**Mitaplatin, a Potent Fusion of Cisplatin and the Orphan Drug Dichloroacetate**

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Detailed Terms
Mitaplatin, a potent fusion of cisplatin and the orphan drug dichloroacetate

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Contributed by Stephen J. Lippard, October 29, 2009 (sent for review August 30, 2009)

The unique glycolytic metabolism of most solid tumors, known as the Warburg effect, is associated with resistance to apoptosis that enables cancer cells to survive. Dichloroacetate (DCA) is an anticancer agent that can reverse the Warburg effect by inhibiting a key enzyme in cancer cells, pyruvate dehydrogenase kinase (PDK), that is required for the process. DCA is currently not approved for cancer treatment in the USA. Here, we present the synthesis, characterization, and anticancer properties of c.t.c-[Pt(NH3)2(O2CCCH2)2Cl]2, mitaplatin, in which two DCA units are appended to the axial positions of a six-coordinate Pt(IV) center. The negative intracellular redox potential reduces the platinum to release cisplatin, a Pt(II) compound, and two equivalents of DCA. By a unique mechanism, mitaplatin thereby attacks both nuclear DNA with cisplatin and mitochondria with DCA selectively in cancer cells. The cytotoxicity of mitaplatin in a variety of cancer cell lines equals or exceeds that of all known Pt(IV) compounds and is comparable to that of cisplatin. Mitaplatin alters the mitochondrial membrane potential gradient (ΔΨm) of cancer cells, promoting apoptosis by releasing cytochrome c and translocating apoptosis inducing factor from mitochondria to the nucleus. Cisplatin formed upon cellular reduction of mitaplatin enters the nucleus and targets DNA to form 1,2-intrastrand (dGpG) cross-links characteristic of its own potency as an anticancer drug. These properties of mitaplatin are manifest in its ability to selectively kill cancer cells cocultured with normal fibroblasts and to partially overcome cisplatin resistance.

Cancer therapy | DNA damage | mitochondrion | platinum | Warburg effect

Normal cells typically use mitochondrial oxidative phosphorylation to metabolize glucose and switch over to glycolysis only when there is little or no oxygen, producing lactate as a byproduct. Cancer cells avidly consume glucose for energy by glycolysis to survive in the hypoxic environment of malignant lesions (1), a phenomenon known as the Warburg effect (2). The dependence of cancer cells on glycolysis comes not only from oxygen deprivation, but also partly from their inability to synthesize ATP in response to cancer cells on glycolysis comes not only from oxygen deprivation, but also partly from their inability to synthesize ATP in response to the mitochondrial membrane potential gradient (ΔΨm) (3). This unique glucose metabolic pathway of cancer cells has identified the mitochondrion as a prime target for cancer therapy (4–7). In addition, cancer cells develop the ability to avoid apoptosis by various pathways that ignore the command to commit cellular suicide (8, 9). Compounds that trigger apoptosis through selective action on mitochondrial target sites of cancer cells bypass defective upstream mechanisms and trigger apoptosis in tumor cells that are otherwise resistant (10).

Dichloroacetate (DCA) is used in humans to treat lactic acidosis (11). DCA inhibits the activity of pyruvate dehydrogenase kinase (PDK), thereby stimulating the mitochondrial enzyme pyruvate dehydrogenase (PDH). When turned off, PDH no longer converts pyruvate to acetyl-CoA required for mitochondrial respiration and glucose-dependent oxidative phosphorylation (12). DCA thus shifts cellular metabolism from glycolysis to glucose oxidation, decreasing ΔΨm (13) and helping to open mitochondrial transition pores (MTPs). This metabolic switch facilitates translocation of proapoptotic mediators like cytochrome c (cyt c) and apoptosis inducing factor (AIF), both of which stimulate apoptosis. DCA thereby drives cancer cells to commit suicide by apoptosis (13). Unlike most other anticancer agents, DCA does not appear to have any deleterious effect on normal cells. DCA reverses mitochondrial changes in a wide range of cancers, making malignant cells more vulnerable to normal cell death programs (14). Being an orphan drug, DCA is both nonpatentable and readily available, but it is not yet approved for use in cancer therapy (15). There is substantial preclinical evidence from both in vitro and in vivo models that DCA might be useful to treat cancer in humans, and a translation to early-phase clinical trials would be of interest (16–18). Funding for such trials would be a challenge because DCA is a generic drug. However, because it withdraws cancer cells from a state of apoptosis resistance, DCA is an attractive sensitizer that could be given concurrently with chemotherapy or radiation therapy. Alternatively, a formulation could be synthesized that incorporates DCA.

Platinum(II) compounds are used in 50% of all cancer therapies (19). Among these, cisplatin, carboplatin, and oxaliplatin have Food and Drug Administration approval and are in the clinic worldwide (20, 21). The use of platinum(II) drugs, cisplatin in particular, to treat malignancies is limited because of side effects and acquired resistance (22). Resistance can emerge from failure to execute apoptosis despite initiation of the apoptotic cascade caused by either the predominance of anti-apoptotic factors or defects in downstream effectors. Cisplatin resistance in ovarian carcinoma cells is associated with a reduced apoptotic response (23). To overcome tumor cell resistance and toxicity to normal tissues, we have been exploring strategies to target platinum constructs to cancer cells. Our tactic has been to employ substitutionally inert platinum(IV) compounds (24), which serve as prodrugs and release clinically effective levels of platinum(II) compounds, such as cisplatin, following cellular uptake (25–27). Appropriately designed platinum(IV) complexes are less likely to be deactivated before reaching their cancer cell destination target. The activity of platinum(IV) complexes generally involves reduction with loss of the axial ligands, affording an active platinum(II) complex that readily binds to DNA. Satraplatin is one such Trojan horse platinum(IV) compound that is currently under investigation for the treatment of patients with advanced prostate cancer (28).

We therefore designed a Pt(IV) compound (mitaplatin, 1) having two DCA moieties (Fig. 1) in the axial positions. We hypothesized that DCA released inside the cells by reduction of the platinum would simultaneously alter mitochondrial metabolism and deliver a dose of cisplatin (Fig. 1). Mitaplatin was thereby expected to have dual killing modes toward cancer cells, one in which cisplatin interacts with its key target, nuclear DNA, and the other, DCA released upon reduction, following a...
**Table 1. Cell killing ability of mitaplatin**

<table>
<thead>
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<th>Cell lines*</th>
<th>Cisplatin</th>
<th>Mitaplatin</th>
<th>DCA</th>
</tr>
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<tbody>
<tr>
<td>NTera-2</td>
<td>0.043</td>
<td>0.051</td>
<td>&gt;0.4</td>
</tr>
<tr>
<td>HeLa</td>
<td>1.2</td>
<td>2.0</td>
<td>&gt;8.0</td>
</tr>
<tr>
<td>U2OS</td>
<td>3.9</td>
<td>6.4</td>
<td>&gt;100</td>
</tr>
<tr>
<td>A549</td>
<td>12.0</td>
<td>14.0</td>
<td>&gt;200</td>
</tr>
<tr>
<td>MCF-7</td>
<td>13.0</td>
<td>18.0</td>
<td>&gt;100</td>
</tr>
<tr>
<td>MRC-5</td>
<td>9.5</td>
<td>18.3</td>
<td>&gt;100</td>
</tr>
<tr>
<td>A2780</td>
<td>0.56</td>
<td>1.1</td>
<td>&gt;120</td>
</tr>
<tr>
<td>A2780/CP70</td>
<td>6.0</td>
<td>3.34</td>
<td>&gt;120</td>
</tr>
</tbody>
</table>

Comparison of IC₅₀ values for mitaplatin, cisplatin, and DCA against cancer and normal cells as determined by the MTT assay.

*NTera-2, human testicular cancer; HeLa, human cervical cancer; U2OS, human osteosarcoma; A549, human lung carcinoma; MCF-7, human breast adenocarcinoma; MRC-5, normal lung fibroblast; A2780, human ovarian carcinoma; A2780/CP70, cisplatin-resistant human ovarian carcinoma.

mitaplatin, a lipophilic cationic dye which, depending on the charge, to enter mitochondria where it accumulates. When a critical concentration is exceeded, J-aggregates form, which is associated with a drop in Δψm. For this reason Δψm is an important parameter of mitochondrial function and has been used to monitor mitochondrial death. 5,5′,6,6′-Tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) is a lipophilic cationic dye which, depending on Δψm, accumulates as a green monomer in the cytoplasm or as red-emitting aggregates in hyperpolarized mitochondria of cancer cell (30). The negative charge established by the mitochondrial membrane potential allows the lipophilic dye, bearing a delocalized positive charge, to enter mitochondria where it accumulates. When a critical concentration is exceeded, J-aggregates form, which fluorescence red. In apoptotic cells, Δψm collapses, and JC-1 cannot accumulate in mitochondria. In these cells, JC-1 remains in the cytoplasm in a green fluorescent monomeric form. Control NTera-2 cells exhibited heterogeneous staining of the cytoplasm with both red and green fluorescence in the same cells (Fig. S3). Treatment of these cells with mitaplatin for 4 h decreased the red fluorescence. Mitochondrial membrane depolarization was detected by a shift in fluorescence emission of JC-1 from red to green. There was no significant effect of mitaplatin on Δψm of normal fibroblasts or of cisplatin on Δψm of NTera-2 cells. The detailed results are given in Fig. S4.

To investigate whether DCA released from mitaplatin can restore the hyperpolarization of cancer cells to the level of normal NTera-2, HeLa, U2OS, A549, and MCF-7 cancer cells as well as MRC-5 normal fibroblasts (Fig. S3). Results are presented in Table 1. Mitaplatin has an IC₅₀ value of 0.051 μM, comparable to that of cisplatin (IC₅₀, 0.043 μM), in cisplatin-sensitive testicular NTera-2 cells and is more toxic than DCA alone. In U2OS osteosarcoma cells, cisplatin has an IC₅₀ of 3.9 μM, whereas that of mitaplatin is 6.4 μM. Similarly, in HeLa cervical cancer cells, comparable IC₅₀ values for mitaplatin and cisplatin were observed, 2.0 and 1.20 μM, respectively. Control experiments with the well known platinum(IV) compound cis-[Pt(NH₃)₂Cl₂(O₂CCH₃)₂], revealed it to be several-fold less active than mitaplatin in all cells (Table S1). Mitaplatin was also established to have cytotoxicity comparable to that of cisplatin in the NCI/DTP 60 cell line growth inhibition assay, exceeding almost all known Pt(IV) compounds. This enhanced potency of mitaplatin is consistent with the expected dual killing mechanism.
Disruption of mitochondrial function and induction of apoptosis in cancer cells by mitaplatin. (A) Changes in the mitochondrial membrane potential as revealed by the JC-1 assay. Treatment with 100 μM mitaplatin dramatically caused the collapse of mitochondrial membrane potentials in NTera-2 cells. In live cells, JC-1 exists either as a green fluorescent monomer at depolarized membrane potentials (positive to ~100 mV) or as an orange-red fluorescent J-aggregate at hyperpolarized membrane potentials (negative to ~140 mV). The shift in membrane charge was observed by disappearance of fluorescent red-orange-stained mitochondria (large negative $\Delta \psi_{m}$) and an increase in fluorescent green-stained mitochondria (loss of $\Delta \psi_{m}$). (B) Reversal of mitochondrial membrane potential by tetramethyl rhodamine methyl ester (TMRM) assay. Mitaplatin significantly depolarized the NTera-2 cells but had no effect on the healthy normal fibroblast cells. Mitochondria were stained with mitotracker red. (C) Cytochrome c release visualized by fluorescence microscopy. Immunolocalization of cytochrome c (green) and mitochondrial morphology (red) shown in untreated NTera-2 cells and in mitaplatin treated NTera-2 cells. Cells were grown for 24 h on glass coverslips and treated with mitaplatin, fixed after treatment, and immunostained with anti-cytochrome c monoclonal antibodies. Mitochondria were stained with mitotracker red, fixed after treatment, and immunostained with anti-cytochrome c monoclonal antibodies. Mitochondria were stained with mitotracker red, fixed after treatment, and immunostained with anti-cytochrome c monoclonal antibodies. Mitochondria were stained with mitotracker red. (D) Translocation of AIF in mitaplatin treated cells. Staining of AIF (Ab) and nuclei (Hoechst) in NTera-2 cells before and after 12 h treatment with mitaplatin. Arrows indicate cells with particularly evident presence of AIF in the nucleus.

In untreated cells, there was no detectable cyt c, whereas in normal fibroblasts, we carried out a TMRM assay (31). All cancer cell lines have significantly more hyperpolarized $\Delta \psi_{m}$ compared to normal cells and therefore exhibit increased fluorescence of the $\Delta \psi_{m}$-sensitive positive dye tetramethyl rhodamine methyl ester, TMRR. Incubation of the NTera-2 cells with mitaplatin for 48 h reversed the hyperpolarization and returned the $\Delta \psi_{m}$ to the level of normal cells (Fig. 2B). In contrast, mitaplatin did not alter the $\Delta \psi_{m}$ of the normal fibroblasts. Because dichloroacetate activates pyruvate dehydrogenase, which increases delivery of pyruvate into mitochondria, DCA released upon reduction of mitaplatin (Fig. 1) increased glucose oxidation, depolarizing the mitochondria and returning the membrane potential to levels of noncancer cells.

Mitochondrial cyt c, which functions as an electron carrier in the respiratory chain, translocates to the cytosol in cells undergoing apoptosis, where it participates in activation of apoptotic proteins (32). The mechanism responsible for this process is unknown. Cyt c release from mitochondria is an early event in the apoptotic process induced by mitaplatin treatment in NTera-2 cells, as visualized by using a FITC-conjugated antibody for the protein and fluorescence microscopy. The cytosol from untreated cells showed no detectable cyt c (Fig. 2C). In contrast, cytosolic cyt c accumulated significantly after 4 h of treatment with mitaplatin.

AIF is a proapoptotic mitochondrial protein (33). Like cyt c, AIF is a bifunctional protein having both electron transfer and apoptogenic functions. AIF is released from mitochondria and translocated to nuclei, stimulating chromatin condensation and DNA fragmentation. We were interested to determine whether mitochondrial outer membrane permeabilization by mitaplatin induces apoptosis only by release of caspase-dependent factors, such as cyt c, or whether caspase-independent processes, such as that mediated by AIF, might be operative. We therefore investigated the location of AIF in the mitaplatin-treated cells. As shown in Fig. 2D, mitaplatin treatment led to translocation of AIF from the mitochondria to nuclei of NTera-2 cells.

To quantify mitaplatin-induced apoptosis in cancer cells, an annexin-V assay was performed by using flow cytometry. With this analysis, we determined the percentage of apoptotic cells at 48 h after exposure to mitaplatin, cisplatin, or DCA. Apoptosis was detected in cancerous U2OS, HeLa, and A549 cells with 10 μM mitaplatin and cisplatin. Cisplatin at 10 μM concentration evoked apoptosis in normal MRC-5 cells whereas mitaplatin did not produce any detectable apoptosis with these normal cells (Table 2).

Visualization of Pt-1,2-d(GpG) Adduct Formation by Mitaplatin. Because the anticancer activity of cisplatin derives from the formation of intrastrand 1,2-d(GpG) cross-links on nuclear DNA (34), we investigated whether cisplatin released by reduction of mitaplatin leads to this signature event by using a monoclonal antibody R-C18 (35) specific for this adduct (SI Text). After 12 h incubation of NTera-2 cells with mitaplatin, formation of 1,2-d(GpG) intrastrand cross-links was observed by antibody-derived green fluorescence in the nuclei of these cells (Fig. S5). These results confirm that mitaplatin has dual cell-killing modes involving DCA, which destroys mitochondrial function, and...
and nuclear extracts were prepared from normal MRC-5 and platin or cisplatin treatment of normal and cancer cells. Cytosolic platinum by atomic absorption spectroscopy (AAS) after mitaplatin uptake, we measured the nuclear and cytosolic concentrations of cells by mitaplatin might be a consequence of its selective conditions. To address whether the selective killing of cancer cells, but not in normal MRC-5 cells under the similar treatment mitaplatin selectively induced cell death in human cancer A549 that, unlike cisplatin, a conventional chemotherapeutic agent, mitaplatin, this study was further extended to a coculture of normal fibroblasts and cancerous NTera-2 cells with mitaplatin, cisplatin, or a mixture of one equivalent of cisplatin and two equivalents of DCA, the stoichiometric composition released upon intracellular mitaplatin reduction. The morphology of these cells at different time points was examined by using bright field microscopy (Fig. 3A). The two types of cells in the coculture are clearly visible because of differences in their morphology. In the coculture, cisplatin and the mixture of cisplatin and DCA killed both the fibroblasts and NTera-2 cancer cells, whereas mitaplatin selectively killed the cancer cells. The results obtained in this study provide compelling evidence that mitaplatin can selectively kill cancer cells, leaving normal cells untouched.

To verify that preferential cancer cell killing occurs with mitaplatin, this study was further extended to a coculture of human lung cancer A549 cells and normal human lung fibroblasts MRC-5.

The selective killing of normal cells by mitaplatin was demonstrated by using a LIVE/DEAD viability assay, which allowed for the simultaneous determination of live and dead cells in a coculture by labeling live cells with calcein AM dye, which fluoresces only when cleaved by intracellular esterase enzymes, and ethidium heterodimer (EthD-1), which only enters dead cells by targeting PDK, we investigated whether mitaplatin would also display specificity for cancer. We therefore treated a coculture of normal fibroblasts and cancerous NTera-2 cells with mitaplatin, cisplatin, or a mixture of one equivalent of cisplatin and two equivalents of DCA, the stoichiometric composition released upon intracellular mitaplatin reduction. The morphology of these cells at different time points was examined by using bright field microscopy (Fig. 3A). The two types of cells in the coculture are clearly visible because of differences in their morphology. In the coculture, cisplatin and the mixture of cisplatin and DCA killed both the fibroblasts and NTera-2 cancer cells, whereas mitaplatin selectively killed the cancer cells. The results obtained in this study provide compelling evidence that mitaplatin can selectively kill cancer cells, leaving normal cells untouched.

Mitaplatin Action on Cisplatin-Resistant Cells. Although very little is known about the effects of cisplatin on the mitochondria of tumor cells (36), a recent study showed that it might have direct impact on mitochondria in head and neck cancer (37). Mitochondrial defects are associated with the cisplatin resistance phenotype (38), and several hypotheses have been suggested to explain this observation. A more negative membrane potential might promote translocation of the active, cationic form of cisplatin from the cytoplasm to mitochondria, thus diminishing platination of nuclear DNA. This effect would suggest that a combination of cisplatin with a mitochondrial targeting moiety would be an attractive therapeutic strategy for attacking cisplatin-resistant tumors. We therefore studied a pair of cisplatin sensitive A2780 and resistant A2780/CP70 ovarian cancer cells (Table 1 and Fig. S6). As controls we used cisplatin and cis-[Pt(NH3)2Cl2(O2CCH3)2]. The cells displayed a low level of resistance to mitaplatin (IC50 for A2780, 1.1 μM; IC50 for A2780/CP70, 3.34 μM) compared to cisplatin (corresponding IC50 values of 0.56 and 6.0 μM). Results for the A2780/CP70 cells indicate that DCA plays a role in making cisplatin-resistant cells susceptible toward mitaplatin treatment. A2780/CP70 cells were

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<th>U2OS</th>
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<th>MRC5</th>
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<td>Apoptosis, %</td>
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<tr>
<td>Necrosis, %</td>
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<td>11.2</td>
<td>10.6</td>
<td>0.1</td>
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HeLa, human cervical cancer; U2OS, human osteosarcoma; A549, human lung carcinoma; MRC-5, normal lung fibroblast. [Mitaplatin], 10 μM; [Cisplatin], 10 μM; [DCA], 20 μM, incubation time 48 h.

![Fig. 3](https://www.pnas.org/cgi/doi/10.1073/pnas.0912276106)
much more resistant to the control platinum(IV) compound \( \text{c.c.c.-}[\text{Pt}(\text{NH}_3)_2\text{Cl}_2(\text{O}_2\text{CCH}_3)_2] \). These data suggest mitaplatin as a promising candidate for further development in the treatment of cisplatin-resistant cells.

**Summary.** In conclusion, mitaplatin displays a dual-killing mode that can only be effective in cancer cells. The platinum center interacts with its own target, nuclear DNA, and DCA released upon reduction attacks mitochondria. These results support the utility of mechanisms targeting cancer cell-specific pathways as an avenue for developing selective anticancer agents. Mitaplatin offers a formulation for future studies incorporating the orphan drug DCA to further its use in the clinic.

**Materials and Methods**

The complexes cis-[Pt(NH3)2Cl2] (39) and c.c.c-[Pt(NH3)2Cl2(OH)2] (40) were synthesized as described. Distilled water was purified by passage through a Millipore Milli-Q Biocel water purification system (18.2 MΩ) containing a 0.22-μm filter. Anti-cytchrome c (Ab-1) sheep polyclonal antibody was procured from Calbiochem. Alexa Fluor 488-labeled secondary antibody donkey anti-(sheep IgG) was obtained from Invitrogen for cytochrome c detection. For AIF detection, we used a rabbit polyclonal IgG antibody from Santa Cruz Biotechnology, Inc. Alexa Fluor 546-labeled secondary antibody goat anti-rabbit IgG was purchased from Invitrogen. The detection of the cisplatin 1,2-d(GpG) intranast adduct was carried out using a monoclonal adduct-specific antibody R-C18 which was kindly provided by Jürgen Thomale (University of Duisburg-Essen). FITC labeled secondary antibody rabbit anti-(rat IgG) was obtained from Invitrogen. Specific adhesion slides for immunofluorescence were purchased from Squarix Biotechnology. JC-1 (5,5′,6′,6′-tetraethylbenzimidazolylcarbo-cyanine iodide) was obtained from Cayman Chemicals. [1H, 13C, and 195Pt NMR spectra were recorded on a Bruker DMEM. The medium was changed and mitaplatin was added to a final concentration of 1 cm at a confluence of 1,600 cells per slip and incubated overnight at 37 °C in DMSO-d6:

\[
\text{[Pt(NH}_3)_2\text{Cl}_2\text{]} \quad \text{(DMSO-d6): dried. Mitaplatin (light yellow solid, which was washed several times with diethyl ether and temperature for 4 h. Diethyl ether was added to the mixture to precipitate a anhydride (0.28 g, 1.5 mmol) and the reaction mixture was stirred at room}
\]

**JC-1 Assay.** GM61889 and Ntera-2 cells were cultured on cover slips to a density of 1 × 10⁶ cells/mL and incubated overnight at 37 °C. Cells were then treated with 100 μM mitaplatin for 4 and 48 h at 37 °C. A solution of JC-1 reagent (Cayman Chemicals; 10 μg/mL in DMEM) was added and incubation was carried out at 37 °C for 30 min. The cells were washed with PBS five times, fixed in 4% paraformaldehyde, and mounted onto glass slides using the procedure described above.

**TMRM Assay.** Analysis of mitochondrial membrane potential (ΔΨm) was carried out by using TMRM. A similar procedure as mentioned above for the JC-1 assay was followed. Before fixing the cells, they were treated with 2 μM TMRM for 30 min at 37 °C.

**LIVE/DEAD Assay.** In vitro selective killing was performed using the LIVE/DEAD Viability/Cytotoxicity Assay (Molecular Probes). A549 and MRC-5 cells were cultured on sterile glass cover slips as subconfluent monolayers for 24 h at 37 °C in 5% CO₂ and grown in DMEM supplemented with 10% FBS and 10 μM penicillin/streptomycin. The cells were then treated with 100 μM cisplatin or mitaplatin for 24 h at 37 °C in 5% CO₂. The cells were washed with Dulbecco’s PBS (D-PBS) to remove serum esterase activity generally present in serum-supplemented growth media before the assay. Calcein AM (4 mM in anhydrous dimethyl sulfoxide, DMSO) and Ethd-1 (2 mM in DMSO/water, 1:4 vol/vol) were added to PBS (1:1,000 ratio) to produce a LIVE/DEAD working solution. Flow cytometry was performed on a BD FACSDiva (BD Biosciences) and data were analyzed on BD FACS diverta (BD Biosciences).

**Annexin-V Assay.** Flow cytometry with a Vybrant Apoptosis Assay kit (annexin V conjugated to allophycocyanin, Invitrogen) was used to determine whether treatment specifically induces apoptosis. Briefly, 5 × 10⁵ cells for each treatment group were added to six-well tissue culture plates and incubated overnight to 60–70% confluence under standard growth conditions. Media for the cell lines were then replaced with fresh growth media with and without a 10 μM dose of cisplatin, mitaplatin, and a 20 μM dose of DCA. Treatment groups for each cell line were replicated three times. The cells were then incubated for 48 h at 37 °C and harvested with 0.25% trypsin-EDTA. Cells were washed with PBS and subsequently stained by annexin V as per the manufacturer’s protocol. Flow cytometry was performed on a BD LSR II flow cytometer (BD Biosciences) and data were analyzed on BD FACS diverta (BD Biosciences).

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