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

Matthew D. Hall1, Katherine A. Telma1, Ki-Eun Chang1, Tobie D. Lee1, James P. Madigan1, John R. Lloyd2, Ian S. Goldlust3, James D. Hoeschele4, Michael M. Gottesman1

1 Laboratory of Cell Biology, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892
2 Advanced Mass Spectrometry Facility, National Institute of Diabetes & Digestive & Kidney Diseases, National Institutes of Health, Bethesda, MD 20892
3 Division of Preclinical Innovation, National Institutes of Health Chemical Genomics Center, National Center for Advancing Translational Sciences, National Institutes of Health, Rockville, MD 20850
4 Department of Chemistry, Eastern Michigan University, Ypsilanti, MI 48197

Corresponding author:
Michael M. Gottesman, Laboratory of Cell Biology, National Cancer Institute, NIH, 37 Convent Drive, Rm. 2108, Bethesda, MD 20892
Email: gottesmm@mail.nih.gov, Phone: 301 496 1921, Fax: 301 402 0450

Running title: DMSO inactivates platinum drugs

Key words: cisplatin, formulation, mechanism of action

Conflicts of interest: None declared
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 dimethylsulfoxide (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’-cdbca)(NH₃)₂], cbdca = cyclobutane-1,1-dicarboxylate) and oxaliplatin ([Pt(R,R-cyclohexane-1,2-diamine)(O,O’-ethanedioato)]) 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, up-regulating 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 in order 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) (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 mM) to prevent drug aquation prior to administration, stabilizing the drug and preventing side-reactions prior to 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 mM). In laboratory and drug screening settings, stock solutions of organic-based drugs are predominately prepared in the solvent dimethyl sulfoxide (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 mM, for example) (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 et al. first reported that DMSO reduced cisplatin’s cytotoxicity towards cultured thyrocytes (17), and Dernel et al. reported that a polymer-based drug delivery system limited activity of cisplatin against stage IIb 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 biological 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 assessed to gauge the solvent types employed in studies of cisplatin. We then assessed the impact of DMSO versus clinical formulations of a number of platinum complexes on the cytotoxicity,
cellular recognition of DNA damage, and cell death signaling. Mass spectrometry was used to
directly assess the interaction of DMSO and clinical formulations with each platinum complex.
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 (http://jpet.aspetjournals.org), and the Public Library of Science (http://www.plos.org) journals. In each case, the word ‘cisplatin’ was entered as a search term on the respective journal web site 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 \textit{in vitro} data. These papers were then assessed for the solvent or solution employed 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 employed was not mentioned in the Materials & 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 Journal of Pharmacology and Experimental Therapeutics, where only twenty-eight relevant articles were identified.

Materials

Cisplatin, carboplatin, DMSO, DMF, DMA, formamide, acetamide and cremophor EL were purchased from Sigma-Aldrich (St. Louis, MO). Oxaliplatin was purchased from LC Laboratories (Woburn, MA). Satraplatin was purchased from Sequoia Research Products
(Pangbourne, UK). [PtCl$_2$(en)] and transplatin were purchased from Alpha Aesar (Ward Hill, MA). Clinical formulations of cisplatin, carboplatin, and oxaliplatin were kindly provided by Dr. Tito Fojo, Clinical Center, National Cancer Institute. Compounds were assessed in various solvents systems as described below.

**Cell lines and cell culture**

This study employed the DLD1 human colorectal carcinoma cells, parental human cervical carcinoma cell line KB-3-1 (a sub-line of HeLa), and its cisplatin-resistant sub-line KB-CP.5. KB-CP.5 cells were originally selected in a single step in 0.5 μg cisplatin/mL (1.6 μM) in our laboratory, as described previously (20, 21). KB lines were originally generated in the laboratory of MMG. DLD-1 cells were provided by the National Cancer Institute (part of the NCI-60 collection). All cell lines were thawed immediately prior to experimentation, and cell lines are characterized by NCI using short tandem repeat profiling. The cisplatin stock solution used for culturing CP.5 cells were prepared in PBS. The cisplatin-resistant cells were maintained in the presence of cisplatin, which was removed from growth medium three days prior to all experiments. All cell lines were grown as monolayer cultures at 37°C in 5% CO$_2$, using either Dulbecco’s modified Eagle medium (DMEM, KB cells), or Roswell Park Memorial Institute medium (RPMI, DLD1 cells) with 4.5 g/L glucose (both from Invitrogen, Carlsbad, CA), supplemented with L-glutamine, penicillin, streptomycin and 10% fetal bovine serum (BioWhittaker, Walkersville, MD). 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 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (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 h. Serially diluted (1:3, RPMI or DMEM used as diluent, chloride concentration ~120 mM) compound was added to give the intended final concentrations. Solvent tolerance testing up to 0.5% under identical conditions confirmed growth all cell lines was unaffected. Cells were then incubated an additional 72 h, and the MTT assay was performed according to the manufacturer's instructions (Molecular Probes, Eugene, OR). Absorbance values were determined at 570 nm on a Spectra Max 250 spectrophotometer (Molecular Devices, Sunnyvale, CA). All MTT assays were performed in triplicate. The 50% inhibitory concentration (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, La Jolla, CA) software for graphs and statistics. All data are expressed as mean ± standard deviation (SD). For bright-field imaging, DLD-1 cells were seeded at a density of 3 × 10⁵ cells per well in 6-well plates and incubated at 37°C in humidified 5% CO₂ for 24 h. Drug was added directly to each well and cells were incubated for another 72 h. Media was aspirated and cells were rinsed with PBS prior to imaging.

**H2AX immunofluorescence staining**

Fixed DLD-1 cells were stained with Phospho-Histone H2A.X (Sε139) (20E3) Rabbit mAb (Alexa Fluor 488 Conjugate) (all components from Cell Signaling Technology, Danvers, MA) following 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 h. Cells were then incubated with Pt complexes for 24 h 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 DAPI 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 mM Tris/HCl, pH 8.0, 150 mM NaCl, 1% NP-40 and supplemented with Complete Protease Inhibitor Cocktail tablets (all from Sigma-Aldrich) and PhosStop phosphatase inhibitor tablets (Roche, Indianapolis, IN)] 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 4-12% Bis-Tris gels (Invitrogen) and transferred onto Immobilon PVDF membrane (Millipore, Billerica, MA). Membranes were blocked in 5% (w/v) non-fat dried skimmed milk powder in TBS-Tween 20 (TBST) [20mM Tris/HCl, pH 7.6, 137 mM 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, Santa Cruz, CA) or Immobilon Western Chemiluminescent HRP Substrate (Millipore) and the signal developed on HyBlot ES film (Denville Scientific, Metuchen, NJ). All primary antibodies were from Cell Signaling Technology: PARP (#9542), cleaved Caspase-3 (#9664), phospho-H2A.X (#9718) and β-actin (#3700).
Mass spectrometry

Accurate mass data was obtained on a Waters (Waltham, MA) Premiere LCT time-of-flight mass spectrometer operated in the positive ion W-mode at 10K resolution. The HPLC solvent pump was operated at 200uL/min and the solvent composition was 50:40:10 water:methanol:acetonitrile. All solvents were LC/MS grade and were purchased from Sigma-Aldrich. Samples were flow injected via a sample loop and ionized in the electrospray ion source. The electrospray capillary was operated at 3.5 kV and at 350 °C. The desolvation gas was purified nitrogen. The MS data was analyzed using Waters MassLynx 4.1 software.
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, Journal of Pharmacology and Experimental Therapeutics (JPET), and the Public Library of Science (PLoS) journals. Thirty-five papers reporting in vitro data were assessed for each journal (twenty-eight for JPET), and the solvent employed 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), dimethyl formamide (DMF), dimethyl sulfoxide (DMSO), saline, phosphate-buffered saline (PBS) and water (Table 1). Where the solvent used was not identified in the Materials & 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 employed assigned for each. While only in vitro data was assessed, it should be noted that cisplatin dissolved in DMSO has also been employed 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 mM) of cisplatin dissolved in 150 mM saline. The saline prevents aquation of the complex in solution prior to administration to the patient (24). Clinical formulation was employed for \textit{in vitro} experiments in 11 – 23\% of papers. However, in some studies cisplatin was dissolved in PBS (chloride concentration 140 mM – phosphate has been reported to not appreciably alter cisplatin aquation (25)) or saline (chloride concentration 154 mM). When these chloride-containing solutions are considered along with clinical formulation, their use was predominant, accounting for 20 – 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 dimethyl formamide (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).

\textbf{In vitro assessment of effect of DMSO on platinum complexes}

In order 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 (\textit{trans}-[PtCl\textsubscript{2}(NH\textsubscript{3})\textsubscript{2}]) and [PtCl\textsubscript{2}(en)] (structures shown in Figure 1) were assessed. Satraplatin is a stable, lipophilic platinum(IV) complex designed to be orally available
(28), and while 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 employed in experimental papers, and like cisplatin contains two cis chlorido leaving groups (8).

The activity of clinical formulations of the drugs cisplatin (3.3 mM in 0.9% saline with 10 mg/mL mannitol), carboplatin (27 mM in 5% glucose solution (31)) and oxaliplatin (12.6 mM in 5% glucose solution (32)) were 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 mM 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 sub-line 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, was affected by DMSO (Table 2, Figure 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, Figure 2, A-C), and by dose-response cell killing (Figure 2, D-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 μM, whereas its IC₅₀ when dissolved in DMSO was 178 ± 9.2 μM. Carboplatin demonstrated reduced cytotoxicity compared with cisplatin (34.2 ± 3.4 μM against KB-3-1 cells), consistent with its diminished reactivity (32), but DMSO reduced its cytotoxicity further (3 to 10-fold, 243 ± 11.2 μM against KB-3-1 cells). In contrast, DMSO appeared to have a slight potentiating effect on oxaliplatin, with both KB-3-1 (aqueous = 3.0 ± 0.5 μM, DMSO = 0.7 ± 0.3 μM) and DLD-1 (aqueous = 3.9 ± 0.6 μM, DMSO = 1.6 ± 0.2 μM) cells being slightly more sensitive to the DMSO-formulated compound. DMSO had no effect on satraplatin cytotoxicity (for example, IC₅₀ for the clinical formulation against DLD-1 = 1.5 ± 0.2 μM, in DMSO = 1.6 ± 0.2 μM).

While DMSO disrupted the activity of cisplatin, cisplatin-resistant cells retained cross-resistance towards the DMSO-formulated drug. CP.5 cells demonstrated 10-fold resistance to cisplatin (CP.5 IC₅₀ = 34.3 ± 1.6 μM vs. KB 3-1 IC₅₀ = 1.9 ± 1.6 μM), 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 appear to have greater potency against CP.5 cells than when formulated in aqueous media (water and saline, respectively). By way of example, the IC₅₀ of transplatin dissolved in DMSO was very high (433
± 30.6 μM) but lower against CP.5 cells (134 ± 80.3 μM). This collateral 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 is 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 biological activity (33).

In order 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 H2A.X. H2A.X 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, H2A.X 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 γH2A.X foci were evaluated by immunofluorescence microscopy after 24 h (Figure 3a-c). Cells treated with cisplatin (5 μM) in saline or carboplatin (5 μM) in water clearly elicited a DNA-damage response, whereas cells treated with the compounds dissolved in DMSO resulted in a striking diminution of γH2A.X staining. Despite being approximately equitoxic with cisplatin, oxaliplatin resulted only in low-level staining. Satraplatin in water and DMSO showed equivalent γH2A.X staining, whereas transplatin and [PtCl2(en)] dissolved in DMSO elicited weaker γH2A.X staining than their saline-dissolved comparison (not shown).

Immunoblot analysis of γH2A.X supported immunofluorescence microscopy staining (Figure 3d). To directly study if DMSO affected cell death, we incubated DLD-1 cells with cisplatin (5
µM), carboplatin (50 µM) and oxaliplatin (5 µM) in either DMSO or water, and whole-cell lysates were collected after 48 h for Western analysis (Figure 3B). Induction of cleaved PARP and cleaved caspase 3 were 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 Pt complexes by DMSO and other solvents

To assess the nature of the interactions between cisplatin and DMSO, we compared electrospray ionization mass spectra (ESI-MS) 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− with DMSO (m/z = 343), and replacement of a Cl− and an NH3 ligand by two DMSO molecules (m/z = 404) (38, 39). We also observed a higher molecular weight species (m/z = 665.9) corresponding to a bridged form of DMSO-substituted cisplatin, \( \mu\text{NH}_2\left[\text{Pt(NH}_3\text{)(Cl)(DMSO)}\right]^{+} \) (Supplementary Table S7) not previously reported. These are shown schematically in Supplementary Figure S3. Carboplatin, transplatin, and \([\text{PtCl}_2(\text{en})]^{-}\) produced reaction products with DMSO, all involving replacement of a Cl− 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 biological 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 mM in 154 mM saline), followed by serial dilution and dosing to cells. DMSO did not have any effect on cisplatin’s cytoxicity on KB-3-1 or DLD-1 cells (e.g., DLD-1 cisplatin saline IC_{50} = 3.3 ± 0.5 μM, cisplatin saline and DMSO IC_{50} = 3.4 ± 0.3 μM) (Table 2). Despite the lack of biological 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 Pt complexes

One limitation of aqueous formulations of cisplatin is its maximal solubility of just over 3 mM, whereas DMSO solutions can be prepared of greater than 10 mM. We examined whether solvent
systems had a similar effect on cisplatin. Stock solutions of cisplatin were prepared in amenable solvents: dimethylformamide (DMF), dimethylacetamide (DMA), formamide, acetamide, cremophor EL (all 10 mM stock solutions), and water (3.3 mM) (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 while not statistically significant, DMF resulted in the greatest decrease in cytotoxicity of cisplatin (IC$_{50}$ = 5.0 ± 1.3 μM compared with 3.8 ± 0.6 μM for cisplatin in saline).

However, each organic solvent alone (at a high concentration of 3% in media, consistent with the lowest dilution of 10 mM cisplatin in media required to perform a dose-response) demonstrated cytotoxicity towards DLD-1 cells (Table 3). The exception was acetonitrile, with the limitation that acetonitrile was one of 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). While 3% solvent is a high concentration, the IC$_{50}$ concentration for cisplatin (~1-3 μM) in our toxicity assays occurs at a drug dilution that is in the presence of 0.2 – 0.05% solvent – a concentration at which no toxicity towards 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 spectrometry. 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, 44). 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 biological testing, five (25%) did not state the solvent used, and three (15%) used DMSO (Table S6). Alternative solvents such as DMF have been reported not to
cause cytotoxicity below 0.5% (45), and solutions of cisplatin dissolved in DMF retain activity (46). However, while DMF is recognized as cytotoxic towards cells (47), there appears 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 DMSO-cisplatin deactivation was first reported over twenty years ago, and the underlying chemistry had been generally well defined (13, 16). Jones et al. demonstrated that co-administration of cisplatin with DMSO in Sprague-Dawley rats reduced nephrotoxicity (48), and Massart and co-workers observed that DMSO inhibited cisplatin cytotoxicity towards 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 (49). Windebank and co-workers later demonstrated that while 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 labs, 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 (50). Uribe et al. reported _increased_ sensory hair cell death in zebrafish co-treated 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 biological activity (51).

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 mM), and carboplatin (27 mM) and oxaliplatin (12.6 mM) 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

None were disclosed.

Authors’ contributions

Conception and design: MDH, MMG, JDH

Acquisition of data: MDH, KAT, KEC, TDL, JL, IG, JPM

Analysis and interpretation of data: MDH, KAT, KEC, TDL, JL, JPM

Writing, review, and/or revision of the manuscript: MDH, KAT, MMG, IG, CT

Acknowledgements

We thank George Leiman for editorial assistance.

Grant support

This research was supported by the Intramural Research Program of the National Institutes of Health, National Cancer Institute. K-EC is a National Institutes of Health (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.
References


50. Tanley SW, Schreurs AM, Kroon-Batenburg LM, Meredith J, Prendergast R, Walsh D, et al. Structural studies of the effect that dimethyl sulfoxide (DMSO) has on cisplatin and

Figure legends

**Figure 1.** Structures of the platinum complexes used in this study.

**Figure 2.** Comparison of the effect of DMSO on the biological activity of cisplatin, carboplatin and oxaliplatin. Bright-field images (A-C), and dose-response curves (D-F) of DLD-1 cells exposed to platinum drugs (cisplatin, carboplatin, oxaliplatin) in different solvents (water/saline, clinical formulation, DMSO).

**Figure 3.** Comparison of the effect of DMSO on cisplatin, carboplatin and oxaliplatin-mediated DNA damage and cell death. Cellular DNA damage recognition caused by platinum drugs observed via confocal images (63 x) of DLD-1 cells exposed to platinum drugs as indicated, and stained with an antibody that recognizes phosphorylated (γ) H2A.X (A). Immunoblot (B) of protein lysate from DLD-1 cells treated as described for (A) and probed for expression of total PARP, cleaved PARP, cleaved caspase-3 and phosphor (γ) H2A.X, with β-actin probed as a control for protein loading.
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></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></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 (6)</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>11 (4)</td>
<td>7 (2)</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>3 (1)</td>
<td>3 (1)</td>
<td>3 (1)</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>
Table 2. Cytotoxicity (IC\textsubscript{50}, \(\mu\)M) of platinum complexes against parental (DLD-1, 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 (384 mM)</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>1014 ± 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>
<td></td>
<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><strong>[PtCl\textsubscript{2}(en)]</strong></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>
Table 3. Cytotoxicity (IC$_{50}$, $\mu$M) of cisplatin, dissolved or suspended in solvents, against DLD-1 cells

<table>
<thead>
<tr>
<th>Cisplatin</th>
<th>IC$_{50}$ (μM)</th>
<th>Stock (μM)</th>
<th>Soluble</th>
<th>3% solvent viability (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td>Y</td>
<td>100</td>
</tr>
<tr>
<td>Clinical</td>
<td>3.3 ± 0.5</td>
<td>3.3</td>
<td>Y</td>
<td>-</td>
</tr>
<tr>
<td>Saline</td>
<td>3.8 ± 0.6</td>
<td>3.3</td>
<td>Y</td>
<td>96 ± 10</td>
</tr>
<tr>
<td>DMSO</td>
<td>177.6 ± 9.2</td>
<td>20</td>
<td>Y</td>
<td>90 ± 5</td>
</tr>
<tr>
<td>DMF</td>
<td>5.0 ± 1.3</td>
<td>10</td>
<td>Y</td>
<td>79 ± 5</td>
</tr>
<tr>
<td>DMA</td>
<td>3.5 ± 0.8</td>
<td>10</td>
<td>Y</td>
<td>24 ± 3</td>
</tr>
<tr>
<td>Formamide</td>
<td>3.7 ± 1.8</td>
<td>10</td>
<td>Y</td>
<td>57 ± 2</td>
</tr>
<tr>
<td>Cremophor EL</td>
<td>2.5 ± 0.2</td>
<td>10</td>
<td>N</td>
<td>0.7 ± 0.1</td>
</tr>
<tr>
<td>Acetonitrile</td>
<td>3.4 ± 0.3</td>
<td>10</td>
<td>N</td>
<td>103 ± 6</td>
</tr>
<tr>
<td>Water</td>
<td>4.0 ± 0.5</td>
<td>3.3</td>
<td>Y</td>
<td>106 ± 7</td>
</tr>
</tbody>
</table>
Figure 1

Cisplatin

Carboplatin

Oxaliplatin

Satraplatin

\([\text{PtCl}_2(\text{en})]\)

Transplatin
Figure 3

A

Cisplatin | Carboplatin | Oxaliplatin
---|---|---
Aqueous
5 μM, saline | 50 μM, water | 5 μM, water
DMSO
5 μM, DMSO | 50 μM, DMSO | 5 μM, DMSO

B

<table>
<thead>
<tr>
<th>Control</th>
<th>Cisplatin (DMSO)</th>
<th>Cisplatin (saline)</th>
<th>Oxaliplatin (DMSO)</th>
<th>Oxaliplatin (water)</th>
<th>Carboplatin (DMSO)</th>
<th>Carboplatin (water)</th>
</tr>
</thead>
</table>

- Total PARP
- Cleaved PARP
- Cleaved caspase-3
- Phospho-H2A.X
- β-actin
Say No to DMSO: Dimethylsulfoxide Inactivates Cisplatin, Carboplatin and Other Platinum Complexes

Matthew D Hall, Katherine A. Telma, Ki-Eun Chang, et al.

Cancer Res  Published OnlineFirst May 8, 2014.