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Enolate-Forming Compounds Provide Protection From Platinum Neurotoxicity

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Abstract

Cisplatin (CisPt) and other platinum (Pt)-based antineoplastic drugs (e.g., carboplatin, oxaliplatin) are highly effective and widely used in the treatment of solid tumors in both pediatric and adult patients. Although considered to be life-saving as a cancer treatment, Pt-based drugs frequently result in dose-limiting toxicities such as chemotherapy-induced peripheral neuropathies (CIPN). Specifically, irreversible damage to outer hair cells and injury of sensory neurons are linked to profound sensorineural hearing loss (ototoxicity), which complicates tumor management and can lead to a poor clinical prognosis. Given the severity of CIPN, substantial effort has been devoted to the development of neuroprotective compounds, regardless clinical results have been underwhelming. It is noteworthy that Pt is a highly reactive electrophile (electron deficient) that causes toxicity by forming adducts with nucleophilic (electron rich) targets on macromolecules. In this regard, we have discovered a series of carbon-based enol nucleophiles; e.g., N-(4-acetyl-3,5-dihydroxyphenyl)-2-oxocyclopentane-1-carboxamide (Gavinol), that can prevent neurotoxicity by scavenging the platinum ion. The chemistry of enol compounds is well understood and

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Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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mechanistic research has demonstrated the role of this chemistry in cytoprotection. Our cell-derived data were corroborated by calculations of hard and soft, acids and bases (HSAB) parameters that describe the electronic character of interacting electrophiles and nucleophiles. Together, these observations indicate that the respective mechanisms of Pt neurotoxicity and antitumor activity are separable and can therefore be affected independently.

Keywords

chemotherapy-induced peripheral neuropathies (CIPN); HSAB parameters; platinum electrophile; enol neuroprotection

1.1 INTRODUCTION

Cisplatin (CisPt) and other platinum (Pt)-based antineoplastic drugs (e.g., carboplatin, oxaliplatin) are highly effective and widely used in the treatment of solid tumors of childhood and adulthood^{4,62}. The antineoplastic mechanism of CisPt involves formation of intrastrand cross-links that disrupt the DNA helical structure necessary for transcription^{9, 10, 13, 67}. This initiates apoptotic cancer cell death through DNA damage-recognition pathways^{2, 73060}. Although considered to be life-saving chemotherapies, the platinum (Pt)-based drugs are frequently (50% - 60% incidence) associated with toxicity²⁸. In adults and children, treatment can be accompanied by irreversible injury of sensory dorsal root ganglion (DRG) neurons^{13, 33, 50, 54}. The signs and symptoms of this neurotoxicity (NTX) are sensory-based and include tingling, paresthesia, neuropathic pain and loss of vibratory sense^{15, 16, 54, 69}. In children, CisPt chemotherapy is linked to severe sensorineural hearing loss (ototoxicity) mediated by irreversible damage to outer hair cells¹⁰. Hearing loss is lifelong and can lead to social stunting and learning disorders³⁵⁷. Depending upon the severity of intoxication, CisPt neurotoxicity can be therapy-limiting, which complicates tumor management and can lead to a poor clinical prognosis²⁸. Accordingly, substantial effort has been devoted to the development of neuroprotective compounds that prevent CIPN, while preserving the antineoplastic activity of Pt-based drugs.

Contemporaneous research has suggested that changes in cell signaling pathways (e.g., mitogen-activated protein kinase; STAT6 signalling pathway) mediate or otherwise modify CisPt-induced cellular damage^{30,31,34}. However, physicochemical research has shown that the square planar geometry of CisPt is an electrophile (electron deficient species) that causes cell damage via formation of irreversible covalent adducts with functionally critical nucleophilic (electron-rich) sites on cellular macromolecules^{6,17,27,40;58,51,70}. In biological systems, these nucleophiles are primarily reactive anionic thiolate sites on cysteine residues of proteins^{11,20,42,43,44,60,74,75}. Corroborative physicochemical evidence suggested that the Pt electrophile caused cytotoxicity by arresting the function of macromolecules through adduction of critical thiol nucleophiles^{17,40,58,70}. Given this understanding, early pre-clinical studies evaluated cysteine candidates that could act as surrogate nucleophile Pt targets; e.g., N-acetyl cysteine (NAC)^{1,3,4,19,22,60,61,74} and sodium thiosulfate (STS)^{10,19,45,53}. Early research showed that these thiol-based nucleophiles could prevent CisPt neurotoxicity without disrupting antineoplastic efficacy^{8,9}. However, despite encouraging pre-clinical

findings, relatively few effective pharmacological strategies were identified in subsequent human clinical trials^{3,28,57,10}. The development of an efficacious neuroprotectant, therefore, remains an obvious unmet clinical need^{1,4,10}.

We have discovered a series of polyphenol derivatives; e.g., 2',4',6'-trihydroxyacetophenone (THA); N-(4-acetyl-3,5-dihydroxyphenyl)-2-oxocyclopentane-1-carboxamide (Gavinol); that can prevent acetaminophen (APAP)-induced liver damage and other drug-induced toxicities involving electrophilic metabolites (e.g., valproic acid; cyclophosphamide)^{24,73}. The aforementioned phenols are carbon-based nucleophiles (Fig. 1) that, like thiol nucleophiles, can scavenge (react with) Pt-based electrophiles. The chemistry of polyphenol compounds is well understood^{11,20,41,44, 75} and corroborative mechanistic research has demonstrated the role of this chemistry in cytoprotection^{24,35, 42, 73}. In our studies, cytoprotection was related to the ability of these compounds to ionize in biological solutions thereby forming enolate nucleophiles^{11,75}. These nucleophiles have been shown to form complexes (adducts) with a variety of electrophiles such as N-acetyl-p-benzoquinone imine (NAPQI) and the unsaturated aldehydes that play a role in secondary oxidative stress; e.g., acrolein, 4-hydroxy-2-nonenal (HNE). The enolate moiety can also chelate metal ions involved in the free-radical generating Fenton reaction and aromatic polyphenol analogues (e.g., NAHA, gavinol) can trap free radicals^{11,20,40,44}. Thus, the multifaceted protective modes of enol analogues are compatible with the presumed molecular mechanism of cisplatin neurotoxicity. The ability of enols to reduce cell damage in experimental models is based on the fact that the associated toxicities are mediated by a common pathophysiological process involving electrophile-based injury^{37,38,39,41,43}. Thus, like NAPQI, the APAP electrophilic metabolite, Pt is a reactive electrophilic metal that causes neurotoxicity through enzyme inactivation, mitochondrial dysfunction and secondary oxidative stress^{33,50,59}. Based on the stated mechanism of cytoprotection, enol derivatives could directly form Pt-enol complexes which would prevent initiation of the injury cascade³³.

Based on the multifunctional cytoprotective properties of the enols, we have hypothesized that these compounds represent a useful pharmacotherapeutic approach to the clinical management of CIPNs. In this paper, we present evidence which supports the neuroprotective efficacy of polyphenol compounds in CysPt neurotoxicity. We gained significant mechanistic insight by calculating quantum mechanical parameters based on the hard and soft, acids and bases (HSAB) theory of Pearson^{40, 41, 44}. These electron descriptors can provide detailed information regarding the electron character (softness, hardness) and electrophilic reactivity (electrophilicity) of the of Pt metal ion interaction with various nucleophilic biological targets (nucleophilicity). From a mechanistic perspective, the nucleophilic polyphenol compounds can provide neuroprotection by forming a covalent adduct with the Pt electrophile. The resulting complex is non-toxic and will therefore not initiate the neuropathogenic CIPN cascade. We also show that the antineoplastic activity of CisPt is not affected in the presence of polyphenol compounds.

2.1 MATERIALS and METHODS

Chemical Reagents and Animal Use

Unless otherwise stated all biochemicals and reagents were of the highest grade commercially available and were purchased from Sigma-Aldrich (St. Louis, MO). N-(4-acetyl-3,5-dihydroxyphenyl)-2-oxocyclopentane-1-carboxamide (Gavinol) and 4-N-acetyl-2,6-dihydroxyacetophenone (NAHA) are enol-based analogues synthesized by The Chemical Synthesis and Biology Core Facility, Albert Einstein College of Medicine (Dr. Lars Nordstroem, Member Chemist).

Cell Preparations and In Vitro Analyses

All aspects of animal use in this study were in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals and were approved by the Montefiore Medical Center Animal Care Committee. Three month old male C57BL/6N mice (mean weight 27gm) were purchased from Charles River Laboratory (Wilmington, MA). Mice were housed individually in polycarbonate boxes, and filtered drinking water and Purina Rodent Laboratory Chow (Purina Mills, Inc., St. Louis, MO) were available *ad libitum*. The animal room was maintained at approximately 22° C and 50% humidity with a 12 hr light/dark cycle. Prior to each experiment, mice were fasted overnight and livers were excised at 0800 the following morning. Hepatocyte suspensions were prepared from anesthetized mice (isoflurane inhalation) using the collagenase perfusion method of Geohagen et al.^{24,25}. Briefly, to separate dead hepatocytes, isolated cells were centrifuged (140g x 8 mins) in a Percoll gradient and then washed in media (140g x 3 mins) to remove Percoll. The isolation procedure yielded ~30-40 million cells with 80-90% viability as determined by PrestoBlue® (see ahead). Hepatocytes (100,000 cells/ml) were incubated in covered 35mm plastic dishes containing supplemented RPMI-1640 media at 37°C in a humidified atmosphere of 95% O₂/5% CO₂. The concentration-dependent cytoprotection of individual enol and thiol compounds was determined in isolated hepatocytes exposed to CisPt. Control conditions included: vehicle alone (0.1% DMSO in media), vehicle plus CisPt and cytoprotectants alone. PrestoBlue® was used as a sensitive index of cell lethality²⁴ and is based on an oxidized cell-permeant compound (blue resazurin) that is converted exclusively in viable cells to a reduced (red resorufin) form. Cell viability assays were performed according to manufacturer's specifications. Preliminary studies were conducted to establish experimental conditions such as Prestoblue® concentration, cell number and incubation times. Isolated hepatocytes were stained by adding Prestoblue® (50nM final concentration) directly to individual samples followed by incubation for 30 mins at 37°C in an atmosphere of 95% O₂/5% CO₂. At the completion of sample incubation, absorbance was determined spectrophotometrically in a SpectraMax M5 plate reader at a wavelength of 570nm (reduced form) and 600nm (oxidized form). A relative decrease in absorbance ratio (570nm/600nm) represented a corresponding degree of cell lethality.

CisPt cytotoxicity and the corresponding protective efficacies of enol compounds were also determined in cultured rat dorsal root ganglion neurons (DRG; Lonza Walkersville, Inc.). As per manufacturer's instructions, cells were incubated in PNGM Bulletkit that contained Primary Neuron Basal Medium (PNBM) SingleQuots. To promote neuron adherence, plates

were coated with Poly-D-Lysine and Laminin. In general, cells were plated for 48 hrs and then incubated with graded-concentrations of CisPt and/or putative protectant. Drug-induced changes in cell viability were determined using PrestoBlue®. Both DRG and hepatocytes are clinically relevant since these cells are primary *in vivo* targets for CisPt^{15,1632}

Cancer cell lines were used to quantify the effects of Pt-based chemotherapy in the presence or absence of enol compounds. Thus, SH-SY5Y (ATCC CRL-2266) is a human neuroblastoma cancer cell line (bone marrow-derived), U-87 MG (ATCC HTB-14) is a human glioblastoma (brain derived) and HOS (ATCC CRL-1543) is a bone-derived human osteosarcoma. Cells were maintained in ATCC-formulated Eagles's Minimum Essential Medium with F-12 medium and fetal bovine serum (10% final concentration). Cell lines were plated on day 1 at 1500 cells per well. At 48 hours post-plating cells were incubated with graded concentrations of Pt antineoplastic agent and or enol/thiol protectant. All cultures were maintained at 37°C in a humidified atmosphere with 5% CO₂ and cell lethality was determined using the PrestoBlue® method. Our working premise is that the soft nucleophilic enolate moiety forms stable complexes with the soft electrophilic attributes of the Pt ion. This subsequently prevents soft electrophile-mediated cellular toxicity.

Tandem Mass Spectrometry

To provide direct evidence for our hypothesis, tandem mass spectrometry was used to identify enol/Pt complexes. Selected enol compounds (NAHA, THA and gavinol) were incubated *in chemico* with CisPt (2 hr)¹⁸. Flow injection analysis was performed with an HP1100 HPLC using a mobile phase consisting of 50% acetonitrile/water containing 0.1% formic acid and flowing at 75 uL/min. Samples, 40 uL, were diluted with 140 uL of deionized H₂O. After injecting 20 uL of the diluted samples the effluent was connected to an Orbitrap Velos mass spectrometer using positive electrospray ionization and scanning from m/z 110 to 1600. Scans were averaged over the top half of the elution peak and background subtracted to provide the mass spectra of each compound or complex²¹.

HSAB Parameters- Quantum Mechanical Calculations.

To calculate Hard and Soft, Aacids and Bases (HSAB) parameters for the electrophiles and nucleophiles involved in this study, the respective energies of the Highest Occupied Molecular Orbital (E_{HOMO}) and the Lowest Unoccupied Molecular Orbital (E_{LUMO}) were derived using Spartan18 version 1.2.0 software (Wavefunction Inc., Irvine CA). For each chemical structure, ground state equilibrium geometries were calculated with Density Functional B3LYP 6-31G* in water starting from 6-31G* geometries. Global (whole molecule) hardness (η) was calculated as $\eta = (E_{LUMO} - E_{HOMO})/2$ and softness (σ) was calculated as the inverse of hardness or $\sigma = 1/\eta$. The nucleophilicity index (ω^-) was calculated as $\omega^- = \eta_A(\mu_A - \mu_B)^2/2(\eta_A + \eta_B)^2$, where $\mu = (E_{LUMO} + E_{HOMO})/2$, A = reacting nucleophile and B = reacting electrophile; see LoPachin et al.,^{40,41}.

Statistical Analyses

All statistical analyses were conducted using Prism 6.0 (GraphPad software; San Diego, CA) with significance set at the 0.05 level of probability. In all studies, cell viability and other toxic measures (see ahead) were determined in at least n = 4-6 independent experiments.

Concentration-response data were fitted by nonlinear regression analyses^{24,25}. Derived IC₅₀ values for were compared statistically by Student's t test. For analyses of cytoprotective and toxicity indices, statistically significant differences between group mean data were determined by a Bonferroni test for multiple comparisons.

3.1 RESULTS

HSAB Analyses of Pt-Based Chemotherapy and Toxicity.

Our results demonstrate that the cytoprotection afforded by carbon (enolate)-based nucleophiles involves the covalent scavenging of electrophiles (e.g., NAPQI, acrolein) that mediate many types of drug-induced toxicity. However, these electrophile-nucleophile reactions do not occur indiscriminately and instead exhibit a significant degree of selectivity as defined by the HSAB theory of Pearson. Based on relative electron mobility (polarizability), electrophiles and nucleophiles are classified as being either soft (polarizable) or hard (non-polarizable) and, in accordance with HSAB principles, electrophiles will react preferentially with nucleophilic targets of comparable softness or hardness. The “hard” or “soft” nature of a chemical can be calculated based on inherent electronic characteristics; i.e., respective frontier molecular orbital energies. These values can be used to quantify corresponding electrophilicity (ω) or nucleophilicity (ω^- ; reviewed in^{39,43}). Our HSAB calculations indicate (Table 1) that the Pt metal ion is a highly reactive soft electrophile; e.g., Pt ω 127.869 = vs. CisPt ω = 3.428. However, ligation of the Pt with Cl and amine groups yields a square planar compound, *cis*-diaminedichloroplatinum (II; CisPt), with substantially lower softness (σ = 1.639 $\rightarrow$ 0.400) and electrophilicity (ω = 127.869 $\rightarrow$ 3.428).

Although the transport mechanism is unknown, CisPt entry into the cell (Fig. 2) is associated with aquation, where the facile chlorine (Cl) leaving groups of CisPt are displaced by water molecules. This process hardens the soft electrophilic character of CisPt which redirects targeting toward hard DNA sites (Table 1). In an attempt to reduce adverse outcomes mediated by electrophilic CisPt, the ligands of many Pt-based chemotherapeutics are, by design, poor leaving groups. For example, the 1,2-diaminocyclohexane leaving group of oxaliplatin lowers electrophilicity (ω = 4.375 $\rightarrow$ 2.444), which might be related to the lower side effects during the course of treatment (Table 1). Similarly, the bidentate dicarboxylate ligand of carboplatin is a poor leaving group and, as a result, the respective electrophilicity is reduced substantially (aquated ω = 4.375 $\rightarrow$ 1.289). The hardness of carboplatin is significantly increased whereas the softness is decreased when compared to CisPt. (Table 1). The carboplatin electronic profile would thus preferentially target hard DNA sites, whereas CisPt would interact with soft cellular sites.

Mass Spectrometry Analysis of Enolate-cisplatin complex formation.

Our working premise is that the soft nucleophilic enolate moiety forms stable complexes with the soft electrophilic attributes of the Pt ion. This subsequently prevents soft electrophile-mediated cellular toxicity. To investigate this hypothesis, tandem MS was used to identify enol/Pt complexes. Selected enol compounds (NAHA, THA and gavinol) were incubated *in chemico* with CisPt. Results showed that CisPt and, for example, NAHA were

observed separately at 306.01 and 210.07 (Fig. 3;)⁷¹ However, tandem MS (insert) using a wide isolation window revealed complexes composed of Pt/Cl/NAHA (m/z 444) and Pt/Cl/NH₃/NAHA (m/z 463). MS analyses of the other listed enol compounds indicated similar complexation⁷¹. These data suggest that enolate-forming chemicals can form varied organometallic adducts with CisPt and therefore support our mechanistic hypothesis.

Enolate-Forming Compounds Prevent Platinum-Based Cytotoxicity.

The preceding MS analyses support the contention that the soft nucleophilic enolates prevent toxicity by forming complexes with soft CisPt electrophiles. To test this possibility further, CisPt (1 μ M) and graded concentrations of enol (gavinol, NAHA and THA)- or thiol (NAC)-compounds were pre-incubated together for 30mins^{18,52}. Neonatal rat DRG cells (Fig. 5A) and isolated mouse hepatocytes (Fig. 5B) were exposed to the CisPt/protectant pre-mixtures. Results indicated that CisPt caused concentration-dependent lethality in both cell models. Co-exposure of CisPt with gavinol or NAHA demonstrated that these enol derivatives provided concentration-dependent protection that was significantly more efficacious than thiol (NAC) protectants (Fig. 4). The prevention of CisPt cytotoxicity represents inhibition of soft-soft interactions between CisPt (soft electrophile) and cysteine thiolate sites (soft nucleophiles) that mediate cytotoxicity. The remaining capabilities for hard-hard interactions represent a chemotherapeutic mechanism that involves the binding of cisplatin to, for example, hard N(7) purine bases³⁰. The subsequent formation of stable inter- and intra-strand DNA crosslinks block replication and transcription which ultimately leads to selective cancer cell lethality.

Toxicity of Pt Complexes.

To exclude the possibility that the complexes themselves were toxic, enol compounds were pre-incubated with CisPt (30 min). DRG or isolated hepatocytes were exposed (4 hr) to enol/Pt complexes and respective changes in cell lethality were determined. Results (Fig. 5) were compared to respective data from hepatocytes similarly exposed to CisPt, carboplatin or oxaliplatin. Findings indicated that exposure to the enol-Pt complexes (NAHA-platin; gavinol-platin) did not cause hepatocyte or DRG toxicity over a broad concentration range. Carboplatin-enol produced intermediate cytotoxicity relative to that caused by CisPt or oxaliplatin. Our results suggest that enol compounds prevent toxicity by forming non-toxic complexes with Pt-based congeners. In contrast, exposure of normal cells to CisPt or oxaliplatin complexes, caused substantial concentration-dependent DRG/hepatocyte lethality. The rank order of cytotoxicities across aquated CisPt, oxaliPt and carboPt was consistent with their respective electrophilicity order (Table 1). The enol-Pt complexes are due to the interaction of soft enol ligands (e.g., NAHA, Gavinol) with the soft attributes of Pt. Although the exact structure of the enol-Pt complex has not been determined, as predicted by the HSAB quantum parameters (Table 2), the presumed Pt complexes with NAHA and gavinol are clearly non-toxic. As indicated, the enol is soft and does not block the hard reactions of Pt with hard DNA targets (Fig. 2). By complexing Pt, the enol acts as a soft target and thereby prevents soft Pt-based cytotoxicity. Carboplatin was less toxic than the other platinum analogues. This partial effect is likely to be due to the fact that carboplatin is a hard drug with relatively low electrophilicity (Table 1). The limited electrophilic reactivity is likely related to ligand retention strength. That is, CisPt carries 2

chlorines and 2 amine groups, whereas the carboplatin leaving group is a cyclobutane-1,1-dicarboxylic acid. The CisPt chlorines are excellent leaving groups, whereas the ligand retention strength for cyclobutane is very high which will, in turn, lower the effective electrophilic reactivity. The partial toxicity of carboplatin is also consistent with fact that this congener is a modestly reactive soft electrophile (Table 1).

Enol Modulation of CisPt Chemotherapeutic Efficacy.

It is possible that application of a putative enol neuroprotectant might reduce or otherwise modify CisPt chemotherapeutic efficacy. To address this possibility, different cancer cell lines [glioblastoma (U87 MG), osteosarcoma (HOS) and neuroblastoma (SH-SY5)] were coincubated with graded concentrations of CisPt in the presence or absence of an enol (NAHA or Gavinol). Results (Fig.6) indicate that enol-based compounds did not alter the ability of CisPt to kill glioblastoma (Fig. 6A) or neuroblastoma (6C) cells as evidenced by the quantitatively similar IC_{50} 's. In osteosarcoma cells (Fig. 6 B), however, CisPt complexes of gavinol or NAHA significantly lowered the respective IC_{50} 's indicating a potentiation of cancer cell death. Parallel studies showed that thiol-based protectants (e.g., NAC) also did not modify CisPt-induced cancer cell toxicity (Fig. 6). Additional experiments were conducted to determine the effects of NAHA or gavinol pre-incubation (30 mins) with CisPt (Fig. 7). Results indicate that the presumed CisPt-enol complex formation (e.g., NAHA-Pt; Gavinol-Pt) did not modify the antineoplastic activity of CisPt compounds in glioblastoma (Fig. 7 A), osteosarcoma (Fig. 7 B) or neuroblastoma (Fig. 7 C) cancer cells. However, carboplatin, and to a lesser extent oxaliplatin, both exhibited reduced cancer cell lethality (Fig. 7). This reduced efficacy is related to the lower electrophilicities (ω) of these antineoplastic compounds (see Table 1). Finally, we tested the inherent toxicity of the enol and thiol protectants in different cancer cell cultures (see Figs 6 and 7). Thus, for example, NAC and the selected enols were relatively weak toxicants in neuroblastoma cells (Fig. 8).

4.1 DISCUSSION

Results from clinical and experimental studies indicate that sensory neurons of the DRG and outer hair cells (OHC) of the ear are primary cisplatin (CisPt) targets^{10,15,16, 54,63}. The vulnerability of these cell types to CisPt is not well understood. Nonetheless, the most prominent neuropathological change in DRG neurons from CisPt-intoxicated rats was nucleolar shrinkage and disintegration^{14,15,16,47}. This neuropathological feature in conjunction with selective accumulation of CisPt in DRG suggested that DNA was the common molecular target for both tumor prevention and neuronal injury^{46,47,48,56,64}. However, other research indicated that the mechanism of CisPt neurotoxicity does not involve DNA alterations. For example, there is evidence that neurotoxicity is not correlated with DNA adduct formation^{2,7,36,46} nor does toxicity require the presence of a cell nucleus^{30,49,63}. Thus, although DNA adduct formation is the antineoplastic mechanism of CisPt, the role of these adducts in neurotoxicity is uncertain. This ambiguity suggests that CisPt-induced chemotherapy and neurotoxicity occur via independent mechanisms.

Cisplatin Electrophilic Character.

We have proposed a molecular mechanism for CisPt-induced neurotoxicity based on the electronic character of platinum (Pt) ions and also our research investigating electrophile-mediated toxicities (Fig. 2). Thus, square planar CisPt is a soft electrophile that reacts with soft biological nucleophilic targets. In accordance with HSAB principles, electronic characteristics can be computed from the energies of the respective frontier molecular orbitals; i.e., the highest occupied molecular orbital (E_{HOMO}) and the lowest unoccupied molecular orbital (E_{LUMO}). These energies have been used to develop parameters that define the electrophilicity (ω) and nucleophilicity (ω^-) of chemical species. Thus, CisPt is considered to be a reactive soft electrophile since electrons in the frontier molecular orbitals are polarizable as reflected in the respective softness (σ) and electrophilicity (ω) values (Table 1). The HSAB profile of CisPt is similar to those of acrolein and N-acetyl-p-benzoquinone imine (NAPQI; the toxic metabolite of acetaminophen), both of which are known to be highly reactive soft electrophiles (Table 1). Research over the past decade has demonstrated that soft electrophilic toxicants such as CisPt, acrolein and NAPQI react preferentially with soft cysteine thiolate groups (-S^-) in the active zones of many enzymes and proteins. Such adduct formation causes protein inactivation that disrupts critical cellular processes; e.g., anterograde axonal transport, vesicle-membrane fusion and mitochondrial function, which subsequently leads to secondary oxidative stress and neuronal cell death^{26,37,39,43,47,55}. Pt orbitals also include non-polarizable electron densities that denote hard electrophile character (η ; Table 1). These densities preferentially react with hard nucleophilic attributes. Thus, in Table 2, compare the ω^- values for the reactions of Pt compounds with guanine (hard) vs. NAHA or gavinol (soft). The hard electrophilic character of Pt compounds will preferentially react with hard nucleophiles such as the oxygen and nitrogen atoms of DNA nucleobases. As soft carbon nucleophiles, our enol compounds react slowly with the hard cisplatin orbitals and will not therefore interfere with the hard-hard chemotherapeutic activity of cisplatin with DNA-based nucleophiles²⁷. Consistent with this proposed mechanism, research has shown that enol (e.g., acetylacetone, NAHA) complexation with cisplatin did not impair the corresponding antitumor properties of this metal (Figs. 6 and 7). In addition, early animal studies involving soft thiol nucleophiles (e.g., NAC, DEDTC) demonstrated that these compounds could prevent CisPt neurotoxicity without disrupting antineoplastic efficacy. Together, these observations indicate that the respective mechanisms of neurotoxicity (soft-soft reactions) and chemotherapy (hard-hard reactions) are separable and can therefore be affected independently (see also Figs. 6 and 7). In other words, the CisPt-DNA adducts in non-mitotic (normal) cells are repairable, whereas the adducts in mitotic (cancer) cells are irreparable and lethal.

We have discovered that polyphenolic derivatives (e.g., NAHA and Gavinol) are highly protective in animal models of electrophile-induced toxicities (e.g., acetaminophen overdose^{24,73}) and organ ischemia-reperfusion injury (IRI)³⁵. The chemistry of the enol compounds is well understood^{5,11,20,41,42,75} and corroborative mechanistic research has demonstrated the role of this chemistry in cytoprotection^{24, 25, 35, 39, 41, 42, 44}. In our studies, cytoprotection (e.g., Fig. 2) was related to the ability of these compounds to ionize in biological solutions thereby forming soft enolate nucleophiles¹¹. These soft nucleophiles have been shown to

form complexes or adducts with a variety of soft electrophiles such as NAPQI and the unsaturated aldehydes that play a role in secondary oxidative stress; e.g., acrolein, 4-hydroxy-2-nonenal (HNE)^{24,42}. With respect to CisPt neurotoxicity, we have shown that enols can form soft-soft complexes with CisPt and therefore prevent cytotoxicity (Fig. 4). The enolate moiety can also chelate metal ions involved in the free-radical generating Fenton reaction and aromatic polyphenol analogues (e.g., NAHA, gavinol) can trap free radicals^{39,41,43}. Thus, the multifaceted protective modes of enol analogues are compatible with the presumed molecular mechanism of cisplatin neurotoxicity. In contrast, other classes of putative neuroprotectants, such as antioxidants and thiol-based nucleophiles, are less effective since the corresponding mechanisms of protection are limited. These compounds are, therefore, incapable of directly arresting cisplatin neurotoxicity. The ability of enols to reduce cell damage in experimental models (see above) is based on the fact that the associated toxicities are mediated by a common pathophysiological process involving electrophile-based injury^{41,42}. Thus, Pt is a reactive soft electrophilic metal that causes neurotoxicity through enzyme inactivation, mitochondrial dysfunction and secondary oxidative stress. In addition, CisPt has hard electrophilic character that can mediate interactions with hard nucleophilic sites on DNA; e.g., nitrogen, oxygen residues. This hard-hard interaction causes DNA cross-linking which prevents transcription and thereby mediates cancer cell death. Based on the stated mechanism of cytoprotection, enol derivatives can directly form Pt-enol complexes which can prevent initiation of the injury cascade displayed in Fig. 2. The soft-soft interaction effectively prevents cytotoxicity associated with soft electrophiles. In contrast, the soft enol nucleophile will react slowly with the hard CisPt orbitals (i.e., soft-hard reactions). Thus, the soft enol moiety will not interfere with the hard-hard chemotherapeutic reaction of CisPt with DNA-based nucleophiles. Regarding clinical application, our intention is to co-formulate an enol with CisPt. This complex would therefore represent a safer antineoplastic preparation since it would be rendered incapable of causing soft-soft mediated neurotoxic adverse effects.

Thiol- Centered Nucleophiles as Neuroprotectants.

Sulfur is a reactive soft element that can participate in numerous non-selective side reactions. Most notable, highly toxic thiyl radicals are produced during redox cycling between a thiol and the corresponding disulfide. In this regard, thiol-based compounds can act as pro-oxidants⁶⁸. Furthermore, the pKa values for the majority of tested thiols are basic (e.g., NAC = 9.5; DEDTC = 11.3; STS = 11.5) and therefore the corresponding sulfhydryl groups exist mostly in the non-nucleophilic thiol state, which limits electrophile (platinum analogues) scavenging^{1,61,65}. In contrast to the thiol compounds, enols are soft carbon-based electrophiles that do not participate in undesired side-reactions (e.g., form radicals). Unlike the thiol compounds, the nucleophilic carbon atoms of our enolate-forming drugs have relatively neutral pKa values (e.g., 2-ACP = 7.8; THA = 7.7; NAHA = 7.9), which indicates that at physiological pH significantly more of the carbon compounds exist in the nucleophilic enolate (anionic carbon) state. Recent phase 3 clinical trials by Freyer et al²³ have provided evidence that STS can reduce the incidence of hearing loss in cisplatin-treated children. However, the modest protection achieved required a very high dose; 533 mg/kg given i.v. 6 hrs after cisplatin, which reflects the limited efficacy of thiol protectant. In this regard, STS is negatively charged at physiological pH, which means that this thiol

compound cannot cross cell membranes and gain entry to the internal milieu where Pt is acting to cause cytotoxicity. This latter concept is also a basis for the high doses needed; i.e., the high extracellular STS concentration increases cross-membrane passive entry. Thus, although STS has been shown to be safe in clinical trials, it has low efficacy as a neuroprotectant.

5.1 CONCLUSIONS

Our data suggest that enol neuroprotection can be achieved without reducing the chemotherapeutic efficacy of cisplatin. Because Pt-based chemotherapeutic agents are highly effective in the treatment of solid tumors, development of enolate-based neuroprotectants could improve clinical outcome and patient quality of life by reducing the debilitating, therapy-limiting effects of Pt neurotoxicity.

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Highlights

- Carbon-based enol nucleophiles scavenge the platinum ion *in vivo*
- Enolate-forming compounds provide protection against platinum-induced ototoxicity
- CisPt-associated neurotoxicity and antitumor activity mechanisms are independent

Fig. 1. Chemical structures. A: 2-ACP (2-acetylcyclopentanone); B: THA (2',4',6',-trihydroxyacetophenone) and C: NAHA (4-N-acetyl-2,6-dihydroxyacetaphenone).

Fig. 2.

Although the mechanism of CisPt [*cis*-diamminedichloroplatinum(II)] cellular entry (1) is unknown, cell entry is associated with “aquation” (2) where the chlorine (Cl) ligands of CisPt are displaced by water molecules (3). This process hardens the soft electrophilic character of CisPt (Table 1) and, as a result, this harder molecule reacts more favorably with hard guanine DNA targets (4). Non-aquated soft CisPt (5) can also react with guanine, although this soft-hard interaction is not kinetically favored. The chlorinated soft CisPt electrophile (6) can form adducts with anionic sulfur groups on GSH and proteins. The aquated CisPt (7) can also deplete GSH content, although this soft-hard reaction is also not favored. The soft-soft reaction of thiolate targets with CisPt causes (8) protein inactivation, GSH depletion, mitochondrial dysfunction and secondary oxidative stress. This toxic cascade results in sensory neuropathy and/or ototoxicity (9).

Fig. 3.

Flow injection analysis was performed with an HP1100 HPLC using a mobile phase consisting of 50% acetonitrile/water containing 0.1% formic acid and flowing at 75 μ L/min. Samples, 40 μ L, were diluted with 140 μ L of deionized H₂O. After injecting 20 μ L of the diluted samples the effluent was connected to an Orbitrap Velos mass spectrometer using positive electrospray ionization and scanning from m/z 110 to 1600. Scans were averaged over the top half of the elution peak and background subtracted to provide the mass spectra of each compound

Fig. 4.

In this study, we determined the relative abilities of enol (Gavinol, NAHA and THA)- and thiol (STS, NAC)-compounds to prevent CisPt-induced cytotoxicity in neonatal rat DRG cells (Fig. 4A) and freshly isolated mouse hepatocytes (Fig. 4B). Results indicated that CisPt caused concentration-dependent toxicity in both cell models and that coadministration of CisPt with THA, NAHA or Gavinol provided substantial cytoprotection. In these studies, NAC was ineffective, whereas STS provided protection.

Fig. 5.

The preceding data are consistent with the possibility that enol compounds form non-toxic complexes with cisplatin and corresponding analogues. To test this possibility, enol compounds were **pre-incubated with CisPt** (30 mins). DRG cells and isolated mouse hepatocytes were exposed (4 hrs) to enol-Pt complexes and respective changes in lethality were determined. Results were compared to respective data from hepatocytes similarly exposed to CisPt, carboplatin or oxaliplatin. Results indicate that exposure to the enol-Pt complexes (NAHA-platin; gavinol-platin) did not cause hepatocyte or DRG cytotoxicity over a broad concentration range. In contrast, exposure of normal (non-cancer) cells to CisPt or oxaliplatin complexes, caused substantial concentration-dependent DRG/hepatocyte lethality. Carboplatin produced intermediate cytotoxicity relative to that caused by CisPt or oxaliplatin. Our results suggest that enol compounds prevent toxicity by forming non-toxic complexes with Pt-based analogues.

Fig. 6.

To test the possibility that putative enol neuroprotectants might reduce or otherwise modify CisPt chemotherapeutic efficacy, different cancer cell lines were co-incubated with graded concentrations of CisPt in the presence or absence of an enol (NAHA or Gavinol)- or thiol (STS)-nucleophiles. Results indicate that enol-based compounds did not alter the ability of CisPt to kill glioblastoma (6A) or neuroblastoma (6C) as evidenced by the quantitative similarity of respective $IC_{50,s}$. In osteosarcoma cells (6B) however, CisPt complexes of gavinol or NAHA significantly lowered the respective $IC_{75,s}$ indicating a potentiation of cancer cell death. Parallel studies showed that a selected thiol- protectant (STS) also did not modify CisPt-induced cancer cell toxicity (Fig. 6 A-C).

Fig. 7.

Experiments were conducted to determine the effects of NAHA or Gavinol preincubation (30 mins) with CisPt or analogues. Results indicate that the presumed CisPt complex formation (e.g., NAHA-Pt; Gavinol-Pt) did not modify the respective antineoplastic activities of CisPt compounds in any cancer cell line. However, carboplatin, and to a lesser extent oxaliplatin, both exhibited reduced cancer cell lethality (Fig. 7 A-B).

Fig. 8.

Experiments were conducted to determine the individual toxicity of CisPt relative to that of the carbon (NAHA, Gavinol)- and thiol (NAC)- protectants. Results indicate that CisPt is highly toxic at low mM concentrations, whereas NAHA, Gavinol or NAC are not toxic in the concentration-ranges used experimentally.

Table 1.Electrophile HSAB Parameters^a

Compound	η (eV)	σ ($\times 10^{-3}$ eV ⁻¹)	ω (eV)
CisPt	2.5	400	3.43
Aqua-CisPt	2.7	370	4.38
OxaliPt	2.8	360	2.44
CarboPt	5.0	200	1.29
NAPQI	1.9	520	6.83
Acrolein	2.7	370	3.82

^aGround state equilibrium geometries were calculated for each structure with DF B3LYP-6.3G* in water from initial 6-31G* geometries. LUMO and HOMO energies were generated exclusively from corresponding s-cis conformation and were used to calculate η and σ (see LoPachin et al., 2012; 2014 and 2016).

Table 2.Nucleophilicity (ω^- ; eV)^a

Electrophiles	Guanine (hard)	NAHA (soft)	gavinol (soft)
Cisplatin	0.071	0.220	0.359
Aqua-CisPt	0.170	0.343	0.501
OxaliPt	0.024	0.121	0.220
CarboPt	0.010	0.044	0.082
NAPQI	0.322	0.676	0.960
Acrolein	0.096	0.243	0.370

^aThe nucleophilic index (ω^-) indicates the propensity of a covalent reaction between an reacting electrophile with a selected nucleophile as described by LoPachin et al. (2014, 2015 and 2016).