Preclinical Development

Spectrum of Cellular Responses to Pyriplatin, a Monofunctional Cationic Antineoplastic Platinum(II) Compound, in Human Cancer Cells

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Introduction

Three platinum compounds currently in use worldwide—cisplatin, carboplatin, and oxaliplatin (Fig. 1)—have been developed with crucial support from the U.S. National Cancer Institute (NCI), including screening by the 60-cell line panel (NCI60 screen) of NCI (1). This process, together with the NCI COMPARE program, identified clear differences in activity profiles and mechanisms of action between platinum compounds, thus enabling the grouping of platinum compounds according to such characteristics (2). The cisplatin activity profile is similar to that of other diammineplatinum(II) compounds and to alkylating agents such as melphalan and topoiso merase I inhibitors such as camptothecin analogs. The oxaliplatin activity profile is similar to that of other platinum compounds containing the \( R_R-1,2\text{-diaminocyclohexane} \) ligand, including the platinum(IV) drug tetraplatin, and is also similar to those of acridines, organic compounds currently being developed as anticancer drugs (2).

Other classes of platinum compounds with activity different from cisplatin, oxaliplatin, or carboplatin have been defined on the basis of the NCI60 screen. The activity of the platinum–pyridines defines one group, into which some polyplatinum compounds including the clinically tested BBR3464 can be classified (3, 4). The platinum–silanes are another distinct group. Cells resistant to compounds from one group are commonly not cross-resistant to compounds from another. Similarly, because of the different mechanisms of action for each type of compound, it is possible for compounds of different groups to be used in combination with synergistic results, an example being the synergistic effect of...
combining cisplatin and oxaliplatin (2). The development of platinum compounds with mechanisms of action different from those of platinum-based drugs already on the market should facilitate identification of candidate compounds that are active in cancers for which cisplatin, carboplatin, or oxaliplatin are inactive. Unique mechanisms of action may derive from the mode of cellular uptake of the compound (5), preferential localization of the platinum compound to a specific body organ or cell organelle (6), manipulation of the cellular response to enhance cytotoxicity (7), or the prevention or retardation of drug inactivation by biotransformation as occurs for platinum(IV) prodrugs (8–10).

Pyriplatin is a monofunctional, cationic platinum(II) compound that has previously shown antitumor activity in mice (11) and which forms only a single covalent bond with DNA, unlike cisplatin, carboplatin, or oxaliplatin, which bind in a bidentate manner. In addition to this nontraditional structure, there is also evidence for a unique cellular mode of pyriplatin uptake that differs from the uptake of cisplatin or oxaliplatin. Pyriplatin is an outstanding substrate for the organic cation transporters (OCT) 1 and 2 (12, 13), which are associated with improved oxaliplatin uptake (14). In addition, OCT1 has been identified as important to the pharmacokinetics and tissue distribution of pyriplatin (12). The mechanism of RNA polymerase II inhibition by pyriplatin–DNA adducts is dramatically different from the inhibition seen with cisplatin–DNA adducts (15), and the low nucleotide excision repair rates of the pyriplatin–DNA adduct, coupled with its inhibition of RNA polymerase II (13), are very likely important to the cytotoxic mechanism of action. The aim of this study was to further characterize pyriplatin in vitro with direct comparison with cisplatin and oxaliplatin to gain insight into the mechanism of action and potential clinical applications for pyriplatin. We investigated cellular and molecular changes induced by pyriplatin to determine possible response biomarkers and predictive factors of pyriplatin activity. The effects of combining pyriplatin with several anticancer drugs in current clinical use were also investigated.

Materials and Methods

Cell lines

All cell lines were obtained from the American Type Culture Collection and NCI cell collections. Cells were grown as monolayers in RPMI medium supplemented with 10% fetal calf serum (Invitrogen), 2 mmol/L glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin. Cells were split twice a week by using trypsin/EDTA (0.25%/0.02%; Invitrogen) and seeded at a concentration of 2.5 × 10^4 cells/mL. All cell lines were tested regularly for Mycoplasma contamination by PCR using a Stratagene kit.

Single agent evaluation

Pyriplatin was submitted to the NCI (United States) for single agent, single dose testing in 2008. For evaluations done in our laboratory, cells were seeded at 2 × 10^3 cells per well in 96-well plates and treated 24 hours later with increasing concentrations of cisplatin, oxaliplatin, or pyriplatin. After 1, 2, 5, 24, or 72 hours of incubation, the cells were washed and postincubated in platinum-free medium for 72 hours (after 1, 2, or 5 hours) or 48 hours (after 24 or 72 hours). Growth inhibition was then determined by the MTT assay (16). Absorption at 560 nm of the control wells containing untreated cells was defined as 100%, and the viability of treated samples was expressed as a percentage of the control.

Cell cycle analysis

Exponentially growing MCF7 or HCT-116 cells were treated for 24 hours with cisplatin, oxaliplatin, or pyriplatin at the IC_{50} concentrations (Table 1). At the end of treatment and following the 24, 48, or 72 hours drug-washout period, the cells were counted, fixed in 70% cold ethanol, and kept at 4°C. The cells were washed with cold PBS and stained with 5 μg/mL propidium iodide in PBS and 12.5 μL/mL RNase A. Flow cytometric cell cycle analysis was done on a minimum of 2 × 10^8 cells per sample on a FACS Calibur instrument (Becton Dickinson). A 488 nm laser and a dichroic mirror (570 nm) were used and fluorescence emission was detected by using a filter for 620 ± 35 nm.

Evaluation of apoptosis

HCT-116 or MCF7 cells were harvested following 24 hours of treatment with platinum compounds at IC_{50} concentrations and 0, 24, 48, or 72 hours of incubation in platinum-free medium. Cells were washed once with cold PBS, then pelleted and resuspended in 100 μL of a staining buffer containing Annexin V–FITC (fluorescein isothiocyanate) and 0.5 μg propidium iodide. Fluorescence analysis by flow cytometry was done after 15-minute incubation in the dark and dilution of the sample to 500 μL.
Combination studies were carried out as the ovarian cancer line OVCAR-3 and the colon cancer line HT29. Combination evaluation was performed using the MTT assay and analyzed by the Chou and Talalay method, which is based on the median–effect principle and the combination index (CI) is calculated. The CI values less than 1 indicate synergism, values of 1 indicate additive effects, and values greater than 1 indicate antagonism.

Western blotting

HOP-62 cells were treated for 24 hours at the IC₅₀ concentrations of pyriplatin, oxaliplatin, or cisplatin. The platinum-containing medium was removed and cells were lysed either immediately or 24, 48, or 72 hours after removal of the platinum-containing medium. Following protein concentration quantification by the Bradford assay, extracts were analyzed on SDS-PAGE, transferred to polyvinylidene difluoride membranes, incubated with primary antibodies, and revealed by peroxidase-coupled secondary antibodies by using enzymatic chemiluminescence. Antibodies were obtained from commercial sources and used at the following dilutions: Ser139 phospho-H2AX, 1:1,000, mouse monoclonal; Millipore; Thr68 phospho-Chk2, 1:1,000, rabbit polyclonal; Cell Signaling Technology; α-tubulin (1:10,000, mouse monoclonal; Amersham Biosciences); β-actin (1:20,000, mouse monoclonal; Sigma-Aldrich).

Combination evaluation

Antiproliferative effects of pyriplatin, cisplatin, or oxaliplatin in combination with paclitaxel, gemcitabine, SN38, cisplatin, or 5-fluorouracil were investigated in the ovarian cancer line OVCAR-3 and the colon cancer line HT29. Combination studies were carried out as described (17, 18). Briefly, cells were seeded at 2 × 10⁵ cells per well in 96-well plates and incubated for 24 hours before treatment. The combination experiments were conducted on 3 different schedules. Cells were either treated with platinum for 24 hours followed by the combination drug for 24 hours, treated with the combination drug for 24 hours followed by platinum for 24 hours, or treated for 24 hours with pyriplatin and the combination drug simultaneously. Drug and platinum concentrations from IC₅₀ to IC₆₀ were used. Antiproliferative effects were evaluated by the MTT assay and analyzed by the Chou and Talalay method, which is based on the median–effect principle and the combination index (CI) is calculated. The CI values less than 1 indicate synergism, values of 1 indicate additive effects, and values greater than 1 indicate antagonism.

Measurement of platinum content

HCT-116 cells were incubated for 2 or 24 hours with 10 μmol/L cisplatin, oxaliplatin, or pyriplatin. A time course of 2 hours platinum exposure followed by 22 hours of incubation in platinum-free medium (2/22 schedule) was also evaluated. After trypsinization, cytosol and nuclei were separated in a hypotonic buffer. Cell lysis was done in a buffer of 100 mmol/L Tris (pH 7.5) 1 mmol/L EDTA, 1 mmol/L EGTA, 0.5 mmol/L Na₃VO₄, 10 mmol/L sodium β-glycerophosphate, 50 mmol/L sodium fluoride, 5 mmol/L sodium pyrophosphate, and 1% Triton X-100. DNA was purified from nuclear extracts by phenol–chloroform extraction and ethanol precipitation. Cellular lysates, DNA samples, and incubation medium were analyzed for platinum levels by inductively coupled plasma mass spectrometry (ICP-MS).

Quantitative real-time reverse transcriptase analysis

HCT-116 cells were treated for 48 hours with IC₅₀ concentrations of drugs, followed by isolation of mRNA as described (20). Briefly, total RNA was reverse-transcribed before real-time quantitative PCR amplification by using the ABI Prism 7900 Sequence Detection System (Perkin-Elmer Applied Biosystems). The transcripts of the gene coding for the TATA box-binding protein (TBP; a component of the DNA-binding protein complex TFIID) were used as the endogenous control RNA for normalization. Results were expressed as N-fold differences in target gene expression relative to the TBP gene. The mRNA expression of ERCC1, XPA, XPC, PARP1, XRCC1, RAD50, BRCA1, DNA-PKcs, XRCC6, MSH2, MLH1, BCL2, PUMA, COX2, CDKN1A/p21, ABCB1,
ABCC1, and GSTP1 was evaluated in a panel of 10 cancer cell lines. Thermal cycling was done with an initial denaturation step at 95 °C for 10 minutes, and 50 cycles of 15 seconds at 95 °C and 1 minute at 65 °C. Experiments were conducted in duplicate.

Statistical analysis
Statistical analyses were done with Instat and Prism software (GraphPad). Results are expressed as the mean ± SD of at least 3 experiments conducted in duplicate. Means and SDs were compared by using the Student’s t test (2-sided P value).

Results
Antiproliferative effects of single agent pyriplatin in a panel of human cancer cell lines
The potential anticancer activity of pyriplatin was tested at the NCI by using the NCI-60 tumor cell line panel screen. Results are shown in Supplementary Fig. S1. Analysis of these results by using the onlineCOMPARE algorithm revealed that the antiproliferative profile of pyriplatin was not similar to those of cisplatin or oxaliplatin. The best correlation with a platinum compound in the NCI database was with (carboxyphthalato) platinum (NSC #5748451), with a correlation coefficient of 0.396. These data suggest that pyriplatin may have a mechanism of action that differs from classical platinum drugs.

Pyriplatin was further evaluated in comparison with cisplatin and oxaliplatin by using a well-characterized panel (21) of 10 cancer cell lines of different tissue origins (colorectal, breast, melanoma, ovarian, and non–small cell lung). Cells were exposed for 24 hours to pyriplatin (0.46–1,000 μmol/L), cisplatin (0.1–160 μmol/L), or oxaliplatin (0.1–160 μmol/L) and assessed for cytotoxicity by the MTT assay. Cell counting and the sulforhodamine B assay (data not shown) confirmed the results of the MTT-based antiproliferative assays, shown in Table 1 as IC50 concentrations. The cytotoxicity profile for pyriplatin, shown in difference plots in Fig. 2, was clearly different from those of both cisplatin and oxaliplatin.

To study the effects of duration of pyriplatin exposure on cell proliferation, we used the cell line in which pyriplatin showed the greatest antiproliferative effect (Fig. 1). HOP-62 cells were treated for 1, 2, 5, 24, or 72 hours with pyriplatin, cisplatin, or oxaliplatin, and then postincubated for an additional 48 or 72 hour period (as described above) in drug-free medium. Pyriplatin displayed dose- and time-dependent antiproliferative effects in HOP-62 cells, with a 72-hour exposure producing the lowest IC50 value obtained for pyriplatin (IC50 = 24.3, Fig. 3). The IC50 of pyriplatin was only

Figure 3. Antiproliferative effects of pyriplatin, cisplatin, and oxaliplatin in HOP-62 cells over time. After 1, 2, 5, 24, or 72 hours incubation, the cells were washed and postincubated in platinum-free medium for 72 hours (after 1, 2, or 5 hours) or 48 (after 24 or 72 hours). Cell viability was determined by the MTT assay. IC50 concentrations for different incubation times are shown as mean ± SD from at least 3 separate experiments.
and pyriplatin induced only a slight G2-M block in MCF7 exposure to any of the 3 compounds, although oxaliplatin block was apparent in both cell lines after a 24-hour phases (see Fig. 4A and Supplementary Fig. S2). This reversible after treatment with 35 or 70 µmol/L pyriplatin loses efficacy over time relative to cisplatin. The cells were able to repair this block at 72 hours except at high concentrations of platinum. tin, but not after treatment with 140 µmol/L oxaliplatin are in line with previously published results (22, 23). The peak of apoptosis in cells treated with pyriplatin corresponded to the maximal apoptotic response to cisplatin, suggesting that pyriplatin acts more quickly than oxaliplatin to induce cell death.

The effects of pyriplatin treatment on DNA damage response pathways related to cell cycle disruption and induction of apoptosis were explored by measuring levels of H2AX and Chk2 phosphorylation, p21 expression, and PARP-1 cleavage in HOP-62 cells by Western blotting after treatment with platinum at the IC50 concentrations. As a downstream substrate of ATM (24) and ATR (25), Chk2 is phosphorylated as part of the cellular response to cisplatin-induced DNA double-strand breaks (24). Phosphorylated H2AX (γ-H2AX) forms part of the repair complex that assembles at the site of DNA double-strand breaks and serves as a marker of DNA damage signaling (26). Cleavage of PARP-1 is observed in cells undergoing apoptosis (27) and produces 2 fragments of 89 and 24 kDa. Multiple roles of p21 and PARP-1 are also described in the context of DNA repair, regulation of cell cycle, apoptosis, and gene transcription.

As shown in Fig. 4B, increases in p21 and PARP levels as well as slight PARP cleavage were seen after exposure to pyriplatin for 24 hours. γ-H2AX and Chk2 phosphorylation at Thr68 were detected following 24 hours of treatment with all 3 platinum compounds. Two bands were observed for γ-H2AX and persisted 72 hours after removal of the platinum-containing medium, corresponding to γ-H2AX at 15 kDa and ubiquitylated γ-H2AX at 25 kDa. The band at 25 kDa is shown in Fig. 4B and both bands are presented in Supplementary Fig. S3. The high endogenous expression of phosphorylated H2AX in some tumor cell lines, including HOP-62, complicates the identification of induced DNA breaks by using the band at 15 kDa (28, 29). The 25-kDa band is a ubiquitylated form of γ-H2AX, which is induced by the recently identified E3 ubiquitin ligase RNF168 (30). The 25-kDa band appears following cisplatin treatment of HCT-116 cells grown on fibronectin (31), and it is also induced upon cisplatin, oxaliplatin, or pyriplatin treatment of HOP-62 cells. Interestingly, ubiquitin-conjugated

Pyriplatin mechanism of action

To investigate the mechanism of pyriplatin cytotoxicity, cell cycle analyses were done in HCT-116 and MCF7 cell lines. All 3 platinum compounds caused dose-dependent progressive accumulation of cells in the G2-M phases (see Fig. 4A and Supplementary Fig. S2). This block was apparent in both cell lines after a 24-hour exposure to any of the 3 compounds, although oxaliplatin and pyriplatin induced only a slight G2-M block in MCF7 cells. The cells were able to repair this block at 72 hours after washout, except at high concentrations of platinum. In the case of pyriplatin in HCT-116 cells, the effect was reversible after treatment with 35 or 70 µmol/L pyriplatin, but not after treatment with 140 µmol/L.

The implications of cell cycle disruption was explored by staining the cells with FITC-conjugated Annexin V for apoptosis detection and propidium iodide to detect necrosis before flow cytometric analysis. Annexin V binds to phosphatidylserine, a lipid that is present in the cell membrane of apoptotic cells. Cells were treated for 24 hours with IC50 platinum concentrations (Table 1). Apoptosis was determined immediately following 24-hour drug exposure (24hT) and at 24 (24hR), 48 (48hR), and 72 hours (72hR) after drug washout. At 24hT drug exposure, the percentage of apoptotic cells in pyriplatin-, cisplatin-, and oxaliplatin-treated cells was approximately twice the control values (Table 2). The percentage of apoptotic cells peaked at 24 hours after drug washout for both cisplatin- and pyriplatin-treated cells (24hR) and at 48-hour drug washout following oxaliplatin treatment (48hR). The detection of apoptosis 48 hours after the start of exposure to cisplatin or 72 hours after the beginning of incubation with oxaliplatin are in line with previously
H2AX seem to accumulate at sites of DSBs, forming nuclear foci (30, 32). Ubiquitylation of histone H2AX is also critical for recruitment of important mediators of the DNA damage response, such as the MRN complex (MRE11, RAD50, and NBS1), the p53-binding protein 1 (53BP1), and BRCA1 (32, 33). Following DNA damage, the chromatin modifier ubiquitin ligase RNF168 colocalizes with γ-H2AX at DNA lesions and increases ubiquitylation of chromatin-associated proteins at the lesion site, promoting a downstream response to the DNA damage (30). The phosphorylation of γ-H2AX and Chk2 suggests the formation of DNA double-strand breaks and indicates apoptotic DNA fragmentation or early DNA damage signaling events in response to treatment with pyriplatin. The pyriplatin-induced DNA damage–dependent ubiquitination of H2AX that is described indicates induction of a downstream DNA damage response to cell treatment with pyriplatin.

**Molecular determinants of pyriplatin activity**

Platinum levels on nuclear DNA were determined after exposure of HCT-116 cells to 10 μmol/L pyriplatin for 2, 6, 24, and 48 hours. Data were also obtained from cells exposed for 2-hour treatment followed by a 22-hour incubation in platinum-free medium. Table 3 and Supplementary Fig. S4 show platinum content on DNA extracted from HCT-116 cells after exposure to pyriplatin, analyzed by ICP-MS. After a 2-hour exposure, pyriplatin induced 3.1- and 1.3-fold fewer DNA adducts than cisplatin and oxaliplatin, respectively, indicating that pyriplatin is binding DNA quickly and supporting the results of cytotoxicity studies showing cytotoxicity of pyriplatin after only 1 or 2 hours of treatment. Differences in adduct formation increased at 24 hours and again at 48 hours, with cisplatin forming 4- and 25-fold more adducts than pyriplatin at 24 and 48 hours, respectively, and oxaliplatin forming 6- and 48-fold more adducts.

Comparing the 2-hour incubation with the 2/22 schedule, DNA platination induced by pyriplatin and oxaliplatin is decreased by 68% to 70% after the 22-hour washout period, whereas DNA platination in cisplatin-treated cells decreased to only 40%. Comparing the 2/22 schedule with the 24-hour incubation, only slightly more pyriplatin–DNA adducts are observed at the 24-hour mark, whereas larger increases in both cisplatin–DNA and oxaliplatin–DNA adducts are observed. Pyriplatin may be inactivated in the cell at a greater rate than either cisplatin or oxaliplatin, rendering less pyriplatin available for binding over time.

Overall, pyriplatin forms fewer DNA adducts than oxaliplatin and cisplatin, which may play a role in the reduced cytotoxicity of pyriplatin relative to the two established drugs. On the other hand, although pyriplatin is 66- to more than 200-fold less potent than cisplatin and oxaliplatin, respectively, at 24 hours in HCT-116 cells, the difference in DNA adduct formation is not as stark, suggesting that each adduct of pyriplatin is less toxic than adducts of either cisplatin or oxaliplatin.

It was recently shown that exposure of colon cancer cells to oxaliplatin and cisplatin induced significant changes in expression of several genes implicated in drug transport, DNA repair, and cell cycle regulation (34). We compared the genetic effects induced by pyriplatin with those of oxaliplatin and cisplatin in the HCT-116 cell line. The mRNA levels encoded by selected genes involved in nucleotide excision repair (ERCC1, XPA, and XPC), base excision repair (PARP1, XRCC1), homologous recombination (RAD50, BRCA1), mismatch repair (MSH2, MLH1), apoptosis (PUMA, CDKN1A/p21, and COX2), transport (MDR1/ABCB1, ABCB1, and GSTP1) and TOP2A, Ki67, and NEK2 were evaluated by quantitative real-time reverse transcriptase PCR (RT-PCR) after a 48 hours of exposure to pyriplatin, cisplatin, or oxaliplatin at IC50 concentrations. As shown in Fig. 5 CDKN1/p21 mRNA expression was significantly induced following 48-hour pyriplatin exposure (3-fold).

### Table 2.

Apoptosis induction according to Annexin V staining in MCF7 cells treated with pyriplatin, cisplatin, or oxaliplatin for 24 hours followed by incubation in drug-free medium for 24, 48, or 72 hours

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Pyriplatin (25 μmol/L)</th>
<th>Cisplatin (2 μmol/L)</th>
<th>Oxaliplatin (0.4 μmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>24hT</td>
<td>9.63</td>
<td>16.84</td>
<td>22.88</td>
<td>18.02</td>
</tr>
<tr>
<td>24hR</td>
<td>21.40</td>
<td>51.70</td>
<td>48.70</td>
<td>21.94</td>
</tr>
<tr>
<td>48hR</td>
<td>28.53</td>
<td>32.57</td>
<td>29.72</td>
<td>35.42</td>
</tr>
<tr>
<td>72hR</td>
<td>22.26</td>
<td>16.66</td>
<td>13.98</td>
<td>16.76</td>
</tr>
</tbody>
</table>

NOTE: All values are expressed in percentage. According to the experimental schedule, data are expressed as percentage of apoptotic cells. Corresponding data for HCT-116 cells are reported in the supporting information.

Abbreviations: T, immediately after drug exposure; R, after wash-out.

### Table 3.

DNA platinum content (ng Pt/mg DNA) of DNA extracted from HCT-116 cells after exposure to pyriplatin, analyzed by ICP-MS

<table>
<thead>
<tr>
<th></th>
<th>2-h platinum</th>
<th>24-h platinum</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>22-h washout</td>
<td>24-h platinum</td>
</tr>
<tr>
<td>Pyriplatin</td>
<td>2.44</td>
<td>0.74</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>7.49</td>
<td>4.48</td>
</tr>
<tr>
<td>Oxaliplatin</td>
<td>3.07</td>
<td>1.00</td>
</tr>
</tbody>
</table>
mRNA levels were also elevated after exposure to cisplatin and oxaliplatin, which is a well-characterized response to these drugs (35, 36). In addition, induction of p21 is associated with cisplatin resistance in human testicular cancer (37). Pyriplatin also significantly decreased ERCC1 (2-fold decrease) expression. In contrast, exposure to cisplatin slightly increased the ERCC1 mRNA level (Fig. 5), whereas exposure to oxaliplatin had little effect on ERCC1 mRNA. Pyriplatin-DNA adducts are repaired less efficiently by nucleotide excision repair than adducts of cisplatin with DNA (intrastrand d(GpG) crosslink) according to mammalian cell free extract-based assays (13). ERCC1 levels may not be elevated in response to pyriplatin treatment because pyriplatin can largely evade repair by the ERCC1-associated nucleotide excision repair pathway. The mRNA levels of other genes studied were not significantly affected (Fig. 5 and data not shown).

The potential predictive role of various genes associated with pyriplatin sensitivity or resistance was investigated by plotting mRNA expression levels of 21 genes in the panel of 10 cell lines as measured by RT-PCR against pyriplatin IC_{50} values (Fig. 6). In this case, cells were not treated with platinum before RT-PCR analysis. Although low levels of ERCC1 mRNA, but not necessarily the ERCC1 protein, are correlated with favorable responses of patients to a modified FOLFOX (biweekly oxaliplatin plus 5-FU and folinic acid) regimen (38), levels of ERCC1 mRNA were not correlated with response to pyriplatin. Cells with high levels of RAD50 mRNA are more resistant to pyriplatin ($r^2 = 0.35$), suggesting that double-strand breaks may play a role in the cellular consequences of pyriplatin-DNA lesions. Cells with high levels of mRNA coding for GSTP1 are also more resistant to pyriplatin ($r^2 = 0.38$), indicating possible cellular inactivation of pyriplatin by modification with glutathione. Genetic factors GSTP1 and RAD50 are slightly correlated with sensitivity to pyriplatin and may serve as predictive factors of response.

**Pyriplatin in combination with other anticancer agents**

The effect of administering pyriplatin before, subsequently to, and simultaneously with 5 commonly used...
The pyriplatin cytotoxicity profile is distinct from that of both cisplatin and oxaliplatin in a panel of 10 well-characterized cell lines and by the NCI single-dose screen. Although the IC_{50} values are 16 to 270 times higher for pyriplatin than for cisplatin or oxaliplatin, it is clear that the cell lines in which pyriplatin is most active (IGROV1, HOP-92, HOP-62, and COLO205) differ from those in which oxaliplatin is most active (HCT-116, OVCAR-3, HOP-92, and MCF7) or those in which cisplatin is most active (HCT-116, OVCAR-3, HOP-92, and MCF7) or those in which oxaliplatin is most active (HCT-116, OVCAR-3, HOP-92, and MCF7), as shown in the difference plots in Fig. 2 and in Table 1. As is the case for cisplatin, the first cytotoxic effects of pyriplatin are seen as soon as 1 hour after the start of treatment, at which point pyriplatin is only 15-fold less toxic than cisplatin. Pyriplatin activity at 1 and 2 hours contrasts with the relatively low activity of oxaliplatin, which must lose the oxalate before exerting cytotoxic action and is significantly less active after 1 or 2 hours than after 5 hours or longer. Pyriplatin antiproliferative activity at 1 and 2 hours is most likely related to the large number of platinum–DNA adducts observed by ICP-MS at the 2-hour time point and the relatively swift induction of cell cycle arrest and apoptosis at only 48 hours for pyriplatin, as opposed to 72 hours for oxaliplatin. Measured IC_{50} values for pyriplatin decreased from 1 to 72 hours, with efficacy peaking at an IC_{50} of 24 μmol/L after 72 hours in HOP-62 cells, the most sensitive cell line. Relative to cisplatin and based on IC_{50} values in HOP-62 cells, pyriplatin is about 15-fold less toxic than cisplatin after 1 or 2 hours. This difference

### Table 4. Effects of pyriplatin in combination with various anticancer agents in HT29 and OVCAR-3 cancer cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Schedule</th>
<th>Paclitaxel</th>
<th>Gemcitabine</th>
<th>SN38</th>
<th>Cisplatin</th>
<th>5-FU</th>
</tr>
</thead>
<tbody>
<tr>
<td>HT29</td>
<td>A</td>
<td>0.87 (0.75–0.98)</td>
<td>1.66 (0.70–2.76)</td>
<td>0.90 (0.72–1.22)</td>
<td>1.11 (0.89–1.23)</td>
<td>0.94 (0.89–1.03)</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>0.88 (0.69–1.22)</td>
<td>1.09 (0.89–1.38)</td>
<td>2.21 (1.42–3.31)</td>
<td>0.96 (0.94–1.22)</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>0.96 (0.55–1.21)</td>
<td>0.86 (0.45–1.49)</td>
<td>0.81 (0.62–1.34)</td>
<td>0.84 (0.83–0.85)</td>
<td>1.02 (0.87–1.20)</td>
</tr>
<tr>
<td>OVCAR-3</td>
<td>A</td>
<td>0.89 (0.63–0.99)</td>
<td>1.13 (0.69–1.91)</td>
<td>1.08 (1.51–0.77)</td>
<td>1.09 (0.96–1.19)</td>
<td>0.99 (0.89–1.22)</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>1.06 (0.63–1.55)</td>
<td>1.31 (0.60–7.22)</td>
<td>0.95 (0.94–0.97)</td>
<td>0.84 (0.72–1.10)</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>1.19 (0.87–1.38)</td>
<td>1.21 (1.11–1.44)</td>
<td>1.38 (1.07–1.99)</td>
<td>0.79 (0.74–0.85)</td>
<td>1.32 (1.22–1.55)</td>
</tr>
</tbody>
</table>

NOTE: Data are presented as the median combination index value and the 95% confidence interval. Schedule A, 24-hour pyriplatin followed by the 24-hour combination drug; Schedule B, 24-hour combination drug followed by the 24-hour pyriplatin; Schedule C, 24-hour simultaneous exposure.

Abbreviation: ND, not determined.

### Discussion

Platinum complexes are widely used in cancer therapy. The successful clinical applications of cisplatin, carboplatin, and oxaliplatin have inspired the synthesis and investigation of numerous platinum compounds as drug candidates. Of these compounds, those that show the most promise for clinical use have improved cytotoxicity, reduced side effects, or different mechanisms of action when compared with cisplatin, carboplatin, or oxaliplatin.

The cellular and molecular effects of the platinum derivative pyriplatin were investigated and directly compared with those of cisplatin and oxaliplatin. Because many variables such as passage number or culture conditions can affect the characteristics of a particular cell line, we ran parallel experiments with pyriplatin, cisplatin, and oxaliplatin on cells plated at the same time from a single flask. This experimental design produced data showing the unique anticancer profile of pyriplatin as compared and contrasted with those of cisplatin and oxaliplatin. The focus on comparisons between the platinum drugs freed us somewhat in the choice of cell line for various experiments, which were then made on the basis of wt p53 status (for apoptosis and cell cycle studies), sensitivity to pyriplatin (protein assays and cytotoxicity over time), and relevance to potential clinical use (combination assays).

The pyriplatin cytotoxicity profile is distinct from that of both cisplatin and oxaliplatin in a panel of 10 well-characterized cell lines and by the NCI single-dose screen. Although the IC_{50} values are 16 to 270 times higher for pyriplatin than for cisplatin or oxaliplatin, it is clear that the cell lines in which pyriplatin is most active (IGROV1, HOP-92, HOP-62, and COLO205) differ from those in which oxaliplatin is most active (HCT-116, OVCAR-3, HOP-92, and MCF7) or those in which cisplatin is most active (HCT-116, HOP-92, and OVCAR-3), as shown in the difference plots in Fig. 2 and in Table 1. As is the case for cisplatin, the first cytotoxic effects of pyriplatin are seen as soon as 1 hour after the start of treatment, at which point pyriplatin is only 15-fold less toxic than cisplatin. Pyriplatin activity at 1 and 2 hours contrasts with the relatively low activity of oxaliplatin, which must lose the oxalate before exerting cytotoxic action and is significantly less active after 1 or 2 hours than after 5 hours or longer. Pyriplatin antiproliferative activity at 1 and 2 hours is most likely related to the large number of platinum–DNA adducts observed by ICP-MS at the 2-hour time point and the relatively swift induction of cell cycle arrest and apoptosis at only 48 hours for pyriplatin, as opposed to 72 hours for oxaliplatin. Measured IC_{50} values for pyriplatin decreased from 1 to 72 hours, with efficacy peaking at an IC_{50} of 24 μmol/L after 72 hours in HOP-62 cells, the most sensitive cell line. Relative to cisplatin and based on IC_{50} values in HOP-62 cells, pyriplatin is about 15-fold less toxic than cisplatin after 1 or 2 hours. This difference...
increases at 5 and 24 hours, possibly pointing to deactivation of pyriplatin over time by cellular and molecular mechanisms. The affinity of thiols, including glutathione for platinum centers, is one probable method of deactivation of pyriplatin in the culture medium, and the single chloride on pyriplatin makes it more susceptible to complete deactivation by thiol coordination than cisplatin or oxaliplatin, which have 2 leaving groups and can still coordinate DNA if a thiol replaces 1 chloride ligand.

Cell cycle studies done in 2 cell lines with wt p53 status indicate that, similar to cisplatin and oxaliplatin, pyriplatin induces a G2-M block that suggests cell cycle delay for a DNA damage response, DNA repair and/or apoptosis. Likewise, Annexin V staining and Western blots showing activation of proteins related to the cell cycle, DNA damage response and apoptosis indicate that pyriplatin also displays an apoptotic mechanism of action, as occurs for cisplatin and oxaliplatin. The detection of Annexin V bound to early-apoptotic cellular membranes 48 hours after initiating cisplatin exposure or 72 hours after oxaliplatin exposure are in line with previously published results (22, 23). The peak of apoptosis in cells treated with pyriplatin corresponded to the maximal apoptotic response to cisplatin occurring 48 hours after beginning treatment, suggesting that pyriplatin acts more quickly than oxaliplatin to induce cell death. The induction of H2AX and Chk2 phosphorylations confirms an early DNA damage signaling response to treatment with pyriplatin or may indicate the formation of dsDNA breaks in cells because of apoptotic DNA fragmentation.

When the effects of all 3 platinum compounds are compared at the same platinum concentration (10 μmol/L), pyriplatin forms fewer DNA adducts than oxaliplatin or cisplatin after 24 hours of treatment. However, after 2 hours the levels of platinum per nucleotide are similar for pyriplatin and oxaliplatin. On the other hand, although pyriplatin is 66- to more than 200-fold less potent than cisplatin and oxaliplatin, respectively, at 24 hours in HCT-116 cells, the difference in DNA adduct formation is not as obvious, suggesting that each pyriplatin–DNA adduct is less toxic than adducts of cisplatin or oxaliplatin. Although the number of pyriplatin–DNA adducts is relatively similar to the number of cisplatin–DNA and oxaliplatin–DNA adducts, the antiproliferative effect of each adduct is significantly reduced. A promising route for development of cationic platinum anticancer compounds may involve replacing platinum with bulkier heterocyclic amines. The X-ray structure of transcribing RNA polymerase II stalled at a site-specific pyriplatin–DNA adduct (15) is valuable for the purpose of predicting which new compounds will improve the transcription inhibition aspect of pyriplatin, an activity that is crucial to DNA damage signaling and eventual triggering of apoptosis. A research program based on pyriplatin as a lead compound has already yielded compounds with significantly improved cytotoxicity compared with pyriplatin as well as cisplatin (G.Y. Park and S.J. Lippard, unpublished data).

The cellular processing of platinum drugs involves a large number of cellular events that may play a role in the ultimate efficiency of these drugs: uptake and efflux, DNA adduct formation, recognition and repair of adducts, and signal transduction of DNA damage. In terms of molecular determinants of pyriplatin sensitivity, a slight correlation of pyriplatin IC50 with GSTP1 mRNA levels in untreated cells may indicate possible cellular inactivation of pyriplatin by glutathione modification. Levels of ERCC1 mRNA in untreated cells were not correlated with pyriplatin IC50, which contrasts with the fact that low levels of ERCC1 mRNA are used to identify patients who are likely to respond well to a modified FOLFOX (biweekly oxaliplatin plus 5-FU and folinic acid) regimen (38).

Previously it was shown (25) that exposure of colon cancer cells to cisplatin and oxaliplatin can induce expression of several genes implicated in drug transport, DNA repair, and cell cycle regulation. We compared the genetic effects induced by pyriplatin with those induced by cisplatin and oxaliplatin in HCT-116 cells. Significant increases in p21 expression were seen for all 3 platinum compounds, whereas ERCC1 expression decreased in response to pyriplatin and increased in response to cisplatin exposure. Because a high amount of ERCC1 is associated with resistance to cisplatin (39–41), the decrease in ERCC1 mRNA upon treatment with pyriplatin indicates a difference in cellular resistance to the 2 compounds. The difference in ERCC1 expression, coupled with previous results showing that pyriplatin–DNA adducts evade repair by the nucleotide excision repair pathway as compared with repair of cisplatin–DNA adducts (13), supports the case that differential repair of cisplatin and pyriplatin adducts contribute to the different activity in our cell line panel.

The potential for use of pyriplatin in combination with other anticancer compounds was explored in terms of the antiproliferative potential of paclitaxel, gemcitabine, SN38, cisplatin, and 5-FU combinations. In both cell lines tested, pyriplatin was synergistic when administered simultaneously with cisplatin, as is seen when cells are treated with both cisplatin and oxaliplatin (2). Synergy implies a molecular mechanism of action distinct from that of cisplatin.

In conclusion, although pyriplatin is not likely to be developed due to its low cytotoxicity, it remains a promising lead compound for the generation of novel drug candidates with different cytotoxicity profiles from those of platinum drugs currently in use.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References

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