
The authors note that the author name Tara Milton should have appeared as Tara Salley. The author line has been corrected online. In addition, in the author contributions footnote, the initials T.M. should appear as T.S. The corrected author line appears below.

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Non-DNA-binding platinum anticancer agents: Cytotoxic activities of platinum–phosphato complexes towards human ovarian cancer cells

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DNA is believed to be the molecular target for the cytotoxic activities of platinum (Pt) anticancer drugs. We report here a class of platinum(II)- and platinum(IV)-pyrophospho complexes that exhibit cytotoxicity comparable with and, in some cases, better than cisplatin in ovarian cell lines (A2780, A2780/C30, and CHO), yet they do not show any evidence of covalent binding to DNA. Moreover, some of these compounds are quite effective in cisplatin- and carboplatin-resistant cell line A2780/C30. The lack of DNA binding was demonstrated by the absence of a detectable Pt signal by atomic absorption spectroscopy using isolated DNA from human ovarian cells treated with a platinum(II)-pyrophosphate complex, (trans-1,2-cyclohexanediamine)(dihydrogen pyrophosphato)platinum(II), (pyrodach-2) and from NMR experiments using a variety of nucleotides including single- and double-stranded DNA. Furthermore, pyrodach-2 exhibited reduced cellular accumulations compared with cisplatin in cisplatin- and carboplatin-resistant human ovarian cells, yet the IC50 value for the pyrophosphate complex was much less than that of cisplatin. Moreover, unlike cisplatin, pyrodach-2 treated cells overexpressed fas and fas-related transcription factors and some proapoptotic genes such as Bak and Bax. Data presented in this report collectively indicate that pyrodach-2 follows different cytotoxic mechanisms than does cisplatin. Unlike cisplatin, pyrodach-2 does not undergo aquation during 1 week and is quite soluble and stable in aqueous solutions. Results presented in this article represent a clear paradigm shift not only in expanding the molecular targets for Pt anticancer drugs but also in strategic development for more effective anticancer drugs.

Results and Discussion

Platinum(II)- and platinum(IV)-pyrophosphato complexes were fully characterized by spectroscopic and, in some cases, by X-ray crystallographic methods. Detailed structural characterizations

they do not show any evidence of covalent binding to DNA. Although we have synthesized and tested a number of platinum(II)- and platinum(IV)-pyrophosphato-complexes containing a variety of amine ligands that exhibit excellent cytotoxicities, results for (trans-1,2-cyclohexanediamine)(dihydrogen pyrophosphato)platinum(II) (pyrodach-2; Fig. 2) are described in detail in this article. Preliminary data indicate that pyrodach-2 activates fas and fas-related genes along with some proapoptotic genes. Other important superior clinical properties of pyrodach-2 are that, unlike cisplatin, this compound does not undergo aquation during the period of 1 week and is quite soluble and stable in aqueous solutions. The solubility of pyrodach-2 in 100 mM phosphate buffer at pH 7.0 exceeds 50 mM at 25 °C. Moreover, pyrodach-2 is quite effective in the cisplatin- and carboplatin-resistant cell line A2780/C30. To the best of our knowledge, no other Pt compounds reported to date exhibit properties and function in the manner described in this article. Therefore, these pyrophosphate complexes may offer advantages over other Pt drugs in treating ovarian and other types of cancer.

Since the discovery of the anticancer activity of cis-diaminedichloroplatinum(II) (1) (cisplatin), remarkable progress has been made in understanding the cellular and molecular mechanisms of cytotoxicity (2, 3). Cisplatin is widely used in treating ovarian, testicular, small-cell lung, and a variety of other cancers (4, 5). In addition, cisplatin is used in conjunction with other therapeutic regimens, including radiation therapy. This platinum (Pt) chemotherapeutic agent mediates apoptosis at the G2 phase of the cell cycle predominantly through transcription inhibition (6, 7) and through replication inhibition processes, especially at high doses (8). Covalent bonding to DNA through the N7 sites of guanine and adenine bases, both by intra- and interstrand modes (9, 10), is believed to be the key molecular event in transducing a cascade of cellular responses leading to apoptosis. One second and 1 third generation of FDA-approved Pt drugs, carboplatin [diammine(1,1-dicarboxylatocyclobutane)-platinum(II)] and oxaloplatin [trans-1,2-cyclohexanediamine]-oxalatoplum(II)], are also believed to function in a manner similar to that of cisplatin (11, 12) (Fig. 1).

In this report, we present a class of potent Pt pyrophosphato compounds that exhibits cytotoxicity comparable with and, in some cases, better than cisplatin and carboplatin in 3 ovarian cell lines, yet

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Fraction of surviving cells determined by clonogenic assay in human ovarian cancer cells (A2780) with cisplatin, carboplatin, and pyrodach-2 concentrations.

Table 1. IC50 values in micromolar concentrations for phosphaplatins, cisplatin, and carboplatin in A2780, A2780/C30, and CHO cell lines

<table>
<thead>
<tr>
<th>Compound</th>
<th>A2780</th>
<th>A2780/C30</th>
<th>CHO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cisplatin</td>
<td>7 ± 1</td>
<td>100 ± 11</td>
<td>29 ± 3</td>
</tr>
<tr>
<td>Carboxplatin</td>
<td>90 ± 13</td>
<td>&gt;200</td>
<td>&gt;200</td>
</tr>
<tr>
<td>Pyrodach-2</td>
<td>20 ± 4</td>
<td>48 ± 5</td>
<td>35 ± 5</td>
</tr>
<tr>
<td>Pyrodach-4</td>
<td>180 ± 15</td>
<td>155 ± 17</td>
<td>116 ± 17</td>
</tr>
<tr>
<td>Pyroen-2</td>
<td>100 ± 11</td>
<td>&gt;200</td>
<td>120 ± 30</td>
</tr>
<tr>
<td>Pyroen-4</td>
<td>175 ± 22</td>
<td>&gt;200</td>
<td>&gt;200</td>
</tr>
</tbody>
</table>

Errors are given as the SEM. A2780/C30 cell lines were completely refractory up to 30 μM cisplatin. However, the same cells, in our hands, showed up to 20% cytotoxicity at 100 μM carboplatin concentration.

The A2780/C30 cell line is cross-resistant to both cisplatin (30 μM) and carboplatin (100 μM). As can be seen from the figure, pyrodach-2 is extremely effective, as evidenced by IC50 values that are significantly below cisplatin and carboplatin in A2780/C30 cells but somewhat higher than cisplatin in the A2780 cell line. For example, the IC50 value for pyrodach-2 was found to be 48 ± 5 μM, whereas cisplatin and carboplatin were 100 ± 11 μM and >200 μM in A2780/C30 cells. For the A2780 cell line, these IC50 values were found to be 7 ± 1 μM and 20 ± 4 μM for cisplatin and pyrodach-2, respectively. The IC50 value for pyrodach-2 in the resistant cell line is ~2.5 times higher than for the sensitive cells, whereas for cisplatin, the IC50 value is ~15 times higher. It is also interesting to note that IC50 values for pyrodach-4 toward A2780/C30 is less than for A2780 (Table 1). Moreover, the IC50 values for pyrodach-2 in cisplatin-sensitive and cisplatin-resistant human head and neck cancer cell lines, UMSSC10b and UMSSC10/15s, are less than that for cisplatin (data not shown) (R. Krishna, S. Sherjeel, and R.N.B., unpublished results; see also ref. 15). The IC50 values for these cisplatin-sensitive and -resistant cell lines were estimated to be 1.5 and 4 μM, respectively. The IC50 value for cisplatin for UMSSC10b has been reported to be 17 μM. The IC50 values for the other platinum(II)- and platinum(IV)-pyrophosphato compounds with all amine ligands are listed in Table 1.

Cellular accumulations of Pt, as determined by atomic absorption spectroscopy (Fig. 4), revealed that at a given concentration, pyrodach-2 was taken up by cells in reduced quantities compared with cisplatin. For example, at 10 μM concentration, cisplatin accumulation in A2780 cells was 3.0 ng of Pt per 10^6 cells whereas cisplatin accumulation in A2780/C30 was only 3.0 ng of Pt per 10^6 cells. A2780/C30, and CHO cell lines

A2780 cells

A2780/C30 cells

Fig. 2. Structural formulas of sodium salts of pyrophosphato-platinum(II) and -platinum(IV) complexes and their abbreviated names. Note that pyrodach-2 and pyrodach-4 contain (+)-trans-1,2-cyclohexanediamine as the amine ligand.

Fig. 3. Fraction of surviving cells determined by clonogenic assay in human ovarian cancer cells (A2780), cisplatin- and carboplatin-resistant human ovarian cells (A2780/C30) as a function of cisplatin, carboplatin, and pyrodach-2 concentrations.

are reported in refs. 13 and 14. The structural formulas for the pyrophosphato complexes are shown Fig. 2. The trans isomer of trans-1,2-cyclohexanediamine that contains both R,R and S,S optical isomers was used for the preparation of pyrodach-2. No attempts were made to resolve the optical isomers.

Fig. 3 shows the cytotoxicity of pyrodach-2 in human ovarian cancer cells (A2780 and A2780/C30). These data are also compared with cisplatin in the same cells under identical conditions. Note that the
Platinum cellular accumulation

![Graph showing platinum cellular accumulation](image)

**Fig. 4.** Cellular accumulation of cisplatin and pyrodach-2 measured in A2780 and A2780/C30 cells after drug exposure of 1 h.

Cells, whereas pyrodach-2 showed 1.0 ng of Pt per 10^6 cells. A similar trend for Pt accumulations holds for other concentrations. When cellular accumulations of Pt at IC$_{50}$ and IC$_{90}$ values were compared, a similar trend was also observed. For example, Pt accumulation of 2.5 ng of Pt per 10^6 cells for cisplatin was observed compared with 1.5 ng of Pt per 10^6 cells for pyrodach-2 at their corresponding IC$_{50}$ values. Much reduced cellular accumulation of pyrodach-2 was observed in the resistant cell line, yet the pyrophosphato complex exhibited superior cytotoxicity. For example, pyrodach-2 showed 1.4 ng of Pt per 10^6 cells at its IC$_{50}$ value, whereas cisplatin showed >5 ng of Pt per 10^6 cells, yet the IC$_{50}$ value for pyrodach-2 former complex is less than half of cisplatin. Comparative cellular accumulations of Pt for cisplatin and pyrodach-2 for A2780 and A2780/C30 cells are listed in Table 2.

The most important finding, however, is the lack of DNA binding by pyrodach-2. No detectable DNA-bound Pt was observed for the pyrodach-2 compound, even after 24-h treatment of the cells, whereas cisplatin-treated cells revealed Pt-bound DNA species as has been observed. For cisplatin-treated A2780 cells, the amount of Pt-bound DNA was estimated to be higher than that reported in the literature (16, 17). For example, DNA-bound Pt was found to be 100 and 250 pg of Pt per microgram of DNA for the doses of 10 and 30 μM cisplatin, respectively, whereas no detectable Pt-bound DNA was observed even for pyrodach-2 concentrations as high as 50 μM with 24-h treatment of 1 × 10^6 cells. Taking the detection limit of the graphite furnace atomic absorption instrument as 2 μg/L and the amount of isolated DNA present in the 20-μL aliquot used for the Pt analysis, an upper limit of Pt binding can be estimated as 1 Pt per 10^8 bases. This extremely low level of Pt binding, if it were to take place, would represent 2 to 3 orders of magnitude less DNA binding compared with cisplatin. Furthermore, considering the fact that only 1% of the administered cisplatin binds to genomic DNA, <0.01% of the administered pyrodach-2 would be needed to exhibit comparable cytotoxic properties. In such a scenario, pyrodach-2 would become the most powerful Pt anticancer agent discovered to date, based on lowest DNA binding requirements.

To confirm the lack of pyrodach-2 binding to DNA in the human ovarian cells, reactions of this compound with double-stranded calf-thymus DNA, a synthetic oligonucleotide containing a 25-mer (5′-ATGATTTAGTGACACTATAGCAGT-3′), a dinucleotide (dGpG), and nucleotide monophosphates (5′-dGMP and 5′-dAMP) were conducted at concentrations as much as 250 times higher than those cellular doses described above. The extent of DNA binding was monitored by 1H NMR spectroscopy. In parallel, experiments with the same nucleotides with cisplatin were conducted under identical conditions. Covalent binding of Pt to guanine and adenine bases through N7 sites is accompanied by the appearance of new proton resonances for the H8 proton of purine ring (18–20). Results from a typical NMR experiment are displayed in Fig. 5 using a 25-mer oligonucleotide. Fig. 5 clearly shows that pyrodach-2 does not exhibit any measurable DNA binding within 7 days with DNA, whereas cisplatin readily forms covalent adducts with DNA, as evidenced by the formation of new signals in the region 8.4 to 8.95 ppm. Likewise, cisplatin readily forms adducts with all of the nucleotides stated above, whereas no detectable NMR signals for the pyrodach-2 binding to nucleotides were observed even after 7 days.

### Table 2. Comparative cellular accumulations (nanograms per 10^6 cells) of Pt for cisplatin and pyrodach-2 in A2780 and A2780/C30 cell lines

<table>
<thead>
<tr>
<th>Concentration, μM</th>
<th>A2780 Cisplatin</th>
<th>A2780 Pyrodach-2</th>
<th>A2780/C30 Cisplatin</th>
<th>A2780/C30 Pyrodach-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>3.00 ± 0.03</td>
<td>1.00 ± 0.10</td>
<td>0.84 ± 0.14</td>
<td>0.10 ± 0.05</td>
</tr>
<tr>
<td>20</td>
<td>5.00 ± 0.03</td>
<td>1.20 ± 0.15</td>
<td>1.86 ± 0.20</td>
<td>0.55 ± 0.08</td>
</tr>
<tr>
<td>30</td>
<td>6.40 ± 0.13</td>
<td>2.60 ± 0.26</td>
<td>2.40 ± 0.27</td>
<td>1.00 ± 0.01</td>
</tr>
<tr>
<td>50</td>
<td>20.00 ± 0.40</td>
<td>5.40 ± 0.17</td>
<td>5.30 ± 0.68</td>
<td>1.50 ± 0.06</td>
</tr>
</tbody>
</table>

Errors are given as the SEM.
Furthermore, to eliminate the possibility that pyrodach-2 may be metabolized in the cellular milieu by pyrophosphatase, thus creating reactive DNA-binding metabolites, pyrodach-2 and pyroam-2 were subjected to hydrolysis by a pyrophosphatase. Unlike free pyrophosphate, this enzyme did not recognize Pt-coordinated pyrophosphate as a substrate, as evidenced by the lack of detectable hydrolyzed products, as measured by $^{31}$P NMR spectroscopy.

To gain insight into the mechanisms of cytotoxicity, expressions of 84 genes treated by pyrodach-2 were followed by real-time PCR experiments and compared with those treated with cisplatin. These 84 genes (Human Apoptosis PCR Array: PAHS-012; SABiosciences) included antiapoptosis, TNF ligands and their receptors, members of BCL2 family, caspases, and IAP family. The 2 sets of gene expressions clearly show a number of differences. For example, fas and family members of the fas superfamily of genes were overexpressed in cells treated with pyrodach-2, whereas cisplatin marginally affected the expression of these genes. Specifically, fas was overexpressed by 6-fold compared with our control, and TNFR5 genes such as TNFRSF-10B and -21 were overexpressed by 2- to 3-fold fold over controls. These latter receptors belong to the fas superfamily. It is interesting to note that proapoptotic BCL2 family members such as BAK1 and BAX were overexpressed $\approx$3-fold in response to pyrodach-2, whereas cisplatin did not significantly change their levels of expression.

The reduced cellular accumulation of Pt in cisplatin-resistant cell lines compared with cisplatin-sensitive cells has been addressed and correlated with the efficient DNA damage repair processes and increased glutathione synthesis (21, 22). However, the reduced accumulation of pyrodach-2 compared with cisplatin is perhaps related to their reactivity differences toward cellular components rather than efflux associated with DNA repair processes, as explained below. Cisplatin is believed to be transported into cells through a copper transport protein (23), Ctr1, presumably through binding to sulfur donor atoms of cysteine or methionine residues (24) of the protein. We find that pyrodach-2 reacts with cysteine in a consecutive 2-step process to deligate the pyrophosphate moiety from the Pt with a second-order rate constant, $4.5 \times 10^{-3}$ M$^{-1}$ s$^{-1}$ for the first step of the reaction at 25 °C, which is slower than that of corresponding cisplatin reactions with cysteine and a nonapeptide containing 2 cysteine residues (25, 26). Furthermore, pyrodach-2 did not undergo aquation or phosphate hydrolysis in aqueous solution at neutral pH as evidenced by the lack of any detectable products even after up to 7 days. The lack of aquation and phosphate hydrolysis of pyrodach-2 and the second-order reaction of this complex with cysteine might have important implications in exhibiting reduced toxicities compared with cisplatin. For example, cisplatin is readily hydrolyzed to several highly reactive species through consecutive first-order aquation reactions. These aquned products then indiscriminately react with a large number of biomolecules in the cellular milieu. Some of these undesired reactions significantly contribute to a variety of toxicities exhibited by cisplatin including nephro-, renal, and neurotoxicities. Because cisplatin reacts with both sulfur and nitrogen donor atoms, both directly and through the aquned products, whereas pyrodach-2 does not readily react with purine and pyrimidine bases, the reduced cellular accumulation can be correlated with the differential reactivities toward a variety of biomolecules in the cells. In fact, this reduced cellular accumulation, by suppressing undesired reactions, might ameliorate side effects for phosphaplatins compared with cisplatin.

Collectively, the above-mentioned data indicate that pyrodach-2 exhibits distinctly different molecular and cellular mechanisms for cytotoxicity compared with cisplatin and other Pt drugs. First, the absence of a covalent linkage is quite intriguing in that this class of compounds most likely is not functioning through the DNA-binding pathway. Second, pyrophosphato complexes should eliminate or significantly reduce cellular resistance due to the lack of DNA binding. It is believed that the acquired cisplatin resistance is due to the efficient removal of Pt from DNA by the nucleotide excision repair process (22). However, it is well known that DNA-polymerase-β, the primary DNA repair enzyme, is involved (27, 28), bypassing platinated DNA lesions (29) in synthesizing DNA strands. Therefore, the cancer cells overexpressing polymerase-β might also contribute to the resistance mechanism. The lack of DNA binding and hence the absence of DNA repair finds its support from the cytotoxicity data in that pyrodach-2 is remarkably active in both cisplatin- and carboplatin-resistant cell lines. Third, reactions of pyrodach-2 with cysteine and glutathione readily replace the pyrophosphate ligand with concomitant ligation through the thiol, indicating possible protein anchoring sites of the cellular surface proteins through cysteine or methionine residues. Fourth, the overexpression of fas and its associated members implies that these genes are involved in apoptosis mediated by pyrodach-2. Although there are scattered reports (30–35) that suggest that cisplatin might function through several other parallel pathways, including fas-activated pathways, the DNA binding mechanism is commonly accepted to be the main molecular event for exhibiting the antitumor activity by these Pt drugs. Although the involvement of fas and fas-related genes are apparent, we cannot eliminate other possible mechanisms that do not require DNA damage pathways (36–40) including senescence, TNF, protein kinases, and other proapoptotic and antiapoptotic transcription factors. In fact, the overexpressations of the proapoptotic genes stated above might function independently or in concert with fas pathways for pyrodach-2-initiated apoptosis. It should be stressed that >30 years of efforts by numerous groups across the globe have not yet fully solved the detailed molecular mechanisms of cisplatin-induced cytotoxicity. Our suggested pathways involving fas and 2 antiapoptotic genes may not be the only pathway involved with phosphaplatins. We anticipate that many years of diligent efforts from cancer researchers will be needed to solve yet another intriguing metallobiochemistry issue of this class of Pt complexes.

Finally, results presented in this article represent a clear paradigm shift not only in expanding the molecular targets for Pt anticancer drugs but also in strategic development of more-effective and less-toxic anticancer drugs. For example, based on the DNA binding strategy, thousands of Pt compounds have been synthesized and tested for their anticancer activities. However, only a handful of compounds have exhibited comparable antitumor activities to that of cisplatin. Our results perhaps will open opportunities not only to design better drugs but also to develop more effective combinatorial therapies based on the potential targets other than DNA described here.

Materials and Methods
Synthesis of Pt Complexes. Cisplatin and carboplatin were synthesized following literature methods. Five platinum(II)- and platinum(IV) amine-pyrophosphato complexes were synthesized and characterized as described elsewhere (13, 14).

Cell Survival Assay. Human ovarian cancer cells, A2780 and A2780/C03 (cross-resistant to 30 μM cisplatin and 100 μM carboplatin), were obtained from Thomas Hamilton (Fox Chase Cancer Center, Philadelphia, PA). Cells were cultured in monolayer by using RPMI medium 1640 supplemented with 10% FBS, 2 mM glutamine, 0.25 units/mL insulin and penicillin/streptomycin (100 units/ml) in a 37 °C incubator continuously gassed with 5% CO$_2$. To detach the cells from the plates, 0.0625% trypsin was applied to the cell monolayer. The cells collected from the plates were centrifuged, and the trypsin-containing solution was discarded. The pellet of cells was then resuspended in medium. The appropriate number of cells were reintroduced to the flask to ensure an optimal cell density to permit exponential growth of the cells. IC$_{50}$ values were determined by using a clonogenic assay. Briefly, 500 A2780 cells from a single-cell suspension were plated onto 60-mm Petri plates 24 h before drug treatment to permit cell attachment. On the day of drug treatment, the
medium was decanted and replaced with the appropriate concentration of drug, and these treated cells were placed back into the 37 °C incubator for 1 h. Triplicate plates were set up for each drug concentration. Five independent experiments for a given concentration, each with a triplicate set, were carried out in performing the cell survival assay. After 1 h of drug treatment, the medium was decanted and replaced with fresh medium. These plates were returned to the 37 °C incubator for 1 week for colony formation. Colonies were fixed and stained by using 2% crystal violet in absolute methanol. Colonies containing 50 or more cells were scored. The number of scored colonies from the triplicate plates was averaged, and this number was divided by the number of cells plated to obtain a value for the fraction of cells forming colonies. These values for fraction of cells forming colonies were then corrected for plating efficiency by dividing it by the number of cells forming colonies in plates that were not treated with drug. Data reported for pyrodach-2 are the mean of 5 sets of triplicate measurements. For other pyrophosphate complexes, 3 independent sets of experiments, each in triplicate, were carried out.

Cellular Accumulation of Pt and Estimation of DNA-Bound Pt. Pt content was quantitatively estimated on a graphite furnace atomic absorption spectrometer (AA-600; PerkinElmer) from calibration curves established by using cisplatin or pyrodox-2 in water. Cells (5 × 10⁶) were seeded in 7T5 flask sizes. After 24 h, these cells were then treated with 0, 10, 20, 30, and 50 μM cisplatin or pyrodox-2 (0.25 mM) with a synthetic 25-mer 5'-ATGATTTAGGTCACCATATAGCAGT-3' (5 μM) and single- and double-stranded DNA (calf thymus, 50 μM) at pH 7.0 maintained by phosphate and carbonate buffers (10–20 mM). Reactions of cisplatin (0.25 and double-stranded DNA (calf thymus, 5.0 mM) at pH 7.0 maintained by experiments, each in triplicate runs, were performed to estimate Pt binding absorption at 260 nm by using a NanoDrop UV-Vis instrument. Duplicate removed and cells were washed with ice-cold PBS. The cells were then trypsinized and centrifuged into a pellet. Cell pellets were digested in concentrated HNO₃ and H₂O₂ before analysis, according to the method of McGahan (41). Data reported in Table 2 were collected from 3 independent experiments, and each was carried out in duplicate. For the cisplatin accumulation, data were obtained from triplicate runs from a single sample at a given concentration.

For DNA–Pt measurements, 1.0 × 10⁶ cells were seeded in 7T5 flask sizes. After 24 h, the Pt compounds were added at 0, 10, 20, 30, and 50 μM. Cells were treated with the Pt compounds for 24 h. After treatment, the medium was removed and cells were washed with ice-cold PBS. The cells were then trypsinized and centrifuged into a pellet. DNA was extracted by using a Wizard SV DNA purification kit (Promega). The DNA was quantitatively estimated from the absorbance at 260 nm after using a NanoDrop UV-Vis instrument. Duplicate experiments, each in triplicate runs, were performed to estimate Pt binding to DNA.

Pt–DNA Binding by NMR. Samples contained cisplatin (2.0 mM) or pyrodox-2 (2.0 mM) and nucleotides [5′-dGMP, 5′-dAMP, dGpG (5.0 mM)], and single- and double-stranded DNA (calf thymus, 5.0 mM) at pH 7.0 maintained by phosphate and carbonate buffers (10–20 mM). Reactions of cisplatin (0.25 mM) and pyrodox-2 (0.25 mM) with a synthetic 25-mer 5′-ATGATTTAGGTCACCATATAGCAGT-3′ (0.25 mM) were also performed under identical conditions. Reactions were carried out up to 7 days, and 1H and 31P NMR spectra were recorded at regular time intervals. Proton NMR spectroscopic experiments were conducted with a triple-resonance indirect probe on a Bruker 500-MHz NMR instrument using a water suppression pulse sequence. Usually, a pulse width of 5 μs with a 1.0-s repetition delay was used for the measurement. Typically, a sweep width of 10,000 Hz and 32,768 data points were selected to collect the free induction decays. A line-broadening factor of 1 Hz was applied to collect Fourier transformation. The chemical shifts, with reference to H-D-O peak, are at 4.67 ppm.

Proton decoupled phosphorus-31 NMR spectra were recorded at 202.45 MHz by using a broadband probe on a Bruker 500-MHz NMR instrument. Typically, a pulse width of 26 μs with a 1.0-s pulse repetition delay, 4,000 data points, and 4,000-Hz sweep width were used in recording spectra. The chemical shifts are with respect to 85% H₃PO₄ in D₂O (0.0 ppm). Reagent concentrations and conditions are the same as stated for the proton NMR experiments for the nucleotide binding experiments. Typical reaction mixtures for pyrophosphate cleavage contained either tetrasodium pyrophosphate (4.0 mM) or pyrodox-2 (4.0 mM), MgCl₂ (2.0 mM), and 8 units of pyrophosphatase in 100 mM Tris·HCl buffer (pH 7.24; 90% H₂O and 10% D₂O vol/vol) in a total volume of 0.5 ml.

Apoptotic Gene Expression by RT-PCR. Human ovarian cancer cells (A2780) were trypsinized and washed with PBS and then frozen at −80 °C. RNA was isolated by using the PureLink Microto-Midi Total RNA Purification System (Invitrogen). Residual genomic DNA was removed with RQ1 RNase-Free DNase (Promega). cDNA synthesis was performed by using DNase-treated RNA and 1 μg of random decamer primers in a final volume of 12 μl. The samples were heated for 5 min at 95 °C and then placed on ice for 5 min. Synthesis of cDNA used the SuperScript III Platinum SYBR green One-Step qRT-PCR kit (Invitrogen) as follows. The 12 μl of sample described above was mixed with 12.5 μl of 2× SYBR green reaction mix and 0.5 μl of SYBR enzyme mix containing SuperScript III Reverse Transcriptase and Platinum TaqDNA polymerase. The reaction mixture was incubated for 10 min at room temperature and then at 50 °C for 30 min and 95 °C for 2 min in a thermocycler.

The cDNA generated above was used as a template for quantitative real-time PCR (QPCR). For a negative control, DNased-RNA (which lacked reverse transcriptase) was used to assess contamination from genomic DNA (data not shown). QPCRs were carried out in an MX3000P real-time PCR system (Stratagene). A mastermix was prepared by using 11.25 μl of water, 0.25 μl of 100× dilution (1×) reference dye R4526 (Sigma), 12.5 μl of SYBR green JumpStart Taq ReadyMix (S4438; Sigma) or 12.5 μl of Brilliant SYBR green QPCR Master Mix (Stratagene), and 1 μl of 10× dilution of cDNA prepared from untreated cells or cells that were treated with cisplatin or pyrodox-2. This mixture was added to each of 96 wells in a human apoptosis PCR array (PAHS-012A; SuperArray). Two independent experiments, each starting with fresh cells and the isolation of fresh RNA, were conducted. Each independent experiment was performed in duplicate.

The standard cycling conditions were as recommended by the ReadyMix or Master Mix suppliers. Data were collected at the end of the annealing step. The cycle threshold (Ct) for each sample was generated by the MxPro software. The Ct values for each sample correspond to the point at which the fluorescence crosses the threshold. Subsequent to amplification, characterization of product was performed by melting and melting-curve analysis. Fluorescence data were continuously collected as the temperature ramped up from 55 °C to 95 °C. The dissociation curve for each sample was generated by the MxPro software to determine the melting temperature (Tm) of the reaction product or products by using the value –ln (Rn(T)) (the first derivative of the normalized fluorescence reading multiplied by –1). Fold differences in the expression of each gene between the test and control samples were normalized based on 2 reference genes, RPL13A and GAPDH.