Say No to DMSO: Dimethylsulfoxide Inactivates Cisplatin, Carboplatin, and Other Platinum Complexes

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Abstract

The platinum drugs cisplatin, carboplatin, and oxaliplatin are highly utilized in the clinic and as a consequence are extensively studied in the laboratory setting. In this study, we examined the literature and found a significant number of studies (11%-34%) in prominent cancer journals utilizing cisplatin dissolved in DMSO. However, dissolving cisplatin in DMSO for laboratory-based studies results in ligand displacement and changes to the structure of the complex. We examined the effect of DMSO on platinum complexes, including cisplatin, carboplatin, and oxaliplatin, finding that DMSO reacted with the complexes, inhibited their cytotoxicity and their ability to initiate cell death. These results render a substantial portion of the literature on cisplatin uninterpretable. Raising awareness of this significant issue in the cancer biology community is critical, and we make recommendations on appropriate solvation of platinum drugs for research.

Introduction

Cisplatin ([cis-[PtCl2(NH3)2]], carboplatin ([Pt(O,O’-cdbea)(NH3)2], cdbea = cyclobutane-1,1-dicarboxylate), and oxaliplatin ([Pt(RR-cyclohexane-1,2-diamine)(O,O’-ethanediato)]) are used in combination with other agents to treat a range of malignancies including testicular, ovarian, head and neck, bladder, esophageal, and small cell lung cancer (1-4). With the exception of testicular cancer, acquired or intrinsic resistance generally occurs and tumors are not eliminated by treatment (3-5). Cisplatin exerts its toxicity by DNA binding and downstream apoptotic signaling, and resistance is conferred by reducing apoptotic signaling, upregulating DNA damage repair mechanisms, altering cell-cycle checkpoints, and disrupting assembly of the cytoskeleton (4, 6). The pleiotropic mechanisms underlying platinum resistance are well described, but their clinical significance is uncertain (4, 6). These factors have prompted continued experimentation with platinum drugs to understand their mechanisms of action alone and in combination with other therapeutics (7), as well as the synthesis and testing of new platinum complexes with the prospect of uncovering compounds with promising activity (8), some of which have entered clinical trials (e.g., picoplatin, satraplatin and BBR3464; ref. 9).

A critical aspect of the activity of platinum drugs in vitro and in vivo is their interaction with the solvent environment. For example, platinum drugs are activated via replacement of leaving groups with water inside the cell, a process termed aquation (10, 11). For cisplatin, this is the loss of a chloride ligand and its replacement with water, which is a more reactive leaving group. For this reason, cisplatin is formulated for clinical use in saline solution with a high chloride concentration (154 mmol/L) to prevent drug aquation before administration, stabilizing the drug and preventing side reactions before cell entry. A limiting factor for platinum drugs is their relatively low solubility, and clinical cisplatin is formulated at a concentration of 1 mg/mL (3.3 mmol/L). In laboratory and drug screening settings, stock solutions of organic-based drugs are predominately prepared in the solvent DMSO (O = S(CH3)2), which is viewed as a virtual “universal solvent” able to solubilize most small molecules at high concentrations (up to 100 mmol/L, for example; ref. 12). DMSO contains a nucleophilic sulfur, which allows it to coordinate with platinum complexes, displacing ligands and changing the structure of the complexes (13-16). This renders platinum complexes unstable in DMSO. Massart and colleagues first reported that DMSO reduced cisplatin’s cytotoxicity toward cultured thymocytes (17), and Dernel and colleagues reported that a polymer-based drug delivery system limited activity of cisplatin against stage Ib appendicular osteosarcoma in dogs (18). Little information exists on the effect of DMSO on other platinum drugs and complexes.

Yet, as discussed in this article, cisplatin and other platinum complexes are regularly dissolved in DMSO for biologic experiments, both in vitro and in vivo in experimental models, and DMSO solutions of cisplatin have been utilized in the clinical veterinary setting (19). This use may be due to the lack of a comprehensive understanding of the effect of DMSO on the
activity of platinum complexes in the cancer biology community. Irrespective of the reason, the implications for published studies on cisplatin’s mechanism using DMSO solutions are profound.

We sought to examine the range of solvent systems utilized for platinum drugs in peer-reviewed research, and the effects of DMSO on platinum drug activity. A number of journals were reviewed to identify and assess only those papers reporting in vitro data. These papers were then assessed for the solvent or solution used for dissolving cisplatin, and these were determined and recorded; similarly, it was noted if the solvent system was not disclosed. In the majority of cases, if the solvent used was not mentioned in the Materials and Methods section, it was not explicitly disclosed anywhere in the manuscript in a manner that allowed unambiguous determination of the experimental strategy used—this was recorded as “Not reported.” Thirty-five manuscripts were assessed for each journal, with the exception of the JPET, where only 28 relevant articles were identified.

Materials and Methods

Literature review

Five journals that regularly publish studies on small-molecule therapeutics and their mechanism were examined: Cancer Research (http://cancerres.aacrjournals.org), Molecular Cancer Therapeutics (http://mct.aacrjournals.org), Molecular Pharmacology (http://molpharm.aspetjournals.org), Journal of Pharmacology and Experimental Therapeutics (JPET; http://jpet.aspetjournals.org), and the Public Library of Science (PLoS; http://www.plos.org) journals. In each case, the word “cisplatin” was entered as a search term on the respective journal website search engine, restricted to the term appearing in the title or abstract of articles. Manuscripts were then individually reviewed to identify and assess only those papers reporting in vitro data. These papers were then assessed for the solvent or solution used for dissolving cisplatin, and these were determined and recorded; similarly, it was noted if the solvent system was not disclosed. In the majority of cases, if the solvent used was not mentioned in the Materials and Methods section, it was not explicitly disclosed anywhere in the manuscript in a manner that allowed unambiguous determination of the experimental strategy used—this was recorded as “Not reported.” Thirty-five manuscripts were assessed for each journal, with the exception of the JPET, where only 28 relevant articles were identified.

Materials

Cisplatin, carboplatin, DMSO, dimethylformamide (DMF), dimethylacetamide (DMA), formamide, acetamide, and cremophor EL were purchased from Sigma-Aldrich. Oxaliplatin was purchased from LC Laboratories. Satraplatin was purchased from Sequoia Research Products. [PtCl2(en)] and trans-platin were purchased from Alpha Aesar. Clinical formulations of cisplatin, carboplatin, and oxaliplatin were kindly provided by Dr. Tito Fojo, Clinical Center, National Cancer Institute (Bethesda, MD). Compounds were assessed in various solvent systems as described below.

Cell lines and cell culture

This study used the DLD1 human colorectal carcinoma cells, parental human cervical carcinoma cell line KB-3-1 (a subline of HeLa), and its cisplatin-resistant subline KB-CP.5. KB-CP.5 cells were originally selected in a single step in 0.5 μg cisplatin/mL (1.6 μmol/L) in our laboratory, as described previously (20, 21). KB lines were originally generated in the laboratory of M.M. Gottesman. DLD-1 cells were provided by the National Cancer Institute (part of the NCI-60 collection). All cell lines were thawed immediately before experimentation, and cell lines were characterized by NCI using short tandem repeat profiling. The cisplatin stock solution used for culturing CP.5 cells was prepared in PBS. The cisplatin-resistant cells were maintained in the presence of cisplatin, which was removed from growth medium 3 days before all experiments. All cell lines were grown as monolayer cultures at 37°C in 5% CO2, using either DMEM (KB cells) or RPMI (DLD1 cells) with 4.5 g/L glucose (both from Invitrogen), supplemented with l-glutamine, penicillin, streptomycin, and 10% FBS (BioWhittaker). Resistance of CP.5 cells to cisplatin was confirmed on a regular (at least monthly) basis, using cell viability assays as described herein.

Cytotoxicity and cell growth

Cell survival was measured by the MTT (Invitrogen) assay. Cells were seeded at a density of 5,000 cells per well in 96-well plates and incubated at 37°C in humidified 5% CO2 for 24 hours. Serially diluted (1:3, RPMI or DMEM used as diluent, chloride concentration ~120 mmol/L) compound was added to give the intended final concentrations. Solvent tolerance testing up to 0.5% under identical conditions confirmed growth of all cell lines was unaffected. Cells were then incubated an additional 72 hours, and the MTT assay was performed according to the manufacturer’s instructions (Molecular Probes). Absorbance values were determined at 570 nm on a Spectra Max 250 spectrophotometer (Molecular Devices). All MTT assays were performed in triplicate. The IC50 values were defined as the drug concentrations required to reduce cellular proliferation to 50% of the untreated control well. We used Prism 6 (GraphPad Software) software for graphs and statistics. All data are expressed as mean ± SD. For bright-field imaging, DLD-1 cells were seeded at a density of 3 × 103 cells per well in 6-well plates and incubated at 37°C in humidified 5% CO2 for 24 hours. Drug was added directly to each well and cells were incubated for another 72 hours. Media were aspirated and cells were rinsed with PBS before imaging.

H2AX immunofluorescence staining

Fixed DLD-1 cells were stained with Phospho-Histone H2AX (Se139; 20E3) Rabbit mAb (Alexa Fluor 488 Conjugate; all components from Cell Signaling Technology) following the manufacturer’s instructions and as previously described (22). Briefly, cells were seeded at 5 × 104 cells per chamber in 8-chamber coverslips and incubated for 24 hours. Cells were then incubated with Pt complexes for 24 hours before being washed with PBS, fixed in 4% paraformaldehyde, then blocked with blocking buffer (PBS/5% normal horse serum/0.3% Triton X-100) for one hour. Diluted antibody (1:50 in PBS/1% BSA/0.3% Triton X-100) was then applied, and cells were incubated overnight at 4°C. Cells were then washed and stained with 4’, 6-diamidino-2-phenylindole nuclear DNA stain, and imaged on a Zeiss LSM 710 NLO confocal microscope.
Preparation of cell lysates, quantification of protein, and Western blot analysis

After 48-hour drug treatments, both floating and adherent DLD-1 colorectal cancer cells were washed with PBS and lysed together in NP-40 lysis buffer (50 mmol/L Tris/HCl, pH 8.0, 150 mmol/L NaCl, 1% NP-40), and supplemented with complete protease inhibitor cocktail tablets (all from Sigma-Aldrich) and PhosStop phosphatase inhibitor tablets (Roche)] and centrifuged to remove insoluble material. 2X Laemmli sample buffer (Sigma-Aldrich) was added to equivalent amounts of cellular lysates (40 μg), which were then resolved by SDS-PAGE on 4% to 20% gradient gels and transferred onto Immobilon PVDF membrane (Millipore). Membranes were blocked in 5% (w/v) nonfat dried skimmed milk powder in TBS-Tween 20 (TBST; 20 mmol/L Tris/HCl, pH 7.6, 137 mmol/L NaCl, and 0.2% Tween 20; Sigma-Aldrich; blocking buffer) and probed with appropriate primary antibodies overnight followed by anti-mouse or anti-rabbit IgG horseradish peroxidase (HRP)-conjugated secondary antibody (Cell Signaling Technology) for one hour. Membranes were washed in TBST and incubated in either Western Blotting Luminol Reagent (Santa Cruz Biotechnology) or Immobilon Western Chemiluminescent HRP Substrate (Millipore) and the signal developed on HyBlot ES film (Denville Scientific). All primary antibodies were from Cell Signaling Technology: PARP (#9542), cleaved caspase-3 (9664), phospho-H2AX (#9718), and β-actin (#3700).

Mass spectrometry

Accurate mass data were obtained on a Waters Premiere LCT time-of-flight mass spectrometer operated in the positive flight mass spectrometer operated in the positive mode at 10 K resolution. The high-performance liquid chromatography solvent pump was operated at 200 μL/minute and the solvent composition was 50:40:10 water:methanol:acetonitrile. All solvents were LC/MS grade and were purified to remove insoluble material. 2X Laemmli sample buffer (Sigma-Aldrich) was added to equivalent amounts of cellular lysates (40 μg), which were then resolved by SDS-PAGE on 4% to 20% gradient gels and transferred onto Immobilon PVDF membrane (Millipore). Membranes were blocked in 5% (w/v) nonfat dried skimmed milk powder in TBS-Tween 20 (TBST; 20 mmol/L Tris/HCl, pH 7.6, 137 mmol/L NaCl, and 0.2% Tween 20; Sigma-Aldrich; blocking buffer) and probed with appropriate primary antibodies overnight followed by anti-mouse or anti-rabbit IgG horseradish peroxidase (HRP)-conjugated secondary antibody (Cell Signaling Technology) for one hour. Membranes were washed in TBST and incubated in either Western Blotting Luminol Reagent (Santa Cruz Biotechnology) or Immobilon Western Chemiluminescent HRP Substrate (Millipore) and the signal developed on HyBlot ES film (Denville Scientific). All primary antibodies were from Cell Signaling Technology: PARP (#9542), cleaved caspase-3 (9664), phospho-H2AX (#9718), and β-actin (#3700).

### Table 1. Reported solvent or formulation of cisplatin used for in vitro studies in selected peer-reviewed scientific journals, stated as percentage of total (with number of reports in parentheses)

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Cancer Research</th>
<th>Molecular Cancer Therapeutics</th>
<th>PLoS</th>
<th>Molecular Pharmacology</th>
<th>JPET</th>
</tr>
</thead>
<tbody>
<tr>
<td>Not reported</td>
<td>43 (15)</td>
<td>37 (13)</td>
<td>43 (15)</td>
<td>26 (9)</td>
<td>50 (14)</td>
</tr>
<tr>
<td>DMSO</td>
<td>34 (12)</td>
<td>20 (7)</td>
<td>17 (8)</td>
<td>11 (4)</td>
<td>14 (4)</td>
</tr>
<tr>
<td>Water</td>
<td>—</td>
<td>3 (1)</td>
<td>11 (4)</td>
<td>3 (1)</td>
<td>—</td>
</tr>
<tr>
<td>Buffer</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>3 (1)</td>
<td>—</td>
</tr>
<tr>
<td>DMF</td>
<td>—</td>
<td>3 (1)</td>
<td>3 (1)</td>
<td>3 (1)</td>
<td>—</td>
</tr>
<tr>
<td>Cell media</td>
<td>—</td>
<td>—</td>
<td>3 (1)</td>
<td>6 (2)</td>
<td>—</td>
</tr>
<tr>
<td>Clinical</td>
<td>14 (5)</td>
<td>23 (8)</td>
<td>17 (6)</td>
<td>14 (5)</td>
<td>11 (3)</td>
</tr>
<tr>
<td>Saline</td>
<td>3 (1)</td>
<td>11 (4)</td>
<td>—</td>
<td>20 (7)</td>
<td>14 (4)</td>
</tr>
<tr>
<td>PBS</td>
<td>3 (1)</td>
<td>3 (1)</td>
<td>3 (1)</td>
<td>6 (2)</td>
<td>4 (1)</td>
</tr>
</tbody>
</table>

Results

Solvent utilization in in vitro studies of cisplatin

Five journals that regularly publish studies on small-molecule therapeutics and their mechanism were examined for reports pertaining to the activity and mechanism of cisplatin: Cancer Research, Molecular Cancer Therapeutics, Molecular Pharmacology, JPET, and the PLoS journals. Thirty-five papers reporting in vitro data were assessed for each journal (28 for JPET), and the solvent used for dissolving cisplatin in each case was determined and recorded. Solvent systems utilized were simple salt buffer, cell culture media, clinical formulation (often indicated as sourced from a research hospital’s in-house pharmacy), DMF, DMSO, saline, PBS, and water (Table 1). Where the solvent used was not identified in the Materials and Methods, this was recorded as “Not reported,” with a very small number of exceptions where the solvent was categorically identified elsewhere in the manuscript. Manuscripts assessed are listed in Supplementary Tables S1–S5, with the solvent used assigned for each. Although only in vitro data were assessed, it should be noted that cisplatin dissolved in DMSO has also been used for in vivo studies (23).

As shown in Table 1, for each journal the majority of papers did not report the solvent used (26%–50% of papers). Following this, a DMSO solution or clinical formulation was the second most used solvent. DMSO was the most heavily utilized in-lab solvent. For example, DMSO was used in 34% of Cancer Research and 20% of Molecular Cancer Therapeutics articles that were examined.

In the clinic, cisplatin is typically provided as a lyophilized powder in a vial containing 50 mg cisplatin, 450 mg NaCl, and 500 mg mannitol. When dissolved in 50 mL of water, this results in a 1 mg/mL solution (3.3 mmol/L) of cisplatin dissolved in 150 mmol/L saline. The saline prevents aquation of the complex in solution before administration to the patient (24). Clinical formulation was used for in vitro experiments in 11% to 23% of papers. However, in some studies, cisplatin was dissolved in PBS (chloride concentration 140 mmol/L—phosphate has been reported to not appreciably alter cisplatin aquation; ref. 25) or saline (chloride concentration 154 mmol/L). When these chloride-containing solutions are
considered along with clinical formulation, their use was predominant, accounting for 20% to 40% of reports (Cancer Research, 20%; Molecular Cancer Therapeutics, 37%; PLoS, 20%; Molecular Pharmacology, 40%; JPET, 29%).

Other solvents were used in only a small number of cases. It is possible that DMF (on one occasion) was used as an alternative organic solvent as it is able to solubilize cisplatin and does not contain a sulfur group. Similarly, water can solubilize cisplatin. However, in the absence of chloride, cisplatin and other complexes with chlorido leaving groups become aquated, producing a mixture of species with increased reactivity and altered cytotoxicity (26, 27).

**In vitro assessment of effect of DMSO on platinum complexes**

To assess the effect of DMSO on the activity of platinum drugs and complexes, the activity of the drugs cisplatin, carboplatin, oxaliplatin, and satraplatin, and the experimental complexes transplatin (trans-[PtCl2(NH3)2]) and [PtCl2(en)] (structures shown in Fig. 1) were assessed. Satraplatin is a stable, lipophilic platinum(IV) complex designed to be orally available (28), and although it entered clinical trials against adult and pediatric malignancies (most prominently prostate cancer in combination with prednisone), it has not been approved for use by the FDA (29). Transplatin is the "inactive" geometric isomer of cisplatin (30), and [PtCl2(en)] is regularly used in experimental papers, and like cisplatin contains two cis-chlorido leaving groups (8).

The activity of clinical formulations of the drugs cisplatin (3.3 mmol/L in 0.9% saline with 10 mg/mL mannitol), carboplatin (27 mmol/L in 5% glucose solution; ref. 31), and oxaliplatin (12.6 mmol/L in 5% glucose solution; 32) was compared with analogous solvent preparations to the clinical formulation (i.e., saline for cisplatin, water for carboplatin, and oxaliplatin), and solutions of each drug in DMSO (at 20 mmol/L stock solution). Satraplatin is only available as capsules for oral use, and aqueous solubility is poor. As such, a 4:1 water:DMSO solution was prepared to enable assessment of the effect of water on activity. No clinical formulation exists for transplatin or [PtCl2(en)], but given that both contain chlorido leaving groups (like cisplatin), both complexes were dissolved in saline. The responses of DLD-1 and KB-3-1 parental cells, and the cisplatin-resistant subline of KB-3-1, termed KB-CP.5, were evaluated to examine whether sensitivity to each platinum agent, and cross-resistance in cisplatin-resistant cells, were affected by DMSO (Table 2; Fig. 2). The CP.5 cells demonstrated cross-resistance to all Pt complexes (>10-fold) with the exception of transplatin. The clinical formulations of cisplatin, carboplatin, and oxaliplatin showed equivalent cytotoxicity to their respective saline/aqueous solutions.

DMSO had a significant effect on the cytotoxicity of all complexes with monodentate (singly coordinated) ligands, diminishing the cytotoxicity of cisplatin, carboplatin, and [PtCl2(en)], and to a lesser effect, transplatin. These effects could be easily observed by bright-field microscopy (for cisplatin, carboplatin, and oxaliplatin; Fig. 2A–C), and by dose–response cell killing (Fig. 2D–F). The strongest effects were on cisplatin, with a greater than 40-fold loss of cytotoxicity against both DLD-1 and KB-3-1 cells (Table 2), and [PtCl2(en)], with a greater than 30-fold loss of cytotoxicity, both complexes contain the "classic" cis-dichlorido leaving group arrangement. For example, cisplatin dissolved in saline demonstrated an IC50 of 3.8 ± 0.6 μmol/L, whereas its IC50 when dissolved in DMSO was 178 ± 9.2 μmol/L. Carboplatin demonstrated reduced cytotoxicity compared with cisplatin (34.2 ± 3.4 μmol/L against KB-3-1 cells), consistent with its diminished reactivity (32), but DMSO reduced its cytotoxicity further (3- to 10-fold, 243 ± 11.2 μmol/L against KB-3-1 cells). In contrast, DMSO seemed to
have a slight potentiating effect on oxaliplatin, with both KB-3-1 (aqueous = 3.0 ± 0.5 μmol/L, DMSO = 0.7 ± 0.3 μmol/L) and DLD-1 (aqueous = 3.9 ± 0.6 μmol/L, DMSO = 1.6 ± 0.2 μmol/L) cells being slightly more sensitive to the DMSO-formulated compound. DMSO had no effect on satraplatin cytotoxicity (e.g., IC_{50} for the clinical formulation against DLD-1 = 1.5 ± 0.2 μmol/L, in DMSO = 1.6 ± 0.2 μmol/L).

Although DMSO disrupted the activity of cisplatin, cisplatin-resistant cells retained cross-resistance toward the DMSO-formulated drug. CP.5 cells demonstrated 10-fold resistance to cisplatin in saline compared to the clinical formulation and DMSO. Table 2 summarizes the IC_{50} values for platinum complexes against parental (DLD-1 and KB-3-1) and cisplatin-resistant (KB-CP.5) cell lines.

### Table 2. Cytotoxicity (IC_{50}, μmol/L) of platinum complexes against parental (DLD-1 and KB-3-1) and cisplatin-resistant (KB-CP.5) cell lines

<table>
<thead>
<tr>
<th></th>
<th>DLD-1</th>
<th>KB 3-1</th>
<th>KB CP.5</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cisplatin</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clinical</td>
<td>3.3 ± 0.5</td>
<td>1.1 ± 1.0</td>
<td>33.2 ± 8.5</td>
</tr>
<tr>
<td>Saline</td>
<td>3.8 ± 0.6</td>
<td>1.9 ± 0.4</td>
<td>34.3 ± 1.6</td>
</tr>
<tr>
<td>DMSO</td>
<td>178 ± 9.2</td>
<td>48.1 ± 3.7</td>
<td>376 ± 283</td>
</tr>
<tr>
<td>Saline + DMSO</td>
<td>3.4 ± 0.3</td>
<td>1.1 ± 0.2</td>
<td>ND</td>
</tr>
<tr>
<td><strong>Carboplatin</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clinical</td>
<td>68.8 ± 22.6</td>
<td>41.4 ± 10.8</td>
<td>460.7 ± 54.4</td>
</tr>
<tr>
<td>Aqueous</td>
<td>78.4 ± 5.1</td>
<td>34.2 ± 3.4</td>
<td>1,014 ± 10.4</td>
</tr>
<tr>
<td>DMSO</td>
<td>573 ± 38.0</td>
<td>243 ± 11.2</td>
<td>118 ± 74.3</td>
</tr>
<tr>
<td><strong>Oxaliplatin</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clinical</td>
<td>1.5 ± 0.2</td>
<td>1.14 ± 0.05</td>
<td>ND</td>
</tr>
<tr>
<td>Aqueous</td>
<td>3.9 ± 0.6</td>
<td>3.0 ± 0.5</td>
<td>74.3 ± 34.1</td>
</tr>
<tr>
<td>DMSO</td>
<td>1.6 ± 0.2</td>
<td>0.7 ± 0.3</td>
<td>84.0 ± 72.7</td>
</tr>
<tr>
<td><strong>Satraplatin</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Water; DMSO (4:1)</td>
<td>3.8 ± 0.4</td>
<td>2.4 ± 0.3</td>
<td>37.3 ± 3.2</td>
</tr>
<tr>
<td>DMSO</td>
<td>2.9 ± 0.6</td>
<td>2.5 ± 0.3</td>
<td>8.6 ± 1.6</td>
</tr>
<tr>
<td><strong>Transplatin</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline</td>
<td>335 ± 165</td>
<td>&gt;500</td>
<td>716 ± 287</td>
</tr>
<tr>
<td>DMSO</td>
<td>587 ± 31.8</td>
<td>433 ± 30.6</td>
<td>134 ± 80.3</td>
</tr>
<tr>
<td>[PtCl2(en)]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline</td>
<td>2.7 ± 1.9</td>
<td>11.4 ± 1.5</td>
<td>569 ± 113</td>
</tr>
<tr>
<td>DMSO</td>
<td>87.1 ± 36.3</td>
<td>135 ± 7.1</td>
<td>256 ± 92.2</td>
</tr>
</tbody>
</table>
cisplatin (CP.5 IC\textsubscript{50} = 34.3 ± 1.6 µmol/L vs. KB 3-1 IC\textsubscript{50} = 1.9 ± 1.6 µmol/L), and cross-resistance to aqueous carboplatin (32-fold), oxaliplatin (25-fold), and satraplatin (4-fold). CP.5 cells demonstrated 8-fold resistance to cisplatin formulated in DMSO, and cross-resistance to oxaliplatin and satraplatin was maintained. Both carboplatin and transplatin dissolved in DMSO seem to have greater potency against CP.5 cells than when formulated in aqueous media (water and saline, respectively). By way of example, the IC\textsubscript{50} of transplatin dissolved in DMSO was very high (433 ± 30.6 µmol/L) but lower against CP.5 cells (134 ± 80.3 µmol/L). This differential sensitivity suggests that reaction with DMSO produces a complex (vide infra) with greater activity than cisplatin. The poor cytotoxicity and in vivo activity of transplatin are generally ascribed to its greater reactivity, but it is known that trans Pt complexes with bulkier ligands have reduced reactivity, and with this improved stability comes biologic activity (33).

To assess the effect of DMSO on the DNA damage and cell killing produced by Pt drugs (9), we examined phosphorylation of the histone H2A family member H2AX. H2AX becomes phosphorylated at serine 139 (γH2AX) in response to double-stranded breaks elicited by DNA damage, recruiting repair factors to sites of damage, and is also phosphorylated as a result of apoptosis induced by DNA damage (34). In this way, H2AX has been shown to be phosphorylated in cells exposed to cisplatin, carboplatin, and oxaliplatin (35, 36). Cells were treated with cisplatin, carboplatin, and oxaliplatin formulated in aqueous solution (saline or water) or DMSO, and γH2AX foci were evaluated by immunofluorescence microscopy after 24 hours (Fig. 3A). Cells treated with cisplatin (5 µmol/L) in saline or carboplatin (5 µmol/L) in water clearly elicited a DNA damage response, whereas cells treated with the compounds dissolved in DMSO resulted in a striking diminution of H2AX staining. Despite being approximately equitoxic with cisplatin, oxaliplatin resulted only in low-level staining. Satraplatin in water and DMSO showed equivalent γH2AX staining, whereas transplatin and [PtCl\textsubscript{2}(en)] dissolved in DMSO elicited weaker γH2AX staining than their saline-dissolved comparison (not shown).

Immunoblot analysis of γH2AX supported immunofluorescence microscopy staining (Fig. 3B). To directly study whether DMSO affected cell death, we incubated DLD-1 cells with cisplatin (5 µmol/L), carboplatin (50 µmol/L), and oxaliplatin (5 µmol/L) in either DMSO or water, and whole-cell lysates were collected after 48 hours for Western analysis (Fig. 3B). Induction of cleaved PARP and cleaved caspase-3 was caused by cisplatin, carboplatin, and oxaliplatin, consistent with their role in platinum-mediated apoptosis (37). These events were prevented by formulation of the three drugs in DMSO.

**Modification of platinum complexes by DMSO and other solvents**

To assess the nature of the interactions between cisplatin and DMSO, we compared electrospray ionization mass spectra in positive mode of all six complexes in aqueous and DMSO solutions (Supplementary Table S7, only peaks related to DMSO adducts are shown). Previous chemical analysis has demonstrated that cisplatin produces multiple species upon dissolution in DMSO, by replacement of a Cl\textsuperscript- with DMSO (m/z = 343), and replacement of a Cl\textsuperscript- and an NH\textsubscript{3} ligand by two DMSO molecules (m/z = 404; refs. 38, 39). We also observed a higher molecular weight species (m/z = 665.9) corresponding to a bridged form of DMSO-substituted cisplatin, µNH\textsubscript{3}–[Pt(NH\textsubscript{3})(Cl)(DMSO)]\textsuperscript{2+} (Supplementary Table S7) not previously reported. These are shown schematically in Supplementary Fig. S3. Carboplatin, transplatin, and [PtCl\textsubscript{2}(en)] produced reaction products with DMSO, all involving replacement of a Cl\textsuperscript- ligand (Supplementary Table S7). Carboplatin and oxaliplatin also produced self-association multimers, as previously reported (32). Mass spectra of satraplatin in DMSO did not produce any peaks corresponding to interaction with DMSO, consistent with the inertness of platinum(IV) complexes (40). Oxaliplatin did not produce any observable peaks corresponding to reaction with DMSO.

**The effect of DMSO on aqueous cisplatin**

The evidence thus far indicates that most Pt complexes (including cisplatin and carboplatin) are deactivated upon dissolution in DMSO stock solutions, and aqueous-based solutions are essential for biologic studies. However, cisplatin is usually used in combination in the clinic, and synergy of Pt drugs with experimental and established therapeutics are regularly assessed (41). As organic-based therapeutics are usually dissolved in DMSO, we wondered whether cisplatin in aqueous solution or growth media would be deactivated by a small component of DMSO if it is introduced into the same solution.

We first examined whether DMSO would inhibit cisplatin’s cytotoxicity. To examine this, cisplatin dissolved in saline was diluted into growth medium, followed by 30 µL/mL DMSO (3%, effective concentration 384 mmol/L in 154 mmol/L saline), followed by serial dilution and dosing to cells. DMSO did not have any effect on cisplatin’s cytotoxicity on KB-3-1 or DLD-1 cells (e.g., DLD-1 cisplatin saline IC\textsubscript{50} = 3.3 ± 0.5 µmol/L, cisplatin saline and DMSO IC\textsubscript{50} = 3.4 ± 0.3 µmol/L; Table 2). Despite the lack of biologic effect, mass spectrometry of saline solutions of cisplatin and cisplatin in the presence of 3% DMSO revealed a peak corresponding to the replacement of one chloride ligand with DMSO (Table 3). These data suggest that DMSO introduced into combination studies with cisplatin does not affect its activity. However, researchers should be aware that DMSO does interact with cisplatin even in dilute environments.

**Assessment of alternative solvent systems for platinum complexes**

One limitation of aqueous formulations of cisplatin is its maximal solubility of just more than 3 mmol/L, whereas DMSO solutions can be prepared of more than 10 mmol/L. We examined whether other organic solvents had a similar effect on cisplatin. Stock solutions of cisplatin were prepared in amenable solvents: DMF, DMA, formamide, acetamide, cremophor EL (all 10 mmol/L stock solutions), and water (3.3 mmol/L; Table 3). Cremophor EL and acetonitrile did not fully dissolve cisplatin, but were tested as fine ultrasonicated suspensions. None of the solvents profoundly affected
cisplatin’s cytotoxicity against DLD-1 cells, and although not statistically significant, DMF resulted in the greatest decrease in cytotoxicity of cisplatin ($IC_{50} = 5.0 \pm 1.3 \mumol/L$ compared with $3.8 \pm 0.6 \mumol/L$ for cisplatin in saline).

However, each organic solvent alone (at a high concentration of 3% in media, consistent with the lowest dilution of 10 mmol/L cisplatin in media required to perform a dose–response) demonstrated cytotoxicity toward DLD-1 cells (Table 3). The exception was acetonitrile, with the limitation that acetonitrile was one of the two solvents unable to fully dissolve cisplatin. The most toxic solvent was cremophor EL, a polyethoxylated castor oil used as the excipient for intravenous administration of poorly soluble drugs such as paclitaxel (42). Although 3% solvent is a high concentration, the $IC_{50}$ concentration for cisplatin ($\sim 1–3 \mumol/L$) in our toxicity assays occurs at a drug dilution that is in the presence of 0.2% to 0.05% solvent, a concentration at which no toxicity toward DLD-1 cells was observed.

**Discussion**

We have demonstrated here the profound effects of DMSO on platinum drugs and complexes that contain monodentate ligands. Drug cytotoxicity is diminished, as evidenced by cell growth and DNA damage response. The replacement of ligands on the platinum complexes could be observed by mass
spectrum. This study was prompted by a recognition of the high rate of use of DMSO with cisplatin in the literature, and partly by limitations encountered in developing a high-throughput screen using cisplatin—drugs are transferred by pins and surface tension has been optimized to transfer a fixed volume of DMSO. Using an alternative solvent would not be possible without significant optimization. A significant number of reports in all five journals examined utilized DMSO-dissolved cisplatin, raising questions about how to interpret the results of a large number of previous studies. Our findings also reinforce the current focus on the challenge of reproducing scientific literature (43).

These findings extend to most platinum complexes. Thousands of platinum complexes (and other metal-based complexes) have been synthesized and tested in vitro in an effort to identify complexes with greater potency than cisplatin, a different mechanism of action from cisplatin, and/or an ability to overcome cross-resistance in cisplatin-resistant cells (8, 6). Given the general lack of information about how to formulate experimental agents and the likelihood that complexes will dissolve in DMSO, most compounds for testing against cell lines are also regularly dissolved in DMSO. For example, of the 20 most recent papers reporting new chemical entities based on platinum in the Journal of Medicinal Chemistry, seven (35%) used DMF to dissolve complexes for biologic testing, five (25%) did not state the solvent used, and three (15%) used DMSO (Supplementary Table S6). Alternative solvents such as DMF have been reported not to cause cytotoxicity below 0.5% (44), and solutions of cisplatin dissolved in DMF retain activity (45). However, although DMF is recognized as cytotoxic toward cells (46), there seems to be no comprehensive assessment of DMF's utility as a drug (or platinum complex) solvent. It is critical then, that an understanding of drug interaction with solvent, and solubility in solvent, be ascertained for experimental metal-based therapeutics.

The problem of cisplatin deactivation by DMSO was first reported over 20 years ago, and the underlying chemistry had been generally well defined (13, 16). Jones and colleagues demonstrated that coadministration of cisplatin with DMSO in Sprague–Dawley rats reduced nephrotoxicity (47), and Massari and colleagues observed that DMSO inhibited cisplatin cytotoxicity toward cultured thyrocytes while investigating whether DMSO could act as an antidote for cisplatin-induced toxicities, based on previously reported protective effects of DMSO (17). This work was initiated in the context of the desire to utilize known thiols as reactive "protective agents" to temper the side effects of cisplatin (48). Fischer and colleagues later demonstrated that although the neurotoxic side effects of cisplatin are tempered by DMSO, loss of cisplatin's cytotoxicity offset any potential benefit (38).

It is not clear whether the cytotoxicity observed in DMSO-formulated cisplatin is due to the mixture of chemical entities present in solution, or the unreacted portion of cisplatin remaining in equilibrium. Given varying cross-reaction in solutions utilized in laboratories, it would be expected to be difficult to replicate results with saline, PBS, or clinical formulations of platinum complexes. For example, a crystallographic study of cisplatin and carboplatin binding to histidines of hen egg-white lysozyme found one platinum bound to His15 when cisplatin was dissolved in aqueous medium. When dissolved in DMSO, two platinum atoms bound to His15, leading to the conclusion that DMSO could facilitate platinum-protein binding (49). Uribe and colleagues reported increased sensory hair cell death in zebrafish cotreated with cisplatin and DMSO but not other solvents, which was ascribed to the cell permeabilizing effects of DMSO, and warned that DMSO could produce false-positive effects in other drug screens due to DMSO's biologic activity (50).

The effects of DMSO on platinum drug activity in vitro can be profound. We believe the practice of dissolving platinum drugs in DMSO must cease, and if the solvent is to be utilized, new platinum agents must demonstrate a lack of interaction with DMSO. For experimentation, cisplatin should be prepared in a saline-based solvent (3 mmol/L), and carboplatin (27 mmol/L) and oxaliplatin (12.6 mmol/L) in water. When available, clinical formulation can substitute. It is the hope of the authors that this report will raise awareness of this significant issue in the cancer biology community, introduce caution when interpreting results of published studies utilizing DMSO, inform future experimental design, and guide editorial guidelines for mechanistic and experimental therapeutic research.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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**Table 3. Cytotoxicity (IC$_{50}$, µmol/L) of cisplatin, dissolved or suspended in solvents, against DLD-1 cells**

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<tr>
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<th>3% Solvent Viability (%)</th>
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<td>3.3 ± 0.5</td>
<td>Y</td>
<td>100</td>
</tr>
<tr>
<td>Clinical</td>
<td>3.8 ± 0.6</td>
<td>Y</td>
<td>96 ± 10</td>
</tr>
<tr>
<td>Saline</td>
<td>177.6 ± 9.2</td>
<td>Y</td>
<td>90 ± 5</td>
</tr>
<tr>
<td>DMSO</td>
<td>5.0 ± 1.3</td>
<td>Y</td>
<td>79 ± 5</td>
</tr>
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<td>DMF</td>
<td>3.5 ± 0.8</td>
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Fluorescence spectroscopy. This study was prompted by a recognition of the high rate of use of DMSO with cisplatin in the literature, and partly by limitations encountered in developing a high-throughput screen using cisplatin—drugs are transferred by pins and surface tension has been optimized to transfer a fixed volume of DMSO. Using an alternative solvent would not be possible without significant optimization. A significant number of reports in all five journals examined utilized DMSO-dissolved cisplatin, raising questions about how to interpret the results of a large number of previous studies. Our findings also reinforce the current focus on the challenge of reproducing scientific literature (43).

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Grant Support
This work was supported by the Intramural Research Program of the NIH, National Cancer Institute. K.-E. Chang is an NIH Medical Research Scholars Program scholar, a public-private partnership supported jointly by the NIH and generous contributions to the Foundation for the NIH from Pfizer Inc, The Doris Duke Charitable Foundation, The Alexandria Real Estate Equities, Inc, Mr. and Mrs. Joel S. Marcus, and the Howard Hughes Medical Institute, as well as other private donors.

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Received February 21, 2014; revised April 16, 2014; accepted April 16, 2014; published OnlineFirst May 8, 2014.

References
Say No to DMSO: Dimethylsulfoxide Inactivates Cisplatin, Carboplatin, and Other Platinum Complexes

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Cancer Res Published OnlineFirst May 8, 2014.

Updated version
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