A 50-μmol portion of Fmoc-9-fluorenylketoxime (FkFmoc) on Rink amide resin was placed in a 2.5 ml round bottom vessel. The vessel was shaken at RT for 1.5 hr and the mtPt was cleaved from the resin with a 25% (v/v) mixture of trifluoroacetic acid (TFA) and water (1:3) in the absence of light. The resulting mtPt was precipitated from the cleavage solution with diethyl ether. The resulting solid mtPt was separated by centrifugation and purified by semipreparative HPLC with a C18 reverse-phase column allowing to adhere overnight. Cells were treated with the indicated concentrations of mtPt and [Pt(acac)(NH3)2]SO4.5 and incubated for 24 or 48 hr. The medium was removed and cells were washed with Hank’s buffered saline (HBS) and then incubated with 5 μM MitoSox reagent (Invitrogen) in HBS for 30 min in the absence of light. Cells were washed three times with HBS, harvested by trypsinization, and analyzed via flow cytometry with FACSCanto. At least 10^4 cells were analyzed for each sample.

Analysis of Mitochondrial Superoxide Levels
A2780 cells were plated in 12-well plates at a density of 10^5 cells/ml and allowed to attach overnight. Cells were then treated with the indicated concentrations of mtPt and [Pt(acac)(NH3)2]SO4.5, incubated for 24 or 48 hr. Following incubation, cells were stained at room temperature using Annexin V-FITC for 15 min and 5 nM Sytox Red for an additional 15 min. Analysis was performed via flow cytometry with FACSCanto. At least 10^4 cells were analyzed for each sample.

Annexin V Apoptosis Assay
A2780 cells were plated in a 12-well dish at a density of 10^5 cells/ml and allowed to adhere overnight. PolG WT and m/m cells were plated at a density of 5 × 10^4 cells/well. A2780 Cells were treated with the indicated concentrations of mtPt and [Pt(acac)(NH3)2]SO4.5 and incubated for 24 or 48 hr. Cells were harvested by trypsinization and fixed at 4°C in 70% ethanol for 2 hr. Cells were centrifuged, ethanol was removed, and the remaining cell pellet was washed once with PBS. Cells were resuspended in PBS and incubated with 0.2 mg/ml RNase A for 1 hr at 37°C, then stained with 10 μg/ml propidium iodide and analyzed immediately via flow cytometry with FACSCanto. Quantitation of DNA damage by qPCR
A2780 cells were seeded at 2 × 10^4 cells/well and allowed to adhere overnight. Cells were treated with either 15 μM mtPt, 30 μM [Pt(acac)(NH3)2]SO4.5, or 15 μM cisplatin for 6 hr and harvested after trypsinization. DNA was isolated from cell pellets using the Sigma GenElute mammalian genomic DNA miniprep kit according to the manufacturer’s instructions. Amplification of an 8.9 kb segment of mitochondrial DNA or a 12.2 kb segment of genomic DNA was performed using the Elongase long range PCR enzyme kit (Invitrogen) as described previously (Santos et al., 2006). Quantitation of amplified product was performed by Pico Green staining and normalized to nontreated value.

Cytotoxicity Assays
The colorimetric MTT assay was used to assess cytotoxicity of the compounds using a previously described protocol (Wilson and Lippard, 2012). From the resulting dose-response curves, 50% growth inhibitory concentration (IC50) values were determined by interpolation.

Cell Imaging Studies
A2780 and A2780CP70 cells were seeded in an imaging dish in 2 ml of growth medium at 60% confluence. The growth medium was swapped with premixed medium containing 1 or 10 μM of mtPt-TAMRA, and the cells were allowed to incubate with the dye for 1 hr. Mitotracker Green and Hoechst 33258, were added to the cells at 1.25–2.5 μM concentrations and allowed to incubate for 30 min. At the end of the incubation period, the medium was aspirated, and the cells were washed with 3 × 1 ml PBS and submerged in 2 ml of dye-free DMEM. The imaging experiments were performed using a Zeiss Axiovert 200M inverted epifluorescence microscope equipped with an EM-CCD digital camera (Hamamatsu) and a MS200 XY Piezo Z stage (Applied Scientific Instrumenta). The light source was an X-Cite 120 metal-halide lamp (EXFO), and the fluorescence images were obtained with an oil-immersion objective at 63 × magnification. The microscope was operated with the Velocity software program of Perkin-Elmer. Colocalization of the dyes was quantitated using the program ImageJ (Schneider et al., 2012) using a previously described protocol (French et al., 2008). HeLa cells were imaged using a previously described protocol (Fonseca et al., 2011).

X-Ray Crystallography
Single crystals of succinylacetone and [Pt(succac)(NH3)2]NO3 were grown from hexanes/EtO and MeOH/EtO, respectively. Data were obtained, solved, and refined as previously described (Wilson and Lippard, 2012). Refinement data and thermal ellipsoid plots are given in Table S3 and Figure S1. These data have been deposited in the Cambridge Crystallographic Data Centre (CCDC) under the accession codes of CCDC 938791 and 938792 for succinylacetone and [Pt(succac)(NH3)2]NO3, respectively.

Cell Culture
A2780 and A2780CP70 (wild-type and cisplatin-resistant ovarian cancer) cell lines were obtained from Fox Chase Cancer Center and cultured in RPMI-1640 medium supplemented with 10% FBS and 1% penicillin/streptomycin. PolG WT and m/m MEFs were cultured in high glucose DMEM supplemented with 10% FBS. The cells were incubated in a humidified atmosphere containing 5% CO2 at 37°C.
SUPPLEMENTAL INFORMATION

Supplemental Information includes one note, four figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.chembiol.2013.08.010.

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