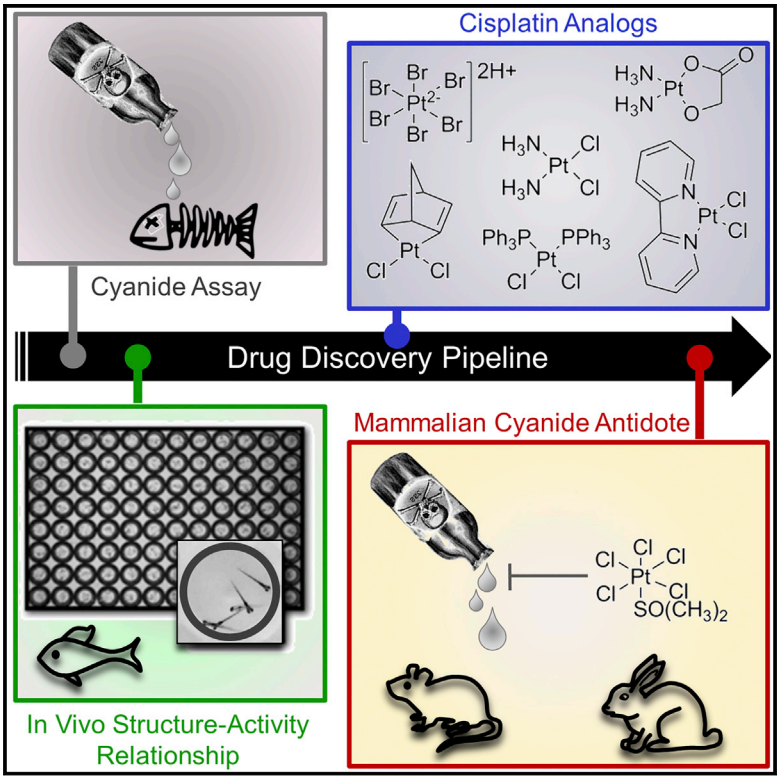
Article

Cell Chemical Biology

Cisplatin Analogs Confer Protection against Cyanide Poisoning

Graphical Abstract



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In Brief

Annually, one million new patients receive the chemotherapeutic drug cisplatin. Here Nath et al. provide proof of concept data that cisplatin analogs are antidotes to cyanide poisoning in three vertebrate species and identify a lead compound for further drug development.

Highlights

- Cyanide antidotes discovered by structure-activity relationships of cisplatin
- Sulfur-containing ligands are a key feature of platinum-based cyanide antidotes
- Cisplatin analogs act as antidotes by binding up to five cyanide anions
- Antidote activity is conserved in zebrafish, mice, and rabbits

Nath et al., 2017, Cell Chemical Biology *24*, 565–575
May 18, 2017
http://dx.doi.org/10.1016/j.chembiol.2017.03.013

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Cisplatin Analogs Confer Protection against Cyanide Poisoning

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SUMMARY

Cisplatin holds an illustrious position in the history of chemistry most notably for its role in the virtual cure of testicular cancer. Here we describe a role for this small molecule in cyanide detoxification in vivo. Cyanide kills organisms as diverse as insects, fish, and humans within seconds to hours. Current antidotes exhibit limited efficacy and are not amenable to mass distribution requiring the development of new classes of antidotes. The binding affinity of the cyanide anion for the positively charged metal platinum is known to create an extremely stable complex in vitro. We therefore screened a panel of diverse cisplatin analogs and identified compounds that conferred protection from cyanide poisoning in zebrafish, mice, and rabbits. Cumulatively, this discovery pipeline begins to establish the characteristics of platinum ligands that influence their solubility, toxicity, and efficacy, and provides proof of concept that platinum-based complexes are effective antidotes for cyanide poisoning.

INTRODUCTION

Historically, cyanide has been an agent of murder, war, and terrorism; however, unintentional exposures are equally possible and lethal. Smoke inhalation is the most common cause of cyanide poisoning in western countries (Barillo et al., 1994). Cyanide reversibly binds to cytochrome *c* oxidase within the mitochondria (Kellin, 1929). Consequently, electron transport and oxidative phosphorylation are halted. If not reversed, the cessation of aerobic metabolism causes a fatal deficit in oxygen consumption. Manufacturers in the United States produce 300,000 tons of hydrogen cyanide annually, which is used in the extraction of gold during mining and in the synthesis of dyes, synthetic fibers, and plastics, as well as in warehouses as a pesticide. The thermal breakdown of materials such as wool, plastic, and synthetic polymers produce cyanide gas in addition to isocyanates (potent respiratory irritants), leading to smoke inhalation fatalities of approximately 5,000–10,000, and injuries of 23,000 per year in the United States (Alcorta, 2004).

Industrial accidents are another major source of cyanide morbidity and mortality. The Bhopal disaster, considered the world's worst industrial accident, occurred when 45 tons of methyl isocyanate and hydrogen cyanide escaped from reservoirs killing nearly 4,000 people immediately, followed by another 15,000–20,000 individuals over the next few weeks, and leaving a half a million survivors with debilitating injuries such as chronic respiratory illnesses and blindness (Broughton, 2005). Although there are two available antidotes for cyanide poisoning, their formulation and mode of action require them to be administered intravenously in hospital settings, therefore they would not be amenable to a mass causality scenario such as the Bhopal disaster (Hall et al., 2009). The large global supply of cyanide, the morbidity and mortality from smoke inhalation and industrial accidents, and the threat to soldiers fighting nonconventional conflicts requires the development of novel classes of antidotes that are amenable to mass distribution in the field. To meet this need, chemical biology and medicinal chemistry approaches are required to discover lead compounds for development into new classes of antidotes to cyanide poisoning.

To identify novel classes of cyanide antidotes, we developed a zebrafish model of cyanide toxicity that is amenable to high-throughput chemical screening (Nath et al., 2013). Upon exposure to cyanide, zebrafish larvae develop stereotypic dose-dependent cyanide pathologies including slow heart rate, deficits in standard neurobehavioral responses, and ultimately death. In addition to its usefulness for chemical screening, the zebrafish is an organism that is uniquely positioned to extend the information gained from classical in vitro structure-activity relationship (SAR) studies by providing insight into some in vivo attributes of compounds, including absorbance, metabolism, and toxicity (Hao et al., 2010). Therefore, this approach represents a powerful tool in the drug discovery arena.

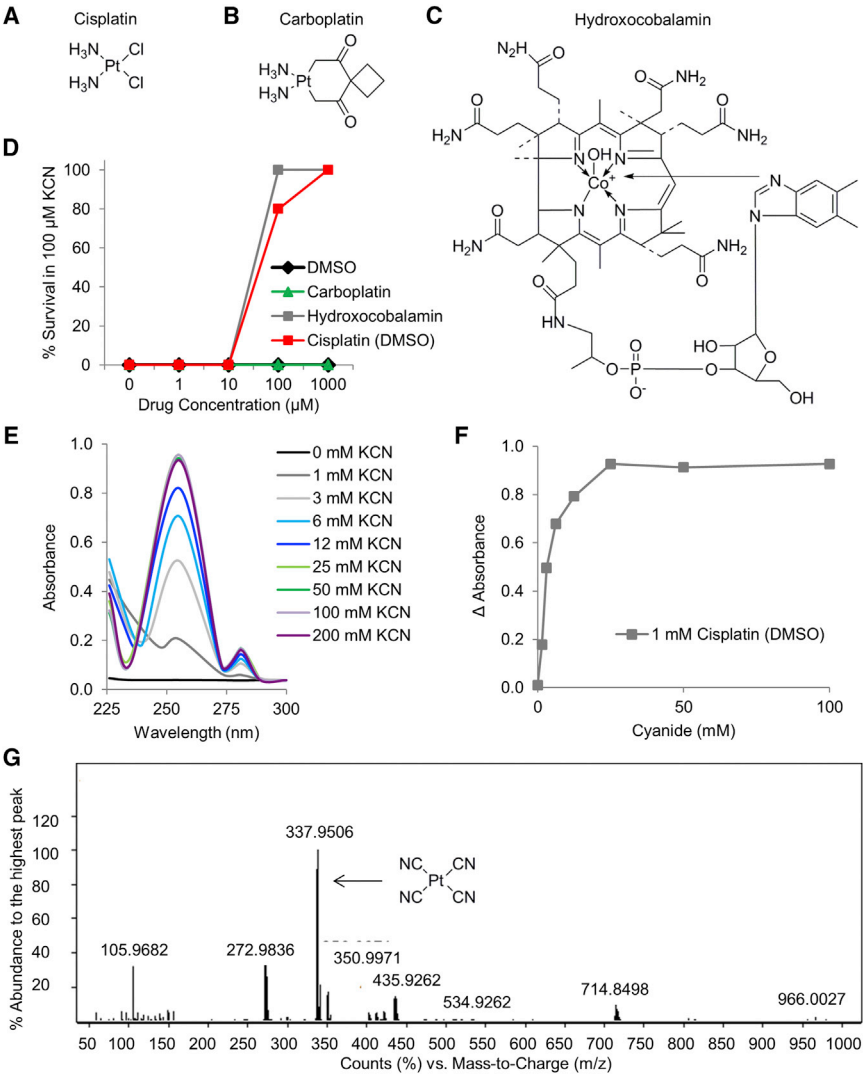

Figure 1. Platinum Complexes Act as Antidotes to Cyanide Poisoning by Binding the Cyanide Anion

(A–C) Chemical structure of (A) cisplatin, (B) carboplatin, and (C) hydroxocobalamin. Compounds were dissolved in DMSO for the assays in this figure.

(D) The effects of compounds on the survival of zebrafish exposed to 100 μM KCN.

(E) UV-vis spectral shift data demonstrating the binding of the cisplatin to cyanide.

(F) Plot of the change in absorbance of 1 mM cisplatin over increasing concentrations of cyanide.

(G) Analysis by mass spectrometry demonstrates that cisplatin binds four cyanide anions. Also see Table S1.

In this study, we used in vivo SAR studies in zebrafish to examine a panel of structurally diverse cisplatin analogs. We reasoned that cisplatin analogs contain a platinum cation, which is known to interact with the cyanide anion; however, it is actually the ligands coordinated to the platinum core that regulate the reactivity of cisplatin analogs and hence modulate their antidote efficacy in vivo. Among the analogs tested, we discovered 22 analogs with effectiveness against cyanide poisoning. To identify high-value candidates to move into mammalian studies, these 22 candidates were then funneled through a series of filters: efficacy in zebrafish, in vivo toxicity in zebrafish, in vitro toxicity in human cells, aqueous solubility, and binding kinetics to cyanide. Based on these selection criteria, three cisplatin analogs were tested in two mammalian models of cyanide poisoning. Finally, detailed temporal measurements of oxidative metabolism were obtained in a rabbit model of cyanide exposure to ascertain the effects of these compounds on known cyanide targets. Cumulatively, this discovery pipeline provides proof of concept that platinum-based complexes have antidote activity against cyanide poisoning in zebrafish, mice, and rabbits, and begin to establish the characteristics of platinum ligands that influence their solubility, toxicity, and efficacy.

RESULTS

Platinum Complexes Act as Antidotes to Cyanide Poisoning by Binding the Cyanide Anion

In a previous chemical screen in zebrafish, we discovered 4 cyanide antidotes within a library of 3,120 U.S. Food and Drug Administration (FDA)-approved drugs (Nath et al., 2013). In that chemical screen, zebrafish were exposed to a dose of cyanide that induces lethality over several hours. In the present study, in order to increase the stringency of the assay, a dose of 100 μM was used, which kills zebrafish in less than 1 hr. Zebrafish larvae were treated with cisplatin, carboplatin, or the known cyanide antidote, hydroxocobalamin, dissolved in DMSO in a 5-point dose-response curve (Figures 1A–1C). Under these conditions, a dose of 100 μM hydroxocobalamin completely blocked cyanide-induced lethality (Figure 1D). Carboplatin was not a cyanide antidote, while cisplatin displayed similar efficacy as hydroxocobalamin. Survival of 80% of zebrafish was observed at 100 μM cisplatin, which is approximately equimolar to the cyanide concentration (Figure 1D).

Cisplatin contains a positively charged platinum core, which indicates that it may act as an antidote by binding the cyanide anion. To test this hypothesis, we conducted an UV-visible (UV-vis) spectrophotometry experiment. Increasing amounts of potassium cyanide were added to a solution of 1 mM cisplatin in 0.1% DMSO/H_2O and the absorbance was measured across the UV-vis spectrum. At concentrations ranging from 1 to 200 mM cyanide a spectral shift was observed at 255 nm (Figure 1E). At approximately 25 mM cyanide, the reaction between cyanide and cisplatin (1 mM) reached saturation (Figure 1F). These results indicate that cyanide binds cisplatin and suggests that the mechanism of action of platinum complexes may involve binding of the cyanide anion to the metal core of cisplatin.

To confirm this hypothesis, we next performed electrospray ionization-mass spectrometry (ESI-MS) to identify the composition of the reaction product created by the addition of cyanide to cisplatin. The spectra contained a major peak with an m/z of 337.9 and minor peaks with m/z values of 272.9 and 350.9 (Figure 1G). Using tandem mass spectrometry and isotope distribution comparison, we identified the major peak as platinum bound to four cyanide anions and the minor peaks as platinum bound to three cyanide anions (Table S1). This finding suggests that platinum complexes may act as antidotes to cyanide poisoning by chelating cyanide anions via the platinum atom.

Cyano-platinum complexes are considered strong metal-cyanide complexes because they do not dissociate easily at physiological pH (Smith and Martel, 1976). To determine if cyanide dissociates from cyano-platinum complexes in vivo and if the released cyanide induces toxicity, zebrafish were treated with 1–1,000 μM potassium cyanide (KCN) or potassium tetracyanoplatinate(II) [$K_2Pt(CN)_4$] for 24 hr. KCN treatment at a dose of 20 μM resulted in 100% lethality. In contrast, zebrafish treated with $K_2Pt(CN)_4$ were alive and active, with no gross morphological defects at all doses of $K_2Pt(CN)_4$ tested. These results suggest that the cyano-platinum species produced by administration of a platinum-based cyanide antidote are relatively non-toxic.

SAR Analysis Identifies Novel Antidotes to Cyanide Poisoning

The finding that carboplatin and cisplatin exhibit differential efficacy as cyanide antidotes suggested that the ligands bound to platinum affect antidote potency (Figure 1D). To explore the potential use of platinum complexes as cyanide antidotes and to mitigate the known insolubility and toxicity associated with cisplatin, an in vivo SAR study was performed.

Cisplatin analogs are coordination complexes containing three components: a positively charged metallic atom that is the coordination center, ligands that are leaving groups, and ligands that remain conjugated to the platinum atom (typically *cis* ammine groups). In the case of cisplatin, upon exposure to the intracellular aqueous environment, an associative substitution reaction occurs in which the chloride leaving groups are replaced by water molecules (Siddik, 2003). This complex enters the nucleus and a second associative substitution reaction occurs. Purine bases displace the water leaving groups, generating two DNA adducts per cisplatin molecule (Fichtinger-Schepman et al., 1985). In the case of cyanide exposure, the carbon of the cyanide anion is the nucleophile that forms a bond with the platinum atom and displaces a ligand of the platinum complex.

A panel of 35 structurally diverse cisplatin analogs was assembled, and doses of 1–1,000 μM were tested (10-point dose-response curve). The doses that rescued 100% of zebrafish (EC_{100}) from a challenge with 100 μM KCN were determined. In a separate assay, the doses that caused 100% lethality (LD_{100}) in the absence of KCN were determined. The EC_{100} was determined in both DMSO and PBS solvents (Figure 2, blue and red, respectively). The LD_{100} was determined for complexes dissolved in DMSO (Figure 2, black). NA indicates instances in which the complex did not induce any toxicity or did not rescue cyanide lethality at any of the doses tested. The cisplatin analogs spanned the following classes: platinum(IV) (1–6), square planar (7–13), FDA-approved drugs (14–19), pyridine (20–24), triphenylphosphine (25–28), alkene (29–32), and sulfur-containing complexes (33–35). The SARs of complexes solvation in DMSO are discussed below (Figure 2, blue), subsequently the SAR results after solvation in PBS are discussed (Figure 2, red).

Platinum(IV) Complexes

Tetravalent platinum complexes have an octahedral geometry and a coordination number of 6. Complexes with hydroxyl groups (1 and 2) displayed no toxicity in zebrafish and were not cyanide antidotes at any of the doses tested. Increasing the number of chloride ligands from two (7) to four to six (3–5) reduced the efficacy of the antidote from 125 to 250–1,000 μM. The increased efficacy and toxicity of the sodium salt (3) compared with the potassium salt (4) may be due to its greater aqueous solubility. The most effective complex (6) had bromide leaving groups (EC_{100} = 62.5 μM). Platinum IV complexes with ligands that are good leaving groups were more effective cyanide antidotes than those with poor leaving groups (-Br > -Cl > -OH > $-NH_3$). These results suggest that the ease with which the leaving groups are lost is a critical determinant of efficacy of platinum-based cyanide antidotes.

Square Planar Complexes

These complexes have a square planar geometry with a coordination number of 4. Both cisplatin (7) and its *trans* stereoisomer transplatin (8) were equipotent antidotes. In contrast, the cisplatin analog with four ammonia ligands (9) was completely ineffective as an antidote, likely due to the fact that $-NH_3$ is a poor leaving group and typically is considered to be a stable ligand in platinum complexes. The four other compounds in this class (10–13) were more potent than cisplatin with minimal toxicity in zebrafish. The two complexes with nitrile ligands (10 and 11) were effective antidotes at 62 μM. The two least sterically hindered platinum complexes tested in this study had a coordination number of 2, a bent geometry, and either two bromide or two chloride ligands. Both were effective antidotes at 62 μM (12 and 13). As a group, these complexes were a potent class of antidotes, suggesting that square planar or bent complexes can be effective cyanide chelators.

FDA-Approved Drugs and Compounds in Clinical Evaluation

Many of the compounds in this class (14–19) have a bidentate carboxyl ligand which is a moderate leaving group (16–19). Except for satraplatin (14), they all have a coordination number of 4, and are divalent. The lipophilic Pt(IV) complex satraplatin (14) is an orally available prodrug which to be active must be converted to a Pt(II) complex by loss of the two axial acetate groups. In our assay it displayed no toxicity; however, it was not a cyanide antidote. Complex 15 (picoplatin) is a square planar complex that contains a methyl group on the pyridine ring that is perpendicular to the square plane, which reduces the substitution kinetics. It also was not a cyanide antidote. Similarly, carboplatin (16), nedaplatin (17), PHM (JM-74) (18), and oxaliplatin (19) were not antidotes when challenged with 100 μM KCN. As a group, this class of complexes displayed no toxicity in zebrafish; however, none display antidote activity, indicating that compounds with bidentate carboxyl ligands are ineffective as antidotes to cyanide poisoning in zebrafish.

Pyridine Complexes

Pyridine complexes are divalent cisplatin analogs with two chloride leaving groups and either a pyridine or bidentate pyridine

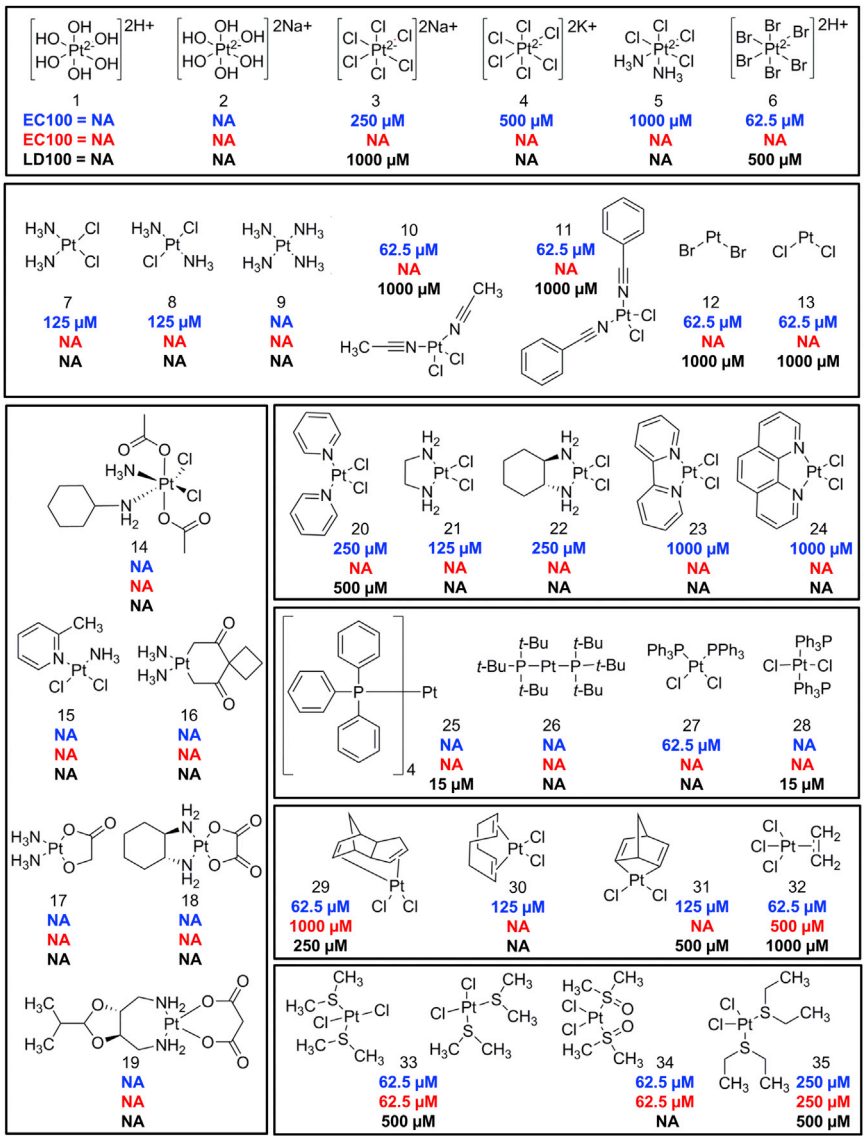

Figure 2. Structure-Activity Relationships of Cisplatin Analogs

A panel of 35 cisplatin analogs was grouped into the following classes: platinum (IV) (1–6), square planar (7–13), FDA-approved (14–19), pyridine (20–24), triphenylphosphine (25–28), alkene (29–32), and sulfur-containing complexes (33–35). A ten-point dose curve ranging from 1 to 1,000 µM was tested. The doses that rescued 100% of zebrafish (EC_{100}) from a challenge with 100 µM KCN were determined. The EC_{100} was determined in both DMSO and PBS solvents (blue and red, respectively). In a separate assay, the doses that caused 100% lethality (LD_{100}) in the absence of KCN were determined for complexes dissolved in DMSO (black). NA indicates instances in which the complex did not induce any toxicity or did not rescue cyanide lethality at any of the doses tested.

ligand. Whereas complex 15 was developed to contain a methyl group on the pyridine ring that is perpendicular to the square plane thereby reducing the substitution kinetics, complex 20 does not contain a methyl group on either of the pyridine rings. The removal of the methyl group in complex 20 converts the complex into a cyanide antidote, although not as effective as cisplatin (250 versus 125 µM). Complexes with bidentate ligands (21–24) displayed decreased efficacy as the size of rings increased, suggesting that these rigid ligands may cause steric hindrance that reduces reactivity (EC_{100} = 1,000 µM). Accordingly the most compact complex (21) in this class was the most effective (EC_{100} = 125 µM). It contains an ethylenediamine group in place of the two ammine groups of cisplatin and displayed no toxicity in zebrafish.

Triphenylphosphine Complexes

These complexes contain lipophilic triphenylphosphine ligands (25–28). One of the four complexes was an effective antidote at 62.5 µM (27). Its *trans* stereoisomer (28) was not an antidote potentially due to the bulky triphenylphosphine ligands reducing access to the platinum atom, whereas in the *cis* position the compact nature of the two *cis* chlorides allows access to the platinum atom. A caveat to this class of complexes is that they are poorly soluble.

Alkene Complexes

Alkene complexes are tetrahedral complexes with chloride and alkene ligands (29–32). Complex 32, also known as Zeise's salt, contains a η2-ethylene ligand, while the other complexes in the group contain cyclodiene ligands. In this class of compounds the platinum is coordinated to one (32) or two alkenes (29–31). They were equipotent or more effective than cisplatin ($EC_{100} \leq$ 125 µM); however, there was some toxicity in this class of complexes as noted by the LD_{100}. Of note, these complexes contain no ammine ligands unlike the majority of the platinum complexes in clinical development. In addition, 1,5-cyclodiene ligands, such as in dichloro(1,5-cyclooctadiene) platinum(II) (30), are often used in organometallic chemistry as these ligands are easily displaced (Elschenbroich, 2006). Although there is some toxicity in this class of complexes, the substitution kinetics of the 1,5-cyclodiene ligand may be a favorable feature for the development of a platinum-based cyanide antidote.

Sulfur-Containing Complexes

Cisplatin analogs containing two sulfur-based ligands and two chloride ligands (33–35) were tested. The three complexes were effective antidotes at 62.5–250 µM. The change from diethylsulfide ligands in complex 35 to sulfoxide ligands in complex 34 increased efficacy from 250 to 62.5 µM and decreased toxicity. The *cis*/*trans* racemic mixture of complex 33, which contains dimethylsulfide ligands compared with complex 35 which contains diethylsulfide ligands, was a more potent antidote (62.5 versus 250 µM). It is unclear if the *cis* or *trans* stereoisomer was conferring the dominant effect.

SAR Summary of DMSO-Solvated Complexes

In a panel of 35 platinum complexes, we identified 22 novel cyanide antidotes with an EC_{100} of 62.5–1,000 μM. The two most effective and least toxic antidotes in zebrafish were the triphenylphosphine complex 27 and the sulfur-containing complex 34 (EC_{100} = 62.5 μM and no observed toxicity in zebrafish). However, complex 27 is poorly soluble in water (0.079 g/L; logP = 8.2), whereas complex 34 is aqueous soluble (84 g/L; logP = −1.35). This is a ∼33-fold improvement in solubility over cisplatin (2.5 g/L; logP = −2.19). In addition, the reported dose of complex 34 that causes acute toxicity in mice is ∼20-fold higher than cisplatin (LD_{50} = 133 versus 6.6 mg/kg intraperitoneally) (Braddock et al., 1975).

SAR Summary of PBS-Solvated Complexes

Surprisingly, when cisplatin analogs were dissolved in PBS, the majority were not cyanide antidotes at any dose tested (Figure 2). However, two of the four alkene complexes (29 and 32) were antidotes, albeit requiring doses 16- and 8-fold higher, respectively, than when solvated in DMSO. All three sulfur-containing complexes (33–35) were equipotent antidotes when dissolved in DMSO and PBS. These findings motivated us to explore the effects of solvation on the efficacy of cisplatin analogs as cyanide antidotes.

cis-Diamminechloro(dimethylsulfoxide)platinum(II), Complex 36, Binds Cyanide Faster than *cis*-Diamminedichloroplatinum(II)

Cisplatin's mechanism of action requires aquation, the replacement of the chloride ligands with water molecules. This generates the active form of cisplatin (37) which is more reactive than the chloride complex (7). Due to the high concentration of chloride ions in the blood (∼100 mM) versus inside the cell (∼4 mM), aquation is favored once cisplatin enters the cell. When cisplatin is dissolved in DMSO, the sulfur in DMSO undergoes nucleophilic attack of platinum. This results in the substitution of a chloride ligand with a DMSO ligand, changing its structure and creating a new chemical species (36). Due to the influence of the leaving ligands on the kinetics of associative substitution, and given the SAR data in PBS versus DMSO, we hypothesized that the DMSO leaving group influences the kinetics of the reaction with cyanide.

To determine if dissolving cisplatin in PBS versus DMSO affected cyanide binding, we conducted UV-vis experiments. Cisplatin was dissolved in water generating the aquated form of cisplatin (37), in PBS preventing the chloride ligands from being displaced by water molecules (7), or in DMSO creating the DMSO adduct species (36) (Figures 3A, 3C, and 3E). In complex 37, cyanide would be predicted to displace a water ligand first (Figure 3A). In complex 7, the cyanide anion would be predicted to displace a chloride ligand first (Figure 3C). In complex 36, based on previous associative substitution studies with platinum complexes, cyanide would be predicted to displace the ammine ligand (Banerjea et al., 1957) (Figure 3E). To determine the binding affinity of cyanide for these three complexes, increasing concentrations of cyanide were added while the absorbance across the UV-vis spectrum was measured (Figures 3B, 3D, and 3F). Both cisplatin (7) and the aquated form of cisplatin (37) did not induce a significant spectral shift when cyanide was added (Figures 3B and 3D). In contrast, complex 36 induced a significant spectral shift in the presence of increasing concentrations of cyanide (Figure 3F). Performing this experiment in a polar, aprotic environment did not affect the binding of cyanide to complex 7. These findings suggest that, during nucleophilic attack, the cyanide anion cannot easily displace the chloride or water ligands in platinum; however, when a DMSO ligand is conjugated to the platinum complex associative substitution is highly favorable.

Next, we performed a time course experiment to evaluate the reaction rate of cyanide with these complexes. To 1 mM of complex, 5 mM of cyanide was added and the absorbance was measured every 1 min for 5 min, followed by every 5 min for 30 min (Figure 3G). Cisplatin (7) dissolved in PBS and assayed in PBS had a reaction rate of 0.021 a.u./min. The aquated form of cisplatin (cisplatin dissolved in water and assayed in water, 37) had a reaction rate of 0.013 a.u./min. Complex 36 (cisplatin dissolved in DMSO and assayed in water), had a reaction rate of 0.367 a.u./min. To create a polar aprotic environment DMF was used as the solvent and assay buffer. Cisplatin dissolved in and assayed in DMF had a reaction rate of 0.019 a.u./min. These results demonstrate that the reaction between cyanide and complex 36 occurred at a rate 17-fold faster than cisplatin (7) and 28-fold faster than the aquated form of cisplatin (37).

Identification of Complex 36 as a Cyanide Antidote In Vivo

We hypothesized that the reaction rate of complex 36 with cyanide, compared to that with cisplatin, drives the different efficacies of these 2 complexes in vivo. Zebrafish treated with cisplatin dissolved in PBS (cisplatin) displayed no activity as a cyanide antidote while cisplatin dissolved in DMSO (complex 36) was an effective antidote (Figure 3H). Theoretically, DMSO could undergo nucleophilic attack of the platinum atom, generating multiple reaction products. To decipher the exact chemical species created when we dissolved cisplatin in DMSO, we used ESI-MS. The most abundant ion signal detected was at m/z = 343, corresponding to the molecular weight of $[Pt(NH_3)_2(Cl)(DMSO)]$ (Figure 3I). In this complex, one chloride ligand was displaced by a DMSO ligand generating *cis*-diamminechloro(dimethylsulfoxide)platinum(II) (36). We observed near complete conversion of cisplatin to complex 36 within a few hours, consistent with the literature (Fischer et al., 2008). Other minor species were detected consistent with published studies (Table S2). Collectively these results demonstrate that the cisplatin analog, *cis*-diamminechloro(dimethylsulfoxide)platinum(II), is a cyanide antidote. Concomitantly, the SAR dataset revealed that complexes dissolved in DMSO were cyanide antidotes, whereas those same complexes dissolved in PBS were not cyanide antidotes, suggesting that sulfur-based ligands are a key feature of platinum-based cyanide antidotes.

Cisplatin Analogs Solvated in DMSO Display Decreased Cytotoxicity in H1975 Cells

The toxic side effects of platinum-based drugs are thought to be due to their mechanism of action, DNA damage leading to cell death. DMSO is known to inactivate chemotherapeutic drugs (cisplatin, carboplatin, and oxaliplatin) by inserting into the complex, disrupting its ability to interact with DNA and hence induce

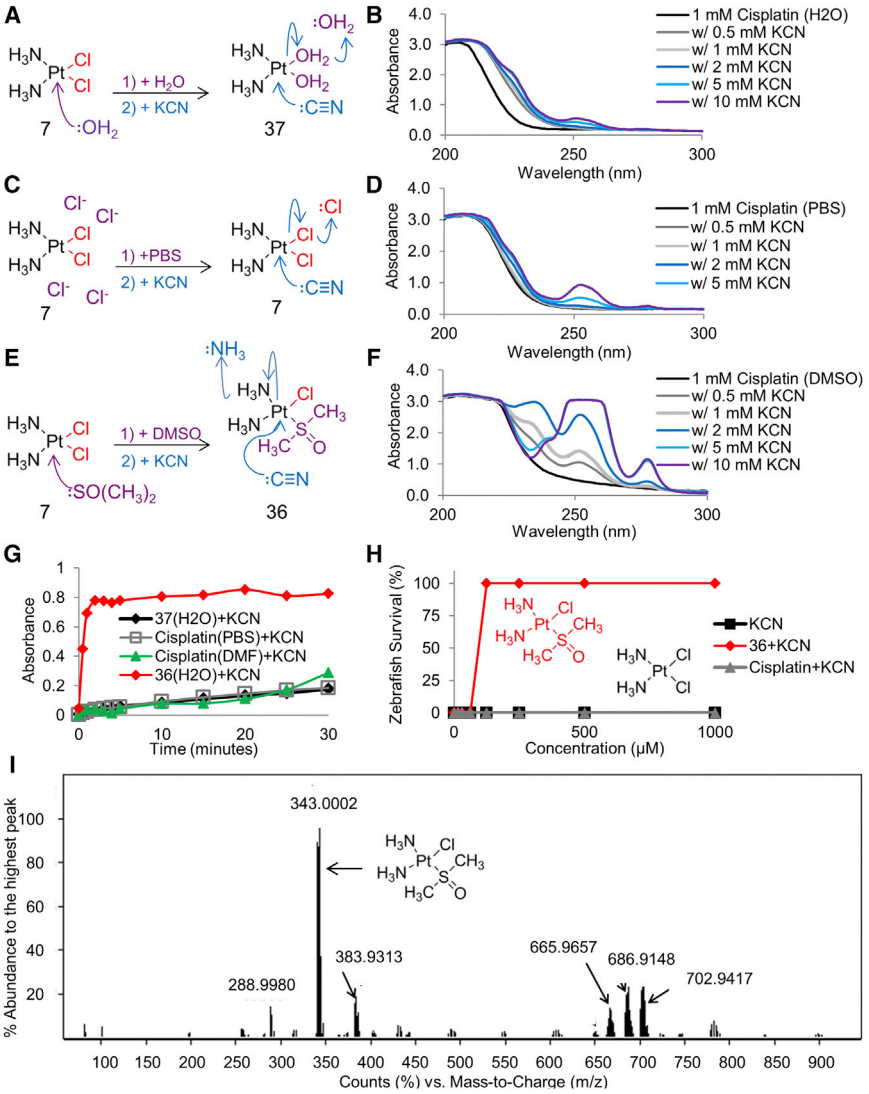

Figure 3. Identification of *cis*-Diammi-nechloro(dimethylsulfoxide)Platinum(II) as a Potent Cyanide Antidote in Zebrafish

(A) Depiction of the associative substitution reaction of water with cisplatin (7), and cyanide with the aquated form of cisplatin (37).

(B) Spectral shift data demonstrating minimal binding of the aquated form of cisplatin to increasing concentrations of cyanide.

(C) Depiction of the solvation effect of PBS on cisplatin, and the associative substitution reaction of cyanide with cisplatin (7).

(D) Spectral shift data demonstrating minimal binding of cisplatin to increasing concentrations of cyanide.

(E) Depiction of the associative substitution reaction of DMSO with cisplatin generating complex 36, and cyanide with complex 36.

(F) Spectral shift data demonstrating binding of complex 36 to increasing concentrations of cyanide.

(G) Graph of the binding of 1 mM complex 37 (black), complex 7 (gray), complex 36 (red), and cisplatin dissolved in DMF (green) to 5 mM cyanide over time, demonstrating the rapid and increased binding rate of complex 36 to cyanide.

(H) Survival assay in zebrafish demonstrating that complex 36 (red) is a cyanide antidote while cisplatin (gray) is not an antidote.

(I) Mass spectrometry identification of *cis*-diamminechloro(dimethylsulfoxide)platinum(II) (complex 36) as the species created by the associative substitution reaction between DMSO and cisplatin. See also Table S2.

cell death (Fischer et al., 2008). To test the cytotoxicity of the compounds under study, cisplatin responsive non-small-cell lung cancer cells (H1975) were used. Cells were treated with 0–300 μM of a platinum complex for 72 hr and cell viability was assessed by measuring ATP levels. Cells treated with 50 μM cisplatin appeared rounded, shrunken, and fragmented, while those treated with complexes 36 or 34 displayed similar morphology to control cells (Figure 4A). At 72 hr, dose-dependent cell killing was observed in cisplatin-treated cells; however, not in cells treated with complex 34 or 36 (Figure 4B). The half maximal inhibitory concentration (IC_{50}) for cisplatin was 62 μM. In an expanded dose-response curve, the IC_{50} values of complexes 34 and 36 were 702 and 689 μM, respectively, an ~10-fold decrease in cytotoxicity.

Activation of p38 MAPK in response to cisplatin-induced DNA damage is a requisite step in the mechanism of action of cisplatin (Galan-Moya et al., 2008). We measured the phosphorylation state of the kinase p38 MAPK in lysates from cells treated with 50 μM of the indicated complexes for 24 hr. As expected, cisplatin activated p38 by inducing phosphorylation (Figure 4C). However, complexes 34 and 36 did not increase activated p38 levels, indicating that these complexes are not initiating the stress-associated signaling pathway that is triggered by cisplatin. These findings are consistent with previous in vitro and in vivo studies, demonstrating the detoxifying effect of DMSO formulations of cisplatin (Hall et al., 2014).

Although the DMSO-bound form of cisplatin undermines the drug's usefulness as a chemotherapeutic drug, the decreased toxicity is a beneficial aspect for its use as a cyanide antidote. Therefore, antidotes identified in the SAR study were solvated in DMSO or PBS and assessed for cytotoxicity in H1975 cells (Figures 4E and S1A–S1D). Several complexes (4, 5, 22, and 31), in both PBS and DMSO formulations, were more cytotoxic than cisplatin. Other complexes (20, 29, 30, 33 and 35) displayed increased cytotoxicity when solvated in DMSO compared with PBS. These two groups of complexes represent compounds with chemotherapeutic potential that may not encounter resistance due to binding with thiol-containing proteins. Several complexes (6, 10, 12, 13, 27, and 32) displayed minimal ability to induce cell death at doses up to 300 μM in both DMSO and PBS formulations. Finally, DMSO inactivated the cytotoxic activity of complexes 3, 11, and 21. These findings indicate that the DMSO adduct form of a few structurally distinct platinum complexes exhibit reduced

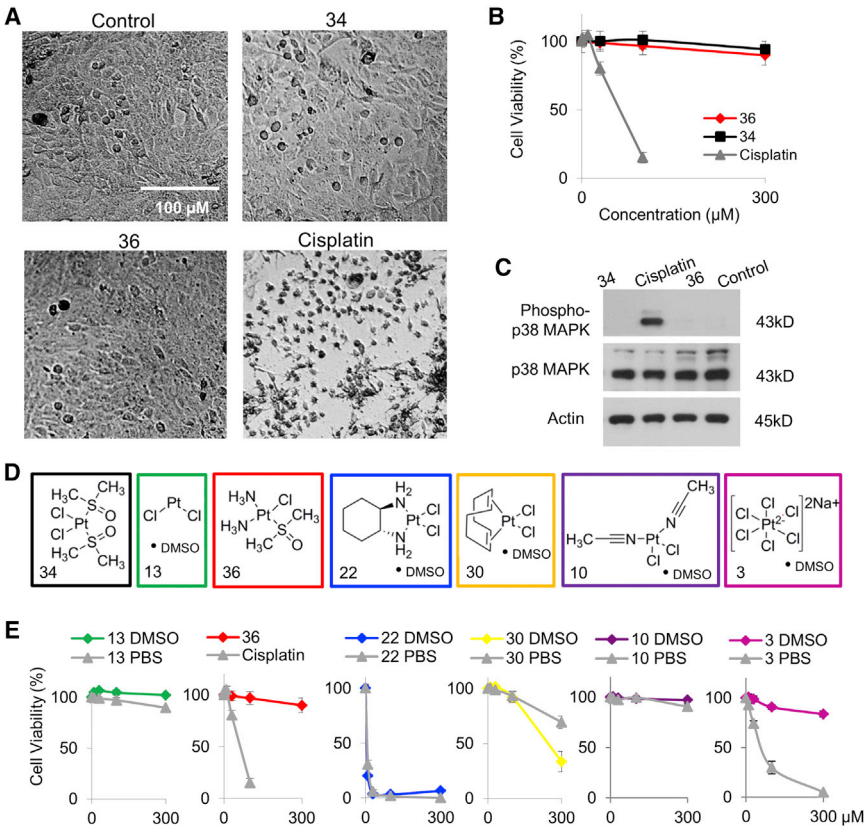

Figure 4. A subset of Cisplatin Analogs Solvated in DMSO Exhibit Decreased Cytotoxicity in Human Cells

(A) Images of H1975 cells treated with vehicle, 34, 36, or cisplatin.

(B) Cell viability over increasing concentrations of 36 (red), cisplatin (gray), and 34 (black). Data represented as the mean ± SD.

(C) Western blots for phospho-p38 MAPK on lysates from cells treated with vehicle, 34, 36, or cisplatin.

(D and E) Complexes from each structural class (D) were dissolved in PBS (gray) or DMSO (color) and cell viability over increasing concentrations was determined (E). Also see Figure S1.

cytotoxicity while maintaining or improving their efficacy as cyanide antidotes.

Cisplatin Analogs Protected Mice Exposed to a Lethal Dose of Cyanide

The successful application of cisplatin analogs as antidotes to cyanide poisoning in zebrafish led us to investigate their effects in a mouse model of cyanide poisoning. In this model, a mouse is placed in a gas-tight chamber and exposed to cyanide gas for 15 min. Subsequently, the mouse is injected intraperitoneally with vehicle or platinum complex and then re-exposed to cyanide gas for 25 min. Thus, total exposure time to cyanide gas is 40 min. All surviving animals are observed for several hours and then euthanized. In this model mice that receive saline consistently died within a 5 min window, 30–35 min after the onset of cyanide exposure (n = 6).

Pt(II) and Pt(IV) cisplatin analogs (36, 34, and 3) were chosen based on efficacy/toxicity in zebrafish, toxicity in human cells, binding kinetics, and solubility. Several other complexes had favorable efficacy and toxicity profiles; however, due to low solubility they will require future formulation studies prior to mammalian testing. Of the mice receiving complex 36 (20 μmol), 83% survived the full exposure period (n = 6) while of those receiving 10 μmol, 33% survived (n = 6) (Figure 5A). Of the mice receiving 20 μmol of complex 34, 100% survived while of those receiving 10 μmol, 33% survived (n = 6) (Figure 5B). For complex 3, 100% of mice receiving 5 μmol and 50% of mice receiving 2.5 μmol survived (n = 6) (Figure 5C). The 4-fold increased potency of complex 3 compared with complex 36 may be because complex 3 binds up to five cyanide anions while complex 36 binds 3–4 cyanide anions (Figure S2). Collectively, these data demonstrate that the effect of cisplatin analogs as countermeasures to cyanide poisoning is conserved in mammals.

To test the hypothesis that formulation in DMSO improves the efficacy of platinum-based cyanide antidotes, we tested complex 3 with or without DMSO. Treatment with 2.5 μmol of complex 3 formulated with DMSO resulted in 50% survival (n = 6). To achieve 66% survival in mice treated with complex 3 formulated without DMSO, a dose of 10 μmol was required (n = 6). These results indicate that DMSO formulation improves the efficacy of complex 3 by ~4-fold and suggests that the improvement in antidote activity is a result of the chemical reaction between DMSO and platinum complexes.

Cisplatin Analogs Reversed Cyanide-Induced Effects on Oxidative Metabolism in Rabbits

Rabbits (n = 5) were infused intravenously (i.v.) with a sub-lethal dose of sodium cyanide (10 mg), while tissue oxygenation in the CNS was monitored in real time using continuous-wave near-infrared spectroscopy (CWNIRS). During the 60 min cyanide infusion, CWNIRS of the CNS detected an increase in the concentration of oxyhemoglobin (red) and decrease in deoxyhemoglobin (blue) (Figure 6A). This occurs as cyanide prevents oxygen offloading from hemoglobin in erythrocytes, thus leading to an increasing fraction of hemoglobin in the oxygenated state. However, when the cyanide infusion stops, both of these curves gradually reverse, indicating oxygen offloading from hemoglobin and an increase in circulating hemoglobin in the deoxygenated state (blue). Thus, the pathophysiological changes associated with sub-lethal cyanide exposure are reversed 30 min following the cessation of cyanide infusion.

To determine if cisplatin analogs alter the kinetics of oxygen offloading from hemoglobin and ameliorate cyanide toxicity in the CNS, rabbits were treated with 15 mg/kg of 36 or 7.5 mg/kg of 3 i.v. after the cyanide infusion (n = 5). There are no rabbit toxicity data on 34, 36, or the DMSO adduct form of

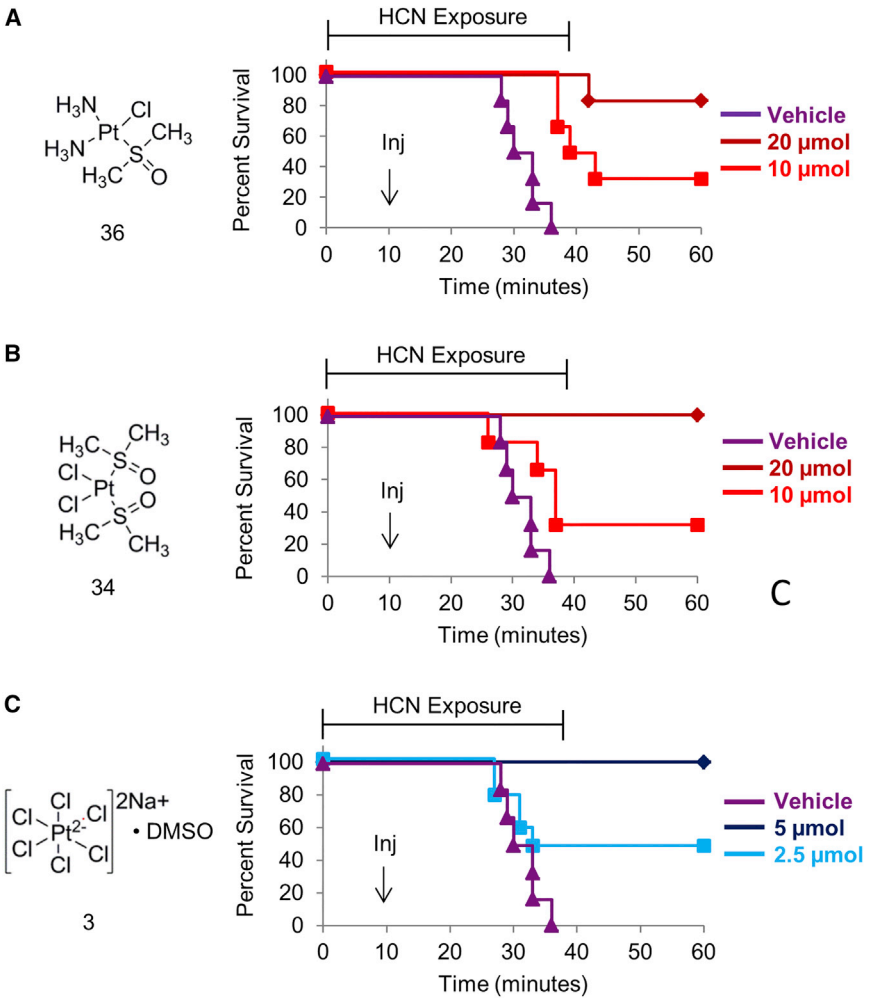

Figure 5. Cisplatin Analogs Protected Mice Exposed to a Lethal Dose of Cyanide

Mice were exposed to cyanide gas for 15 min, injected with the indicated complex (Inj) and placed back in the gas chamber for another 25 min. The data are shown as percent survival versus time. The animals injected with vehicle (purple) consistently died between 30 and 35 min; however, (A) 83% of mice treated with 20 μmol of 36, (B) 100% of mice treated with 20 μmol of 34, and (C) 100% of mice treated with 5 μmol of 3 survived exposure to a lethal dose of cyanide. n = 6. Also see Figure S2.

complex 3 in the literature, but the doses of 3 that were used are well below the lowest dose causing lethality (LDLo) in rabbits (7.5 versus 180 mg/kg) (TOXNET; https://chem.nlm.nih.gov/chemidplus/rn/1307-82-0). As expected, during cyanide infusion, an increase in the concentration of oxyhemoglobin (red) and decrease in deoxyhemoglobin (blue) was detected, indicating cyanide toxicity. However, immediately following the administration of 36 or 3 (Inj), the oxy- and deoxyhemoglobin concentrations rapidly returned to baseline levels (Figures 6C and 6E). Restoration to baseline occurs in less than 10 min compared with vehicle controls in which restoration to near baseline occurs in 30 min (intersection of oxy- and deoxyhemoglobin curves). Further, the time constant (tau) which represents the decay of the oxyhemoglobin was significantly different compared with controls (257 ± 143 min; $p < 0.01$) for both complexes 3 and 36 (6.61 ± 4.41 and 12.15 ± 4.42 min, respectively). Collectively, these changes indicate a reversal of the pathophysiological events induced by cyanide.

We hypothesized that the elimination pathway for cyano-platinum complexes in mammals occurs via the kidneys as both thiocyanate and cisplatin are excreted in the urine. Urine was collected 90 min post antidote injection and cyano-platinum complexes were measured by mass spectrometry. We detected 1.3 ± 0.7 μg/mL of $Pt(CN)_3$ and 0.6 ± 0.3 μg/mL of $Pt(CN)_4$ in the urine of rabbits (n = 3) treated with complex 36. In rabbits (n = 3) treated with complex 3 we detected 30.5 ± 17.6 μg/mL of $Pt(CN)_3$ and 12.9 ± 7.5 μg/mL of $Pt(CN)_4$ in the urine 90 min post injection. These data demonstrate that cyano-platinum species produced by the administration of platinum-based antidotes are excreted into the urine.

Cisplatin Analogs Corrected Cytochrome *c* Oxidase Redox State in Rabbits Exposed to Cyanide

Next, we used diffuse optical spectroscopy to monitor cytochrome *c* oxidase redox state in the muscle of rabbits. During the 60 min cyanide infusion, the cytochrome *c* oxidase redox ratio (black) decreased due to the binding of cyanide anion to iron in cytochrome *c* oxidase and did not return to baseline levels after cessation of the cyanide infusion (Figure 6B). However, when 13 mg/kg of 36 or 7.5 mg/kg of 3 (n = 5) was injected i.v., cytochrome *c* oxidase redox ratio returned to baseline in 10–20 min (Figures 6D and 6F). These findings indicate that cisplatin analogs restore muscle cytochrome *c* oxidase redox state to baseline, indicating that cisplatin analogs are effective antidotes in mammals.

DISCUSSION

Herein we report the SARs of cisplatin analogs and describe their efficacy as cyanide antidotes in zebrafish, mice, and rabbits. Through efficacy, toxicity, and binding studies, we identified the features of cisplatin analogs that confer protection against cyanide poisoning. Ultimately, we identified a Pt(IV) complex (3) as a promising antidote to pursue for further studies.

Serendipitously, we discovered that cisplatin is converted into a cyanide antidote by solvation in DMSO, the standard solvent used to dissolve compounds for chemical screening. After solvation in PBS we identified 5 of 35 complexes (14%) as antidotes, whereas after DMSO solvation we identified 22 of 35 complexes (62%) as antidotes. The increased hit rate in DMSO-solvated complexes occurs because the sulfur atom in DMSO attacks

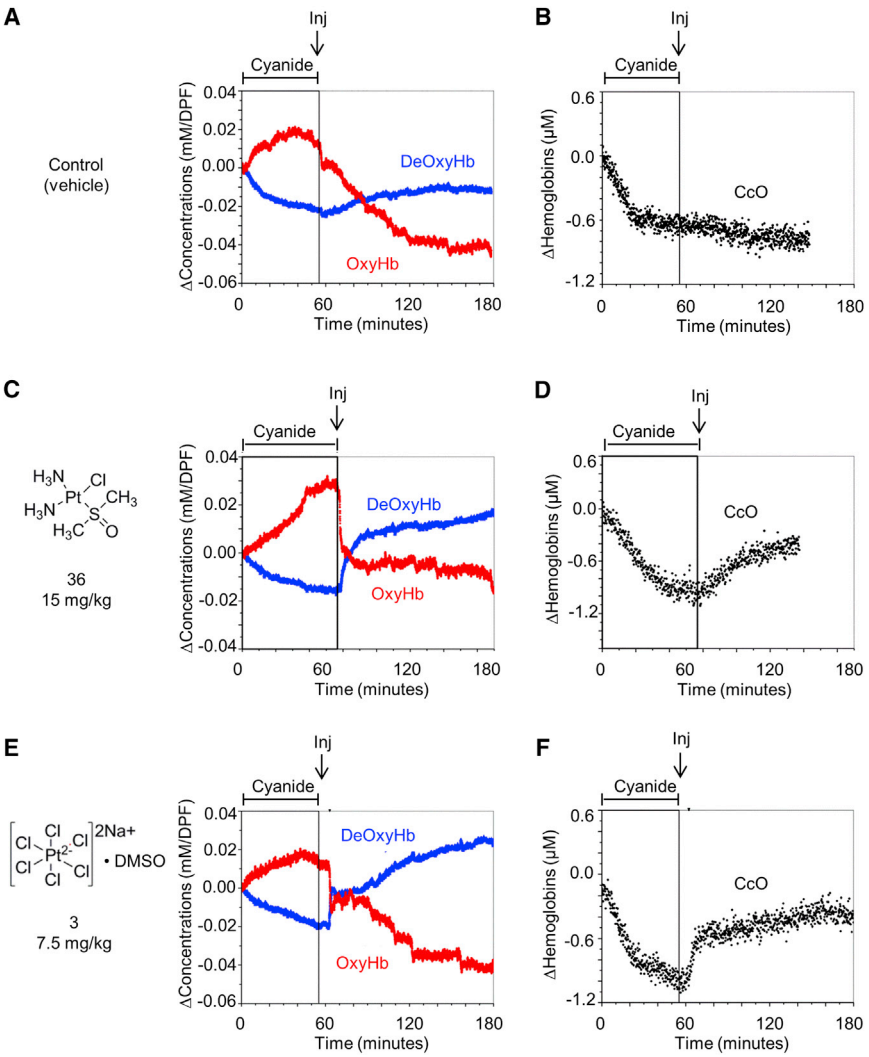

Figure 6. Cisplatin Analogs Reversed Cyanide-Induced Changes in Oxidative Metabolism in Rabbits

(A and B) A representative rabbit injected with cyanide demonstrating (A) increased concentration of hemoglobin in the oxygenated state (red) compared with the deoxygenated state (blue) in the CNS and (B) decreased cytochrome oxidase *c* redox ratio in the muscle (black).

(C–F) Injection of 36 (C and D) or 3 (E and F) after the cyanide infusion results in rapid reversal of cyanide-induced pathophysiologic changes. n = 5.

the platinum atom, displaces ligands, and changes the structure of the complex into a new molecule with different chemical properties.

We speculate that the *trans*-directing effect of sulfur underlies the new substitution kinetics of platinum complexes with DMSO ligands (Basolo and Pearson, 2007). A *trans*-directing ligand affects the lability of the ligand *trans* to itself and directs the positioning of an entering ligand to that position. For instance, complex 36 has one *cis* ammonia ligand, one chloride, and one DMSO ligand, which is *trans* to one of the ammonia ligands. Ammonia ligands are extremely poor leaving groups. However, the potent *trans*-directing effect of sulfur in the DMSO ligand weakens the ammonia ligand bonded *trans* to its position. Thus in this configuration, the ammonia ligand ($-NH_3$) leaves first (Ivanov et al., 1998). Our UV-vis and MS data demonstrate that the cyanide anion rapidly displaces ligands in DMSO-solvated cisplatin analogs. Therefore, the high hit rate of platinum complexes solvated in DMSO compared with PBS suggests that the presence of a sulfur-containing ligand and the *trans*-directing effects of sulfur are key features of platinum-based cyanide antidote activity.

In the SAR study, we identified 22 complexes with antidote activity ranging from an EC_{100} of 62.5–1,000 μM. Cisplatin analogs are capable of binding up to five cyanide anions as demonstrated by our MS data (Figure S2 and Table S2). Their efficacy as cyanide antidotes may be based on the number of ligands displaced by cyanide and the kinetics of displacement of these ligands by associative substitution. In turn, associative substitution reactions are governed by factors including (1) the nature of the metal (in this case platinum), (2) the charge or electrons to be donated by the nucleophile (in this case the cyanide anion), and (3) the characteristics of the ligands coordinated to the metal (the leaving groups). Our SAR study spanned seven structural classes, allowing for the evaluation of the effects of an array of ligands on efficacy.

A number of observations were gleaned from the SAR study regarding the chemical features that conferred cisplatin analogs with the most potency. Critical features included the presence of a sulfur-containing ligand. Further complexes should not be developed to contain ligands that are rigid (multiple-ring structures) or bulky, as steric hindrance may interfere with the molecular rearrangements that occur during nucleophilic attack thereby limiting cyanide anion's ability to access the platinum atom. FDA-approved drugs containing leaving groups such as bidentate carboxyl ligands are less reactive and were ineffective antidotes. Although the prototypic cisplatin analog contains two chloride ligands and two ammine ligands, in 10 of the 14 most potent cyanide antidotes, the platinum was not conjugated to any ammine ligands. Instead they were coordinated to alkene, sulfur, or triphenylphosphine ligands. Bidentate alkene complexes were much more effective antidotes than bidentate ammine ligands. Typically bidentate groups are moderate leavings groups due to the two-step mechanism of ligand loss, but the resonance stabilization properties of alkenes or the *trans* effect of sulfur may facilitate ring opening and subsequent loss of the ligand. Within the Pt(IV) class, four of the seven complexes were antidotes in zebrafish and one was in mammals. Pt(IV) complexes tend to be less toxic than Pt(II) complexes.

Accordingly, complex 3 which was a cyanide antidote at 7.5 mg/kg in rabbits, has an LDLo of 180 mg/kg, subcutaneously, in rabbits compared with cisplatin which has an LDLo of <10 mg/kg in several species (TOXNET; https://chem.nlm.nih.gov/chemidplus/rn/1307-82-0). In summary, complexes should be engineered to have fast substitution kinetics for at least some of the ligands, as cyanide can be lethal within minutes; however, the tradeoff between high reactivity and toxicity must be evaluated carefully. Therefore, Pt(IV) complexes may possess the optimal balance of reactivity, efficacy, and toxicity.

One million new patients in North America receive cisplatin annually even though cisplatin is associated with a number of side effects including nephrotoxicity, neurotoxicity, and ototoxicity (Strumberg et al., 2002). These drugs act by crosslinking DNA strands, which prevents DNA replication and cell division, and ultimately leads to the induction of apoptosis (Jamieson and Lippard, 1999). The histone protein λH2A.X becomes phosphorylated in response to the DNA damage caused by platinum drugs. However, DLD-1 cells treated with cisplatin, carboplatin, transplatin, or [$PtCl_2$(en)] formulated in DMSO do not exhibit this response (Hall et al., 2014). In addition, cleaved PARP and cleaved caspase-3 were not detected, demonstrating the loss of platinum-induced apoptotic events (Hall et al., 2014). In particular one of the most concerning side effects, nephrotoxicity, was decreased in studies with rats using the cisplatin-DMSO adduct species (Fischer et al., 2008). Although the DMSO adduct form of cisplatin undermines the drug's utility as a chemotherapeutic drug, the decreased toxicity is a beneficial aspect for its use as a cyanide antidote because it reduces toxicity while improving interaction with cyanide.

Formal toxicology studies are beyond the scope of this study; however, much can be learned from published efforts to temper the side effects of platinum-based chemotherapeutic drugs. For instance, the decreased toxicity of the cisplatin-DMSO species initiated studies utilizing thiols as reactive "protective agents." Pretreatment with D-methionine or glutathione ester protects against cisplatin-induced toxicity in CNS tissue and kidney, respectively (Anderson et al., 1990; Gopal et al., 2012). Further, the ability of N-acetylcysteine to prevent cisplatin-induced ototoxicity is in phase I clinical trials (NCT02094625). Coordination of protective agents to platinum that could be displaced by the cyanide anion during the associative substitution reaction is an intriguing approach for the development of a dual cyanide antidote and is currently underway in our lab.

Promising strategies to overcome platinum toxicity being pioneered include nanoparticles and targeted encapsulations (Wisnovsky et al., 2013). Lipoplatin is a liposomal-encapsulated formulation of cisplatin that has successfully completed phase III trials for lung cancer and demonstrated significantly reduced toxicity compared with cisplatin, especially in the kidney (6.1% versus 40%) (Stathopoulos et al., 2010). Organelle-specific drug delivery using triphenylphosphine is an effective way to deliver drugs to the mitochondria (Frantz and Wipf, 2010; Zhou et al., 2014). Directly delivering a cyanide antidote to its target organelle could be a significant advance in therapy. Here, complex 22 was shown to be a potent antidote in the SAR study (EC_{100} = 62.5 μM). However, due to the lipophilic triphenylphosphine group, the solubility of complex 22 was in the low micromolar range. To overcome its low solubility, it will likely require specialized formulation. In addition to formulation strategies, medicinal chemistry approaches are being used to mitigate side effects. For instance, replacing the two ammonia groups in cisplatin with a cyclopropanammine group in JM-11 significantly decreased nephrotoxicity in rodents, demonstrating that changes in ligands influence the unique properties of platinum complexes and their in vivo biology (Harrap et al., 1980).

Collectively, using three different vertebrate animal models, the studies reported here represent a proof of concept demonstrating the efficacy of platinum-based complexes as a class of molecules to counteract cyanide poisoning. The SAR data yielded a promising antidote and generated information to rationally design additional platinum antidotes. Future cycles of medicinal chemistry and formulation chemistry are required to bring rationally designed cisplatin analogs through the pipeline that in the future may be first-in-class treatments for cyanide poisoning.

SIGNIFICANCE

Cisplatin is a simple but elegant small molecule that has led to the virtual cure of testicular cancer. Nath et al. discovered that cisplatin analogs are antidotes to cyanide poisoning in three vertebrate species. Using structure-activity relationships they evaluate the efficacy, toxicity, and solubility of cisplatin analogs in human lung cells, zebrafish, mice, and rabbits. These findings lead to the identification of the chemical features of cisplatin analogs that confer protection against cyanide poisoning, thereby contributing to the ongoing effort to discover effective cyanide antidotes and to understand the in vivo biology of cisplatin analogs.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental Information includes two figures and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.chembiol.2017.03.013.

AUTHOR CONTRIBUTIONS

Conceptualization, A.K.N. and R.T.P.; Investigation, A.K.N., J.L., S.M., D.H., A.C., J.M., P.S., and X.S.; Writing – Original Draft, A.K.N.; Writing – Review and Editing, A.K.N., X.S., D.L.H., J.E.M., S.M., A.C., P.S., J.L., C.A.M., G.R.B., M.B., R.E.G., R.T.P.; Supervision, A.K.N., R.T.P., R.E.G., C.A.M., M.B., S.M., and G.R.B.; Resources and Funding Acquisition, R.T.P., R.E.G., C.A.M., M.B., and G.R.B.

ACKNOWLEDGMENTS

This work was supported by NIH U54NS079201.

Received: August 22, 2016
Revised: January 19, 2017
Accepted: March 16, 2017
Published: April 13, 2017

REFERENCES

Alcorta, R. (2004). Smoke inhalation & acute cyanide poisoning. Hydrogen cyanide poisoning proves increasingly common in smoke-inhalation victims. JEMS *29*, suppl 6–15; quiz suppl 16–17.

Anderson, M.E., Naganuma, A., and Meister, A. (1990). Protection against cisplatin toxicity by administration of glutathione ester. FASEB J. *4*, 3251–3255.

Banerjea, D., Basolo, F., and Pearson, R.G. (1957). Mechanism of substitution reactions of complex ions. XII. Reactions of some platinum(II) complexes with various reactants. J. Am. Chem. Soc. *79*, 4055–4062.

Barillo, D.J., Goode, R., and Esch, V. (1994). Cyanide poisoning in victims of fire: analysis of 364 cases and review of the literature. J. Burn Care Rehabil. *15*, 46–57.

Basolo, F., and Pearson, R.G. (2007). The trans effect in metal complexes. In Progress in Inorganic Chemistry, *Volume 4*, F.A. Cottonr, ed. (John Wiley), pp. 381–453.

Braddock, P.D., Connors, T.A., Jones, M., Khokhar, A.R., Melzack, D.H., and Tobe, M.L. (1975). Structure and activity relationships of platinum complexes with anti-tumour activity. Chem. Biol. Interact. *11*, 145–161.

Brenner, M., Kim, J.G., Mahon, S.B., Lee, J., Kreuter, K.A., Blackledge, W., Mukai, D., Patterson, S., Mohammad, O., Sharma, V.S., et al. (2010). Intramuscular cobinamide sulfite in a rabbit model of sublethal cyanide toxicity. Ann. Emerg. Med. *55*, 352–363.

Broughton, E. (2005). The Bhopal disaster and its aftermath: a review. Environ. Health *4*, 6.

Chan, A., Jiang, J., Fridman, A., Guo, L.T., Shelton, G.D., Liu, M.T., Green, C., Haushalter, K.J., Patel, H.H., Lee, J., et al. (2015). Nitrocobinamide, a new cyanide antidote that can be administered by intramuscular injection. J. Med. Chem. *58*, 1750–1759.

Elschenbroich, C. (2006). Organometallics (Wiley-VCH).

Fichtinger-Schepman, A.M., van der Veer, J.L., den Hartog, J.H., Lohman, P.H., and Reedijk, J. (1985). Adducts of the antitumor drug cis-diamminedichloroplatinum(II) with DNA: formation, identification, and quantitation. Biochemistry *24*, 707–713.

Fischer, S.J., Benson, L.M., Fauq, A., Naylor, S., and Windebank, A.J. (2008). Cisplatin and dimethyl sulfoxide react to form an adducted compound with reduced cytotoxicity and neurotoxicity. Neurotoxicology *29*, 444–452.

Frantz, M.C., and Wipf, P. (2010). Mitochondria as a target in treatment. Environ. Mol. Mutagen. *51*, 462–475.

Galan-Moya, E.M., Hernandez-Losa, J., Aceves Luquero, C.I., de la Cruz-Morcillo, M.A., Ramirez-Castillejo, C., Callejas-Valera, J.L., Arriaga, A., Aranburo, A.F., Ramon y Cajal, S., Silvio Gutkind, J., et al. (2008). c-Abl activates p38 MAPK independently of its tyrosine kinase activity: implications in cisplatin-based therapy. Int. J. Cancer *122*, 289–297.

Gopal, K.V., Wu, C., Shrestha, B., Campbell, K.C., Moore, E.J., and Gross, G.W. (2012). d-Methionine protects against cisplatin-induced neurotoxicity in cortical networks. Neurotoxicol. Teratol. *34*, 495–504.

Hall, A.H., Saiers, J., and Baud, F. (2009). Which cyanide antidote? Crit. Rev. Toxicol. *39*, 541–552.

Hall, M.D., Telma, K.A., Chang, K.E., Lee, T.D., Madigan, J.P., Lloyd, J.R., Goldlust, I.S., Hoeschele, J.D., and Gottesman, M.M. (2014). Say no to DMSO: dimethylsulfoxide inactivates cisplatin, carboplatin, and other platinum complexes. Cancer Res. *74*, 3913–3922.

Hao, J., Ho, J.N., Lewis, J.A., Karim, K.A., Daniels, R.N., Gentry, P.R., Hopkins, C.R., Lindsley, C.W., and Hong, C.C. (2010). In vivo structure-activity relationship study of dorsomorphin analogues identifies selective VEGF and BMP inhibitors. ACS Chem. Biol. *5*, 245–253.

Harrap, K.R., Jones, M., Wilkinson, C.R., Clink, H.M., Sparrow, S., Mitchley, B.C.V., Clarke, S., and Veasey, A. (1980). Antitumour, toxic and biochemical properties of cis-platin and eight other platinum complexes. In cis-Platin: Current Status and New Developments, A.W. Prestayko, S.T. Crooke, and S.K. Carter, eds. (Academic Press), pp. 193–212.

Ivanov, A.I., Christodoulou, J., Parkinson, J.A., Barnham, K.J., Tucker, A., Woodrow, J., and Sadler, P.J. (1998). Cisplatin binding sites on human albumin. J. Biol. Chem. *273*, 14721–14730.

Jamieson, E.R., and Lippard, S.J. (1999). Structure, recognition, and processing of cisplatin-DNA adducts. Chem. Rev. *99*, 2467–2498.

Kellin, D. (1929). Cytochrome and respiratory enzymes. Proc. R. Soc. B: Biol. Sci. *104*, 206–251.

Nath, A.K., Roberts, L.D., Liu, Y., Mahon, S.B., Kim, S., Ryu, J.H., Werdich, A., Januzzi, J.L., Boss, G.R., Rockwood, G.A., et al. (2013). Chemical and metabolomic screens identify novel biomarkers and antidotes for cyanide exposure. FASEB J. *27*, 1928–1938.

Siddik, Z.H. (2003). Cisplatin: mode of cytotoxic action and molecular basis of resistance. Oncogene *22*, 7265–7279.

Smith, R.M., and Martel, A.E. (1976). Inorganic ligands. In Critical stability constants. Inorganic Complexes, A.E. Martel, ed. (Plenum Press), pp. 1–129.

Stathopoulos, G.P., Antoniou, D., Dimitroulis, J., Michalopoulou, P., Bastas, A., Marosis, K., Stathopoulos, J., Provata, A., Yiamboudakis, P., Veldekis, D., et al. (2010). Liposomal cisplatin combined with paclitaxel versus cisplatin and paclitaxel in non-small-cell lung cancer: a randomized phase III multicenter trial. Ann. Oncol. *21*, 2227–2232.

Strumberg, D., Brugge, S., Korn, M.W., Koeppen, S., Ranft, J., Scheiber, G., Reiners, C., Mockel, C., Seeber, S., and Scheulen, M.E. (2002). Evaluation of long-term toxicity in patients after cisplatin-based chemotherapy for non-seminomatous testicular cancer. Ann. Oncol. *13*, 229–236.

Wisnovsky, S.P., Wilson, J.J., Radford, R.J., Pereira, M.P., Chan, M.R., Laposa, R.R., Lippard, S.J., and Kelley, S.O. (2013). Targeting mitochondrial DNA with a platinum-based anticancer agent. Chem. Biol. *20*, 1323–1328.

Zhou, W., Wang, X., Hu, M., Zhu, C., and Guo, Z. (2014). A mitochondrion-targeting copper complex exhibits potent cytotoxicity against cisplatin-resistant tumor cells through multiple mechanisms of action. Chem. Sci. *5*, 2761–2770.

STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
phospho-p38 MAPK	Cell Signaling	9211
p38 MAPK	Cell Signaling	9212
β-actin	Cell Signaling	3700
Chemicals, Peptides, and Recombinant Proteins		
Potassium cyanide	Fischer Scientific	P223
Hydroxocobalamin	Sigma-Aldrich	95200
Cisplatin	Sigma-Aldrich	P4394
Potassium tetracyanoplatinate(II)	Sigma-Aldrich	323411
Transplatin	Sigma-Aldrich	P1525
Oxaliplatin	Sigma-Aldrich	O9512
Carboplatin	Sigma-Aldrich	C2538
cis-Dichlorobis(pyridine)platinum(II)	Sigma-Aldrich	400033
cis-Dichlorobis(triphenylphosphine)platinum(II)	Sigma-Aldrich	244945
Dichloro(1,2-diaminocyclohexane)platinum(II)	Sigma-Aldrich	404322
Dichloro(1,10-phenanthroline)platinum(II)	Sigma-Aldrich	400165
Dichloro(ethylenediamine)platinum(II)	Sigma-Aldrich	244929
Nedaplatin	Abcam	ab142046
Picoplatin	Abcam	ab142453
Satraplatin	Abcam	ab142679
Dichloro(dicyclopentadienyl)platinum(II)	Sigma-Aldrich	701203
(2,2′-Bipyridine)dichloroplatinum(II)	Sigma-Aldrich	401609
cis-Bis(acetonitrile)dichloroplatinum(II)	Sigma-Aldrich	277290
Potassium trichloro(ethylene)platinate(II) hydrate	Sigma-Aldrich	244953
Tetraammineplatinum(II) nitrate	Sigma-Aldrich	482293
cis-Diamminetetrachloroplatinum(IV)	Sigma-Aldrich	574759
Hydrogen hexabromoplatinate(IV) hydrate	Sigma-Aldrich	398357
Hydrogen hexahydroxyplatinate(IV)	Sigma-Aldrich	334472
Sodium hexahydroxyplatinate(IV)	Sigma-Aldrich	464708
Sodium hexachloroplatinate(IV) hexahydrate	Sigma-Aldrich	288152
cis-Dichlorobis(dimethylsulfoxide)platinum(II)	Sigma-Aldrich	432849
cis-Dichlorobis(diethylsulfide)platinum(II)	Sigma-Aldrich	767654
cis-Bis(benzonitrile)dichloroplatinum(II)	Sigma-Aldrich	275816
Dichloro(1,5-cyclooctadiene)platinum(II)	Sigma-Aldrich	244937
Dichloro(norbornadiene)platinum(II)	Sigma-Aldrich	763411
Dichlorobis(dimethyl sulfide)platinum(II)	Sigma-Aldrich	732575
Heptaplatin	Abcam	ab142767
trans-Dichlorobis(triethylphosphine)platinum(II)	Sigma-Aldrich	331414
Tetrakis(triphenylphosphine)platinum(0)	Sigma-Aldrich	244961
Bis(tri-tert-butylphosphine)platinum(0)	Sigma-Aldrich	682691
Platinum(II) chloride	Sigma-Aldrich	520632
Platinum(II) bromide	Sigma-Aldrich	206121
cis-Bis(acetonitrile)dichloroplatinum(II)	Sigma-Aldrich	277290
Potassium hexachloroplatinate(IV)	Sigma-Aldrich	379859

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Critical Commercial Assays		
CellTiter-Glo Luminescent Cell Viability Assay	Promega	G7570
Experimental Models: Cell Lines		
H1975 cells	ATCC	CRL-5908
Experimental Models: Organisms/Strains		
Zebrafish: EkkWill	EkkWill Waterlife Resources	NA
Mice: C57/Bl/6J	Jackson Laboratories	000664
Rabbits: New Zealand White	Western Oregon Rabbit Company	NA
Software and Algorithms		
Labview 7.1	National Instrument	http://www.ni.com/download/labview-development-system-7.1/3578/en/
Isotope Distribution Calculator and Mass Spec Plotter	Scientific Instrument Services	http://www.sisweb.com/mstools/isotope.htm

CONTACT FOR REAGENT AND RESOURCE SHARING

Please contact A.K.N. (anjali.nath@aya.yale.edu) for reagents and resources generated in this study.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Zebrafish

Animals were maintained and embryos were obtained according to standard fish husbandry protocols in accordance with the Massachusetts General Hospital Institutional Animal Care and Use Committee. Zebrafish embryos (Ekkwill strain) were grown at 28°C in HEPES buffered Tübingen E3 medium and assayed at 6 d.p.f..

Mice

All studies were carried out according to NIH Guidelines for the Care and Use of Laboratory Animals and approved by the University of California, San Diego Institutional Animal Care and Use Committee. C57/BL6J male (Jackson Laboratories) male mice weighing 20-25 g were used and were fed ad libitum Teklad #7001.

Rabbits

The protocol was reviewed and approved by the University of California Irvine (UCI) Institutional Animal Care and Use Committee (IACUC). Pathogen-free New Zealand White male rabbits (Western Oregon Rabbit Supply), weighing 3.5-4.5 kg were used in this study.

Tissue Culture Cells

H1975 non-small cell cancer cells were grown in RPMI-1640 Medium supplemented with 10% FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 IU/ml penicillin and 100 μg/ml streptomycin.

METHOD DETAILS

In Vivo SAR Studies

The assay was carried out on 6 d.p.f. larval zebrafish loaded in 96-well plates containing HEPES buffered Tübingen E3 medium (n=5 per well). Compounds were screened using a 12 point dose response analysis 0.4-1000 μM. Potassium cyanide was added at a dose of 100 μM which induces 100% death within 1 hour in controls animals. Following the addition of cyanide the plates were sealed with adhesive PCR plate foil and incubated at 28°C. The lowest effective dose to rescue 100% of larvae (EC_{100}) was reported 4 hours post treatment. For assessment of compound toxicity, larvae were treated for 24 hours with compounds and viability was assessed by observing heart rate and response to touch as previously described (Nath et al., 2013). The dose that causes 100% lethality was reported (LD_{100}). All compounds were purchased from Sigma-Aldrich or Abcam. Complex 36 was prepared as previously described and its structure was confirmed by mass spectrometry (Fischer et al., 2008). In the zebrafish assay, each dose of each drug was tested on 5 larvae and these experiments were repeated on 5 separate days.

UV-VIS Spectral Assay

Cisplatin was dissolved in PBS (1 mM), DMSO (1 M), H_2O (1 mM) or DMF (1 mM) and heated for several hours to generate the stock solution at the concentration indicated in parentheses. Subsequently the DMSO stock was diluted to 1 mM in PBS or H_2O. For dose response experiments, from a stock solution of 1M KCN in PBS or H_2O, a cyanide concentration curve from 1-200 mM was generated in PBS or H_2O. The reaction was incubated for 30 minute and the absorbance read over the UV-VIS spectrum on a NanoDrop (Thermo Scientific). For time course experiments, 1 mM complex was reacted with 5 mM KCN and the absorbance was measured every 1 minute for 5 minutes followed by every 5 minutes for 30 minutes. To create a polar aprotic environment, DMF was used as the solvent and assay buffer. All reactions were blanked to a solution of complex only.

Cytotoxicity Assay

Cell viability was evaluated in H1975 cells, a cisplatin responsive non-small-cell lung cancer line. Cells were plated into 96-well plates. When the plates reached 80% confluency, the cells were treated with the indicated compounds for 72 hours. The compounds were dissolved in DMSO or PBS and tested at doses at 0, 3, 10, 30 and 300 μM (eight wells per dose). Viability was assayed using the CellTiter-Glo Luminescent Cell Viability Assay (Promega) by gently removing the media prior to adding CellTiter-Glo reagent to the wells. Three biological replicates were performed.

Western Blot

Cells were treated for 24 hours then lysed in NP-40 lysis buffer [50 mM Tris/HCl, pH 8.0, 150 mM NaCl, 1% NP-40, complete protease inhibitor tablets (Sigma-Aldrich) and PhosStop phosphatase inhibitor tablets (Roche)]. Lysates were ran on NuPAGE Novex 4-12% Bis-Tris Protein Gels (Invitrogen) and transferred to PVDF membranes. The membranes were blocked with 5% BSA for 1 hour at room temperature and probed with antibodies to phospho-p38 MAPK, p38 MAPK and β-actin overnight at 4°C. Three biological replicates were performed.

Mass Spectrometry

The cisplatin-DMSO solution (100 mM) was generated by adding 15 mg cisplatin into 500 μl of DMSO (warmed to 95°C). The solution was incubated in the dark for 1 hour. The hexachloroplatinate-DMSO solution (100 mM) was generated by adding 28 mg hexachloroplatinate into 500 μl of DMSO (warmed to 95°C). The solution was incubated in the dark for 1 hour. Next, a solution of 1M KCN or $K^{13}C^{15}N$ was prepared in water that was adjusted to pH 7.4 with NaOH. Equal volumes of the platinum complexes and the cyanide solution were then combined, vortexed and incubated. The resulting mixture was diluted 10-fold with HPLC-grade methanol and then infused directly into an Agilent 6550 iFunnel Q-TOF Mass Spectrometer equipped with a dual AJS-ESI source at 10 μl/min. The source parameters for acquisition were set as: drying gas temperature and flow was 250°C and 14 L/min respectively; nebulizer was set at 35 psig; sheath gas temperature and flow were 350°C and 11 L/min respectively; Vcap and nozzle voltage were at 1500V and 2000V. The MS-only full scan and/or targeted MS/MS scan in positive mode was acquired through Agilent MassHunter Workstation LC/MS Data Acquisition software (version B.05.01). The mass range of TOF spectra was 70-1700 m/z and the acquisition rate was 1 spectra/s. The reference mass of 121.050873 and 922.009798 was selected for mass correction. The acquired Q-TOF LC/MS data was analyzed with the Agilent MassHunter Workstation Qualitative Analysis software (version B.06.00) for peak identification. To further confirm the structure of identified compounds, the isotope distribution of the observed compound spectra was compared with predicted spectra generated using an Isotope Distribution Calculator and Mass Spec Plotter (http://www.sisweb.com/mstools/isotope.htm).

Mouse Cyanide Inhalation Model

Mice were placed in an acrylic glass chamber and anesthetized by injecting isoflurane into the chamber (2% v/v). The mice become anesthetized within 1-2 min, but 5 min are allowed to be sure they are in a homeostatic state before cyanide exposure. Hydrogen cyanide is generated by injecting 0.1 M KCN into a beaker of 1 M sulfuric acid. Mice are exposed to cyanide gas for 15 min in gas chamber, removed from the chamber, injected with antidote, and re-exposed to cyanide gas for an additional 25 min. The antidotes are prepared in DMSO, diluted 10 fold in saline and injected IP. The animals are anesthetized with isoflurane throughout the cyanide exposure period and all surviving mice are euthanized at the end of the experiment. This model has been previously described (Chan et al., 2015).

Rabbit Cyanide Infusion Model

Animals were anesthetized with an intramuscular injection of ketamine HCl 50mg/kg (Ketaject, Phoenix Pharmaceutical Inc., St. Joseph, MI) and xylazine 5mg/kg (Anased, Lloyed Laboratories, Shenandoah, IA). After the injection, a 23 gauge, 1 inch catheter was placed in the animal's marginal ear vein to administer continuous intravenous anesthesia with ketamine/xylazine. The depth of anesthesia was evaluated by monitoring the animals' physical reflexes and heart rate. Animals were intubated with a 3.0 cuffed endotracheal tube secured by a gauze tie; they were mechanically ventilated (dual phase control respirator, model 32A4BEPM-5R, Harvard Apparatus, Chicago, IL) at a rate of 20 respirations per minute, a tidal volume of 50 cc, and FiO2 of 100%. A pulse oximeter (Biox 3700 Pulse Oximeter, Ohmeda, Boulder, CO) with a probe was placed on the tongue to measure SpO2 and heart rate. Sodium cyanide, 10mg in 60cc normal saline, was infused continuously intravenously at a rate of 1cc/min. Inspired oxygen remained at 100% throughout the experiment. At the end of infusion, antidote compounds were given IV and animals were monitored for an additional

90 minutes. The effects of cyanide toxicity and reversal of toxicity with antidotes were observed in real time using optical spectroscopy. Subsequently, the animals were euthanized with an intravenous injection of Euthasol (1.0 cc, Euthasol, Virbac AH, Inc. Fort Worth, Texas). This model has been previously described (Brenner et al., 2010).

In Vivo Optical Spectroscopy

The details of DOS and CWNIRS methodology have been previously described (Brenner et al., 2010). Briefly, DOS measurements were obtained through a fiber-optic probe placed on the shaved surface of the right inner thigh of the rabbit. The broadband DOS system combines multi-frequency domain photon migration with time-independent near infrared spectroscopy to accurately measure bulk tissue absorption and scattering spectra. Tissue concentrations of oxyhemoglobin, deoxyhemoglobin and cytochrome c redox state (ratio of oxidized to reduced cytochrome c) were calculated by a linear least squares fit of the wavelength-dependent extinction coefficient spectra of each chromophore. CWNIRS penetrates more deeply into tissues than DOS therefore was used to assess oxy- and deoxyhemoglobin effects of cyanide toxicity in the CNS. The CWNIRS system consists of a light source (HL 2000, Ocean Optics, FL), a CCD spectrometer (USB4000, Ocean Optics, FL), and customized optical fiber guides. Continuous wave near infrared light was delivered to the rabbit brain using a fiber optic probe (9mm source-detector separation), and transmitted light intensities at five wavelengths (732, 758, 805, 840, 880 nm) were measured using the CCD spectrometer every second. We quantified changes in oxy- and deoxyhemoglobin concentrations throughout the experiment using a modified Beer-Lamberts' law and those changes are displayed in real time using Labview software (Labview 7.1, National Instrument, TX).

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical parameters are reported in Figure Legends or in Method Details. In the zebrafish assay, each dose of each drug was tested on 5 larvae and these experiments were repeated on 5 separate days. Cell viability experiments were tested three separate times with error bars indicating the SD of 8 wells from one of the three replicates. The western blot experiments were performed on 3 biological replicates. The murine cyanide inhalation model used 6 mice per treatment group. The rabbit cyanide infusion model used 5 rabbits per treatment group.

Cell Chemical Biology, Volume *24*

Supplemental Information

Cisplatin Analogs Confer Protection against Cyanide Poisoning

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Supplemental Figures

Supplemental Figure 1. Related to Figure 4. Cytotoxicity of cisplatin analogues in H1975 cells

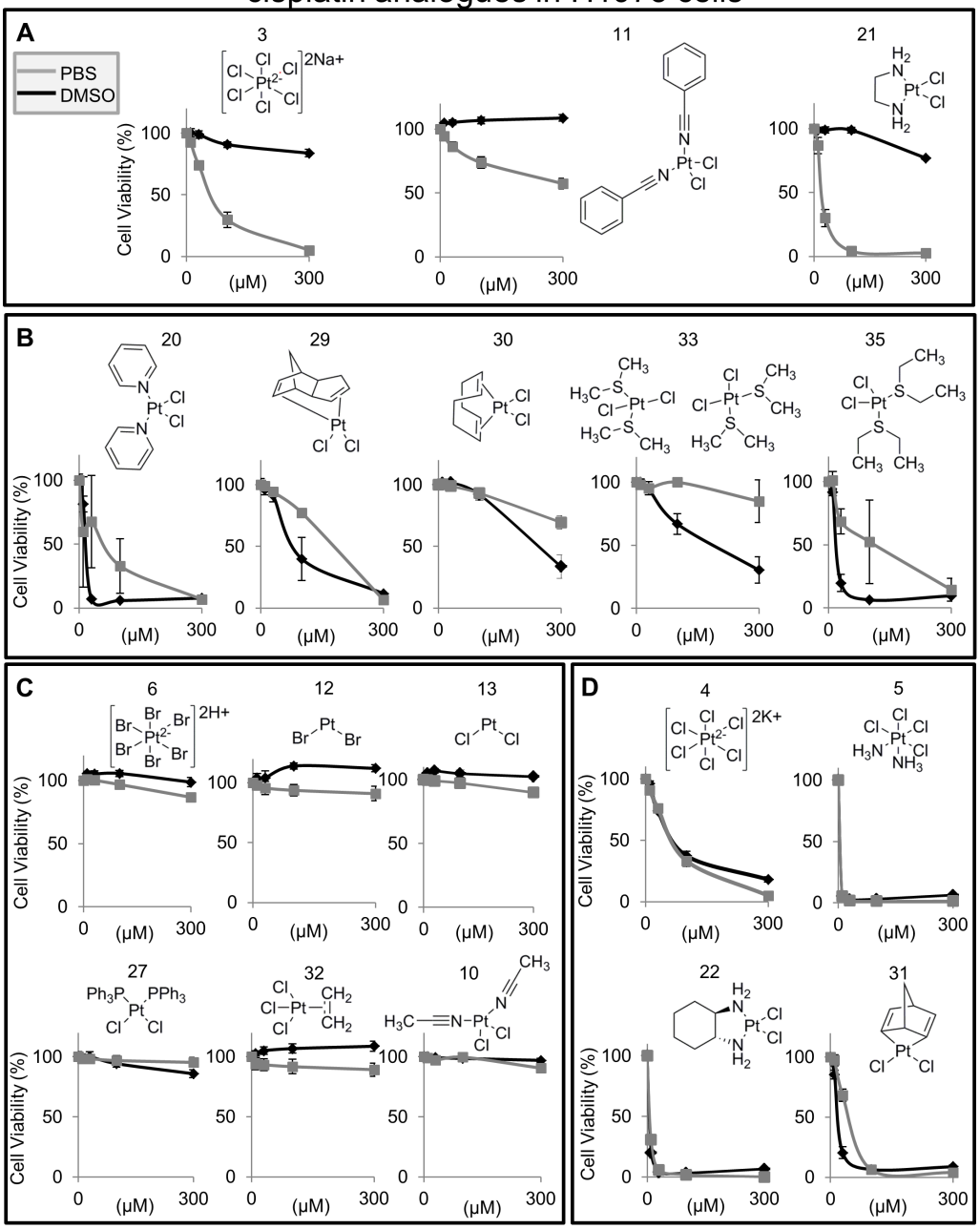

Figure S1 Related to Figure 4. Cytotoxicity of cisplatin analogs in H1975 Cells. Antidotes identified in the SAR study were solvated in DMSO or PBS and assessed for cytotoxicity in H1975 cells. A) DMSO inactivated the cytotoxic activity of complex 3, 11 and 21 while maintaining or improving their efficacy as cyanide antidotes. B) Complexes 20, 29, 30, 33 and 35 displayed increased ability to induce cell death when solvated in DMSO compared to PBS. C) Complexes 6, 10, 12, 13, 27 and 32 displayed minimal ability to induce cell death in both DMSO and PBS formulations at doses up to 300 μM. D) Complexes 4, 5, 22 and 31, in both PBS and DMSO formulations were more cytotoxic than cisplatin. Data represented as the mean ± SEM.

Supplemental Figure 2. Related to Figure 5 and 6. Identification of the products generated by the reaction of hexachloroplatinate(IV) with DMSO and their capacity to bind cyanide anions

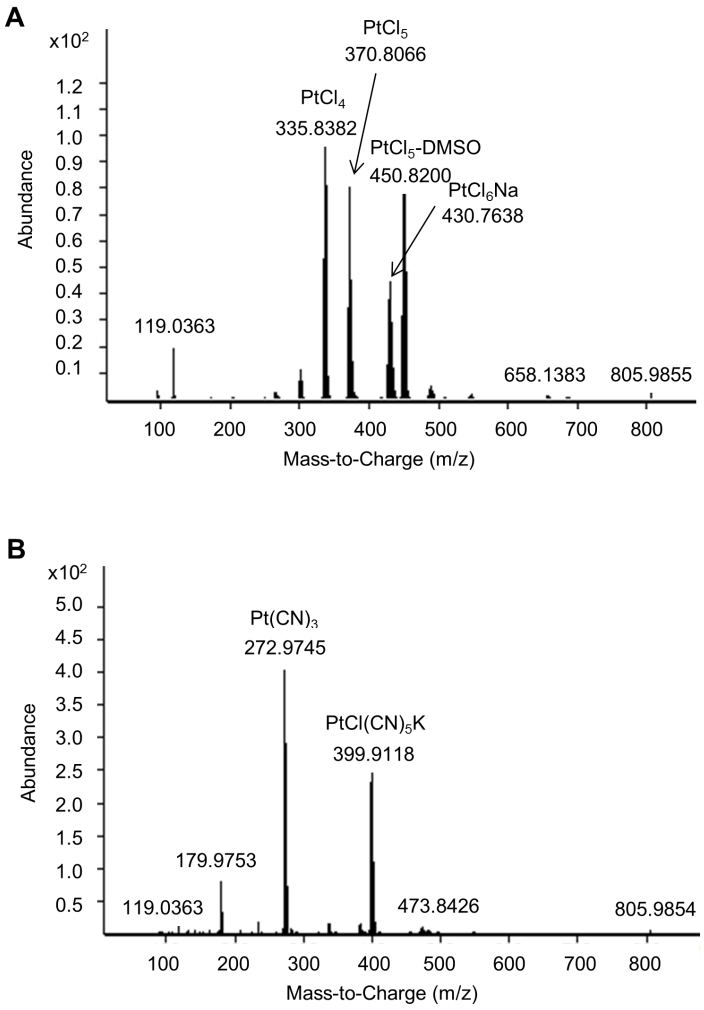

Figure S2 Related to Figure 5 and 6. Identification of the products generated by the reaction of hexachloroplatinate(IV) with DMSO and their capacity to bind cyanide anions. A) To decipher the chemical species created when hexachloroplatinate(IV) is dissolved in DMSO, we used ESI-MS. DMSO undergoes nucleophilic attack of the platinum atom, displacing one chloride ligand and generating $PtCl_5$-DMSO (m/z = 450). An ion signal detected at m/z = 430 corresponds to the starting material ($PtCl_6Na$). B) When cyanide is add to hexachloroplatinate(IV) that has been dissolved in DMSO the most abundant ion signals detected were at m/z = 272 and 399 corresponding to the platinum atom bound to 3 or 5 cyanide anions.

Supplemental Tables

Table S1. Related to Figure 1. Identification of the products generated from the reaction between cyanide and cisplatin (DMSO) using isotope distribution comparison of K12C14N versus K13C15N

Species	Peak in $K^{12}C^{14}N$	Peak in $K^{13}C^{15}N$	Mass Difference
$[Pt(CN)_4]$	337.9	345.9	8
$[Pt(CN)_3]$	272.9	278.9	6
$[Pt(CN)_3DMSO]$	350.9	356.9	6

Table S2. Related to Figure 3. Peaks observed for ESI+ mass spectra of cisplatin dissolved in DMSO

Species	Formula	Observed Mass	Calculated Mass
[Pt(NH3)2(Cl)(DMSO)]+	C2H12ClN2OPtS	342.9996	343.0085
[Pt(NH3)(Cl)(DMSO)2]+	C4H15ClNO2PtS2	403.9868	403.9959
μNH2-[Pt(NH3)(Cl)(DMSO)]2+	C4H20Cl2N3O2Pt2S2	665.9661	665.9670