Supporting Information

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SI Methods.

Chemicals and Reagents. All of the chemicals and reagents [cisplatin (Pt(NH3)2Cl2) (CDDP): N,N′,N′-tetramethyl-p-phenylenediamine (TMPD); etc.] were obtained from Sigma-Aldrich. Unless specifically indicated, TMPD was prepared in pure ethanol (EtOH) at a concentration of 40 mM; cisplatin CDDP was stored at a concentration of 3 mM in pure water.

Absorption/Fluorescence Spectroscopic Measurements. The dissociative electron-transfer (DET) reactions of TMPD with CDDP were studied by steady-state absorption and fluorescence spectrum measurements using a spectrophotometer and a fluorometer.

Agarose Gel Electrophoresis Measurements. Details can be found in ref. 1.

Cell Lines and Culture Condition. The HeLa (ATCC#: CCL-2), NIH:OVCAR-3 (ATCC#: HTB-161), and A549 (ATCC#: CCL-185™) cell lines, together with RPMI 1640 and F-12K culture media, were obtained from the American Type Culture Collection (ATCC); fetal bovine serum (FBS) was obtained from Hyclone Laboratories. HeLa cells were cultivated with MEM (Hyclone) supplemented with 10% FBS, 100 units/mL penicillin G, and 100 μg/mL streptomycin (Hyclone). The complete growth media for NIH:OVCAR-3 and A549 cells were ATCC-formulated RPMI 1640 medium supplemented with 20% and F-12K medium with 10% FBS, respectively.

Assay Kits. The Vybrant 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) Cell Proliferation/Viability Assay Kit, the APO-BrdU™ TUNEL Assay Kit, the Image-iT™ LIVE Green Caspase-3 and-7 Detection Kit, RNase, and propidium iodide (PI) were purchased from Invitrogen.

Cell Viability Assay. The combination effects of CDDP and TMPD on cell viability were determined by the MTT assay, one of the most commonly used cell viability assays. Briefly, cells were plated at a density of 7 × 103 cells per well in 96-well plates. Following overnight incubation, cells were treated with different concentrations of cisplatin and TMPD for 24 or 48 h. After incubation for specified times at 37 °C in a humidified incubator, 20 μL of MTT (5 mg/mL in PBS) were added to each well, and cells were incubated for a further 4 h. After removal of the medium, 100 μL DMSO was added to each well. The absorbance was recorded on a microplate reader at the wavelength of 540 nm. The effects of CDDP and TMPD on growth inhibition were assessed as percent cell viability where cells treated with 0.2% ethanol (vehicle) were taken as 100% viable.

Apoptosis Observation by Fluorescence Microscopy. The Image-iT™ LIVE Green Caspase-3 and-7 Detection Kit (Invitrogen) was used for detecting active caspases and apoptosis, following the vendor’s protocol. The kit is specific for detection of active caspase-3 and-7, and also includes Hoechst 33342 and PI stains, which allow the simultaneous evaluation of nuclear morphology and plasma membrane integrity. Cells were grown to 60% confluence and then treated with CDDP and 100 μM TMPD for 10 h. Fluorescence images were taken with a Nikon TS100 microscope.

DNA Fragmentation Measurements by Flow Cytometry. One late-stage marker of apoptosis is the degradation of DNA into small fragments. An enhancement in DNA fragmentation indicates increases in apoptosis, and thus the potency of the combination therapies. As per manufacturer’s protocol, we detected DNA fragmentation using a standard APO-BrdU TUNEL assay (Invitrogen). The cell cycle distribution can also be detected simultaneously from the ordinate axis of the generated dot plot (with the BrdU-content on the y axis), where the events with fluorescence intensity above the background indicate the cells with fragmented DNA. For our measurements, cells were grown at a density of 60% confluence in T-25 flasks and were treated with CDDP and TMPD for 24 or 48 h. After trypsinized, the cells were washed with PBS, processed for labeling with the deoxothyrimidine analogue 5-bromo-2'-deoxyuridine 5'-triphosphate (BrdU-TP), incubated with an Alexa Fluor 488 dye-labeled anti-BrdU antibody, and stained with PI. The labeled cells were analyzed by flow cytometry.

Cell Cycle Analysis by Flow Cytometry. Cells were plated at a density of 2 × 10^5 per well on six-well plates. After overnight incubation, cells were treated with CDDP and TMPD for 24 h. The cells were trypsinized, washed twice with chilled PBS, and centrifuged. The cell pellet was resuspended in 70% ethanol at −20 °C overnight. The cells were centrifuged at 1,000 rpm for 5 min; the pellet was washed twice with chilled PBS, suspended in 500 μL PBS, and incubated with RNase (20 μg/mL final concentration) at 25 °C for 30 min, and stained with PI (50 μg/mL final concentration) for an additional 30 min. The cells were analyzed by flow cytometry (Becton Dickinson). A minimum of 10,000 cells per sample were counted and the DNA histograms were further analyzed by using FCS Express software for cell cycle analysis.

SI Results.

Determination of the Rate Constant k of the DET Reaction Between CDDP and TMPD. Because the absorption intensity I0 of TMPD in EtOH did not change for days when there was no CDDP added, the rate constant k for the DET reaction (Eq. 1) can simply be calculated by

\[ \frac{I_t}{I_0} = e^{-k[C]t} \text{, or } -\ln \left( \frac{I_t}{I_0} \right) = k[C]t \]  

where \( I_t \) is the absorption intensity at 261 nm at time \( t \) after the CDDP with a concentration \([C]\) was added. The plot of \(-\ln(\frac{I_t}{I_0})\) versus reaction time \( t \) is shown in Fig. S2, in which the slope of the linear fit to measured data gives directly the product of \( k[C] \). The reaction rate constant thus obtained is \( k = 1.7 ± 0.2 \times 10^{-2} \text{ M}^{-1} \text{ s}^{-1} \).

Cell Cycle Assays. The induction of apoptosis may also be related to cell cycle arrest. The apoptotic cells with degraded DNA appear as cells with hypodiploid DNA content and are represented in the sub-G1 peak on a DNA histogram (2, 3). To assess the effect of the combination of cisplatin with TMPD on the distribution of cells in the cell cycle, we performed DNA cell cycle analysis. As shown in Fig. S7 for NIH:OVCAR-3 cells, the treatment of either the vehicle or 100 μM TMPD led to a near absence (≤0.7%) of the sub-G1 peak, whereas the treatments of CDDP with increasing concentrations (10–50 μM) showed an increasing intensity of the sub-G1 peak in DNA histograms. The combination of CDDP with 100 μM TMPD exhibited cell cycle profiles that resembled those of cells treated with CDDP alone. Notably, however, there was a significant increase in the population of
hypodiploid cells (in sub-G1), which, for instance, increased from 3.4% (10.1%) for the treatment of 10 (50) μM CDDP alone to 8.5% (18.5%) for the combined treatment with 100 μM TMPD. The latter values nearly doubled (4.1% and 10.8%) those expected for the additive effect of the two agents. The observed enhancement factors of 1.8–2.5 in the sub-G1 population is smaller than the factors of approximately 3.5 for DSBs of plasmid DNA (Fig. 2), 2–4 for CDDP IC₅₀ (Table S1), and 2–5 for DNA fragmentation in cells (Fig. 5). However, researchers have also noted that the sub-G1 peak on a DNA histogram should not be taken as definite evidence for the apoptotic cell population (4, 5).


**Fig. S1.** Static absorption spectra of 100 μM TMPD (indicated as PM2A in this figure) in EtOH with the addition of various concentrations (0–2.0 mM) of cisplatin (CDDP), measured at 0 min, 30 min, 1 h, 2 h, and 3 h after CDDP was introduced.

**Fig. S2.** Reaction rate constant determination: −ln(I/I₀) as a function of reaction time between 100 μM TMPD and 2 mM CDDP, where I₀ and I are the absorption intensity of TMPD at 261 nm without and with the presence of CDDP, obtained from the absorption spectra shown in Fig. S1. The solid line is the linear fit to observed data; from the slope, the DET reaction rate constant k is obtained.
Fig. S3. Agarose gel densitograms for plasmid DNA treated by cisplatin alone at various concentrations (A) and in combination with 100 μM TMPD (B), where the peaks for supercoiled (SC) DNA, open circular DNA with single-strand breaks (SSBs), and linear DNA with double-strand breaks (DSBs) are indicated.

Fig. S4. Effects of cisplatin CDDP and TMPD on the viability of A549 lung cancer cells. The cells were treated with vehicle alone (0.2% EtOH) and various concentrations of CDDP and 50 μM TMPD for 48 h, and cell viability was determined by MTT assay as detailed in SI Methods. The values are represented as the percent of viable cells, with vehicle-treated cells regarded as 100% viable: mean of triplicate experiments (points); SD (bars). The expected additive cell viability curve (dash line and Δ) was calculated by multiplying the effect of TMPD by that of CDDP at each concentration. Because the observed effect (green circle) is below that of the expected additive line, synergy was concluded.

Fig. S5. Effects of cisplatin CDDP and TMPD on the viability of HeLa cells (A) and NIH:OVCAR-3 cells (B). Unlike other experiments described in the text, here the TMPD stock solution was prepared in pure water instead of pure ethanol for 24 h prior to experiments. The cells were treated with various concentrations of CDDP alone and their combinations with 100 μM so-prepared “TMPD” for 24 h, and cell viability was determined by MTT assay as detailed in SI Methods. The values are represented as the percent of viable cells, with untreated cells regarded as 100% viable: mean of triplicate experiments (points); SD (bars).
Fig. S6. APO-BrdU DNA fragmentation assays for HeLa cells with various treatments for 48 h: (A) control (no drugs); (B) 100 μM TMPD; (C) 10 μM CDDP; (D) 10 μM CDDP + 100 μM TMPD; (E) 25 μM CDDP; (F) 25 μM CDDP + 100 μM TMPD. Density plot illustrates BrdU-positive cells as a function of the DNA content (position in the cell cycle). The dot plots are colored to indicate the density of the data points; red indicates a low density and blue indicates a high density of points. Each percentage is the mean of triplicate experiments (standard deviation indicated).
Fig. S7. Percents of hypodiploid cells (in sub-G1) in NIH:OVCAR-3 cells with various treatments indicated. Cell cycle analysis was made by flow cytometry (SI Methods). FL2-A is the PI channel, giving indication of the cell cycle (DNA content). Each percentage is the mean of triplicate experiments (standard deviation indicated).

Table S1. Cisplatin IC\textsubscript{50} (μM) for treatments of CDDP only, CDDP plus 100 μM TMPD (for HeLa and NIH:OVCAR-3 cells) or 50 μM TMPD (for A549 cells)

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Treatment time</th>
<th>HeLa (24-h incubation)</th>
<th>NIH:OVCAR-3 (24-h incubation)</th>
<th>A549 (48-h incubation)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDDP only</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CDDP+TMPD (expected additive)</td>
<td>35</td>
<td>87</td>
<td>25</td>
<td></td>
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<tr>
<td>CDDP+TMPD (observed)</td>
<td>16</td>
<td>22</td>
<td>11</td>
<td></td>
</tr>
</tbody>
</table>

*The concentration required to kill 50% of untreated cells. IC\textsubscript{50} values for the expected additive effect of CDDP and TMPD are also given.