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A Potential Antidote for Both Azide and Cyanide Poisonings

Linda L. Pearce, Kimberly K. Garrett, Yookyung Bae, Kristin L. Frawley, Samantha Carpenter Totoni, and Jim Peterson

Department of Environmental and Occupational Health, Graduate School of Public Health, The University of Pittsburgh, Pittsburgh, Pennsylvania

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Abstract

There do not appear to be any established therapeutics for treating azide poisoning at this time, and presently available antidotes to cyanide poisoning are far from ideal, being particularly impractical for use if multiple victims present. The cobalt (II/III) complex of the Schiff-base ligand trans-[14]-diene (5,7,7,12,14,14-hexamethyl-1,4,8,11-tetraazaclitotetradeca-4,11-diene; CoN4[14]) is shown to act as an effective antidote to both azide and cyanide toxicity in mice. Groups of animals challenged with an LD50 dose of NaCN (100 μmol/kg i.p.) exhibited significantly faster recovery from knockdown and fewer (zero) deaths if given CoN4[14] (50 μmol/kg i.p.) 2 minutes after the toxicant. Groups of animals challenged with an essentially lethal dose of NaN3 (1.5 x LD50 = 150 μmol/kg i.p.) all survived if given the CoN4[14] (75 μmol/kg i.p.) 5 minutes before the toxicant dose. These data represent improved antidotal capability over the Food and Drug Administration–approved cobalt-containing hydroxocobalamin. CoN4[14] is also antidotal in mice toward azide poisoning, for which there is seemingly no approved therapy currently available. The activity toward cyanide involves a “redox-switching” mechanism that could be a common, but largely unrecognized, feature of all cobalt-based cyanide antidotes in use and under development.

Significance Statement

The Schiff-base complex CoN4[14] is shown to be an effective antidote to cyanide in mice, with improved therapeutic capabilities compared to the Food and Drug Administration–approved cobalt-containing hydroxocobalamin. CoN4[14] is also antidotal in mice toward azide poisoning, for which there is seemingly no approved therapy currently available. The activity toward cyanide involves a “redox-switching” mechanism that could be a common, but largely unrecognized, feature of all cobalt-based cyanide antidotes in use and under development.

Introduction

Worldwide, other options are available, but in the United States, the presently available therapeutics for treating acute cyanide poisonings, through ingestion of cyanogenic substances or smoke inhalation, are Nithiodote, a combination of sodium nitrite and sodium thiosulfate solutions (Geller, 2015), or Cyanokit (Hall and Borron, 2015), containing hydroxocobalamin (vitamin B12). Unfortunately, none of the cyanide antidotes in use are ideal, being especially problematic in situations where there may be mass casualties since delivery of the active agents is slow compared with the rapidity with which cyanide can act. For instance, although hydroxocobalamin (or “cobalamin”) is considered to be safe, particularly for the treatment of smoke inhalation victims (Borron and Baud, 2012; MacLennan and Moiemen, 2015), it requires about 15 minutes to intravenously infuse a single adult dose in humans (and more than one may be necessary) (Hall and Borron, 2015), but deaths from acute cyanide poisonings typically occur within 2 to 3 minutes in our experiments with rodents. From a mechanistic perspective, cobalamin is approved for use as a decaperating agent, that is, scavenging cyanide from the toxicant dose recovered twice as fast as the controls given no antidote. The interactions of cyanide and azide with CoN4[14] in vitro (buffered aqueous solutions) have been further investigated by a combination of spectroscopic approaches. The Co(II) form of the complex is able to bind two CN− anions while only binding a single N3− anion, providing a reasonable explanation for the difference between their therapeutic abilities.

Abbreviations: μ, effective magnetic moment; X, molar susceptibility; A, absorbance; b, path length; ΣCo, CoN4[14] extinction coefficient; CoL, CoN4[14]-ligand concentration; CoN4[14], cobalt trans-[14]-diene (5,7,7,12,14,14-hexamethyl-1,4,8,11-tetraazaclitotetradeca-4,11-diene; CoN4[11.3.1]; cobalt 2,12-dimethyl-3,7,11,17-tetraazaclitocybolo[11.3.1]-heptadeca-1(17)2,11,13,15-pentaenyl cation; [Co], total CoN4[14] concentration; EPR, electron paramagnetic resonance; K, binding constant; K1, binding constant; K1=[Co-L]/[L][Co]; K2, binding constant; K2=[Co-L2]/[L][Co-L]; [L], total ligand concentration.
tissue by binding it in a nontoxic form suitable for excretion. The molecule is excreted in the urine when present in amounts exceeding the binding capacity of the plasma; however, most is secreted into the gastrointestinal tract in bile and then reabsorbed, with only ~10% net excretion daily (Fukuwatari et al., 2009; Doets et al., 2013)—note that if it were not for the long elimination time (half-life of several days or hundreds of days for the liver), some cobalamin (vitamin B_{12}) nutritional replacement supplementation therapies might not work. Thus, although cobalamin may be a reasonable cyanide scavenger, trapping the ligand in a nontoxic coordination complex, it may, in fact, also prolong cyanide’s systemic residence time by preventing its rhodanese-dependent conversion to more rapidly excretable thiocyanate.

In addition to improved antidotal capability and safety compared with cobalamin, desirable characteristics of new cyanide decorating agents include lower cost and greater solubility in aqueous media, allowing higher therapeutic doses to be given in a single administration. Storage of the therapeutics ampules in deliverable form not requiring refrigeration would be an advantage. Since there currently seems to be no available antidote for azide poisoning, dual-purpose agents that may also be able to decorate this species are worthy of investigation.

In an effort to find inexpensive compounds of smaller molecular structure than cobalamin and other cobalt corrinoids/metalloporphyrins, without the drawback of being predisposed to have high affinity for biomolecular sites, an investigation of some simple Schiff-base complexes (less than 600 daltons) potentially able to ameliorate both cyanide and azide toxicity has been initiated (Lopez-Manzano et al., 2016; Cronican et al., 2018; Frawley et al., 2020; Praekunatham et al., 2020). It is probably important that these cobalt compounds are macrocycles as this largely circumvents any possible toxicity that might be associated with the “free” metal ions (Hall, 2015) due to the high affinity of the macrocyclic ligand for cobalt ions in all common oxidation states. In the Schiff-base complexes, the central cobalt ion is coordinated by four nitrogen donors in an approximately square planar arrangement, allowing for the potential coordination of two additional exogenous ligands, such as cyanide and azide anions. In this manuscript, we report the cyanide and azide ameliorating effects of the Schiff-base complex, 5,7,7,12,14,14-hexamethyl-1,4,8,11-tetraazacyclotetradeca-4,11-dienyl cobalt (II) dibromide dihydrate (CoN₄[14] Br₂·2H₂O, Fig. 1) in mice and investigate some of the cyanide/azide interactions facilitating the antidotal activity.

**Materials and Methods**

**Reagents.** All chemical reagents were American Chemical Society grade or better, were purchased from either Fisher or Sigma-Aldrich, and, unless stated otherwise, were used without further purification. Argon gas was purchased from Matheson Incorporated.

**Synthesis of Trans-[14]-Diene.** Trans-[14]-diene (5,7,7,12,14,14-hexamethyl-1,4,8,11-tetraazacyclotetradeca-4,11-diene) was synthesized according to the method first described by Hay et al. (1975). To a stirred solution of 10 mL ethylenediamine and 100 mL methanol cooled in an ice bath, 35 mL (0.32 mol) of 48% hydrobromic acid was added dropwise. The resulting white precipitate of ethylenediamine dibromobromide was recovered by filtration, washed several times with diethylether (50 mL), and dried under vacuum.

To the ethylenediamine dibromobromide (11.1 g, 0.05 mol), 100 mL acetonitrile and 3.33 mL of ethylenediamine were added with stirring. The mixture was heated under reflux at 45°C for 45 minutes, cooled to room temperature, and then filtered. Following washing with ice-cold acetone and then diethylether, the final product was obtained as a white powder (17.23 g, 0.04 mol, 90%) by filtration. Elemental analysis (Atlantic Microlab): 40.4% C; 7.9% H; 11.6% N; 33.5% Br (found); 40.2% C, 8.0% H, 11.7% N, 33.5% Br (calculated for C₁₆H₃₄N₄Br₂×2H₂O). 1H NMR (300 MHz, CD₂OD) δ (ppm) 4.8 (s, 4H), 3.7 (m, 4H), 3.4 (m, 4H), 2.8 (s, 4H), 2.1 (s, 6H), 1.5 (s, 12H). Electrospary ionization mass spectrometry (positive ion mode) calculated for [C₁₆H₃₃N₄]^{2+} (doubly protonated parent) 141.1, found 141.0; calculated for [C₁₆H₃₃N₄]^{+} (loss of proton from parent) 81.3, found 80.9.

**Synthesis of CoN₄[14].** The macrocyclic cobalt complex 5,7,7,12,14,14-hexamethyl-1,4,8,11-tetraazacyclotetradeca-4,11-diene cobalt (II) dibromide dihydrate was prepared under argon. To 20 mL of deoxygenated methanol, the previously prepared ligand trans-[14]-diene (0.9566 g, 0.002 mol) and cobalt acetate tetrahydrate (0.04982 g, 0.002 mol) were added to 20 mL of deoxygenated methanol. The resulting solution was continuously stirred and refluxed under argon at 40°C for 2 hours. Product formation was monitored by the appearance of a characteristic electronic absorption peak at 442 nm, which ceased to grow upon completion of the reaction. The product was obtained as an orange solid (0.856 g, 0.0016 mol, 81%) by slow evaporation overnight. The product was further purified by column chromatography on silica gel using a mixture of hexane and ethyl acetate (1:1) as eluent. Crystallization of the product from methanol provided the essentially quantitative conversion of the free ligand to the complex.

**Cyanide and Azide Titrations of CoN₄[14].** Shimadzu UV-1650PC and UV-2501PC recording spectrophotometers were used for the measurement of electronic absorption spectra in the near-UV to near-infrared range and monitoring titrations. Co(II)N₄[14] was prepared in a Vacuum Atmospheres Omni-Laboratory glovebox under argon operating at ~0.5 ppm O₂ (and ~4 ppm H₂O). Titrations of Co(II)N₄[14] and Co(III)N₄[14] with azide and cyanide were carried out in 100 mM sodium phosphate buffer, pH 7.4, at 25°C. Anaerobic titrations of Co(II)N₄[14] with sodium azide and sodium cyanide were performed using Gastight Hamiltonian syringes. Co(III)N₄[14] was prepared by allowing the cobaltous form to oxidize for several days in air, verifying completion of the oxidation by electronic absorption and electron paramagnetic resonance (EPR) spectroscopies. Sodium cyanide solutions were prepared in septum-sealed vials with minimized head spaces in 50 mM sodium tetraborate buffer, pH 10. The binding constant(s) for these titrations were determined using the method of Hargrove et al. (2010). Equations for either the binding of one or two ligands to the cobalt complex were fit using Kaleidagraph with nonlinear least-squares analysis:

For K = [Co-L/L][L][Co],
A = \left\{ \frac{e_{Co,b} + \frac{e_{Co,b} \cdot b \cdot K \cdot [Co]}{1 + 0.5(-1 - K \cdot [L] + \sqrt{1 - K \cdot [L] + K \cdot [Co]^2} + 4K \cdot [L]}}}{2K} \right\} 

\times \left( \frac{1}{1 + \frac{K \cdot [L] + K \cdot [Co]^2}{2K}} \right) 

(1)

For 2 binding constants, $K_1 = [Co-L]/[L][Co]$ and $K_2 = [Co-L]/[L][Co-L]$

$A = \frac{e_{Co,b} + \frac{e_{Co,b} \cdot b \cdot K_1 \cdot [Co] + e_{Co,b} \cdot b \cdot K_2 \cdot [Co]^2}{1 + K_1 \cdot [L] + K_1 \cdot K_2 \cdot [L]^2}}{[Co]}$ 

(2)

Where at the wavelength in question, $A$ is the absorbance, $e_{Co,b}$ the extinction coefficient, $b$ the path length (1.00 cm), $[L]$ the total ligand concentration, $[Co]$ the total cobalt complex concentration and $e_{Co,b}/b$ the extinction coefficient for the complex with two ligands bound. The extinction coefficient of the complex ($e_{Co,b}/b$) and the binding constant(s) ($K$ or $K_1$ and $K_2$) can be determined from the fit. Reasonable initial guesses of the unknowns for the second equation are important. We refer herein to constants describing associative processes as “binding constants” to avoid use of the term “association constant,” which has conventions/definitions attached that we do not intend. Aqueous solutions of cyanide contain an equilibrium mixture of HCN and CN⁻, predominantly the former at neutral pH, and either, or both, of these can be the attacking species in cyanide-substitution reactions at aqau-metal ions. This represents an unnecessary complication in many discussions of aqueous cyanide chemistry, particularly at a single pH. We have previously suggested (Yuan et al., 2017) that using pseudoequilibrium constants where we write $[cyanide_{total}] = [HCN] + [CN]^{-}$, ignoring $[H^+]$ at neutral pH, provides a practical approach to simplifying some discussions of cyanide reactions. Accordingly, we evaluate “binding constants” for cyanide throughout this manuscript as $K = [Co(II)/III] \cdot CN^{-} / [Co(II)/III] \cdot [cyanide_{total}]^{pp}$

**Job’s Method.** Job’s method (also called the method of continuous variation) was used to determine the stoichiometry of cyanide binding in the low cyanide to CoII/III[N₄][14] ratio (Hill and MacCarthy, 1986; Renny et al., 2013). The total concentrations of cyanide plus CoII/III N₄ [14] were held constant, whereas the relative proportions of sodium cyanide and CoII/III[N₄][14] were varied. Formation of the final product was monitored through absorbance changes at 1064 nm, where there was no interference from other species. The change in absorbance of the CoII/III(N₄)CN⁻ compound (normalized) is plotted (ordinate) versus the volume fraction of CoII/III[N₄][14], $X_m = [A_{(III)} + (B)] / ([A_{(III)}])$ (abscissa). The maximum change in absorbance was found at an $X_m$ value corresponding to the binding stoichiometry of the complex formed; for example, 0.5 for a 1:1 complex and 0.33 for a 1:2 complex.

**EPR Spectroscopy.** X-band (9GHz) EPR spectra were recorded on a Bruker ESP 300 spectrometer equipped with an Oxford ESR-910 liquid helium cryostat for ultralow-temperature measurements. The quantification of EPR signals was performed by stimulating the spectra using SpinCount software and comparison with cupric ethylenediaminetetraacetate standards. Access to the spectrometer and software was provided by Michael P. Hendrich, Carnegie Mellon University. EPR samples of CoII/III[N₄][14] with increasing equivalents of sodium cyanide or sodium azide in 50 mM sodium phosphate buffer, pH 7.4, +/- 10% glycerol as a glassing agent, were frozen and then stored in liquid nitrogen prior to the recording of the spectra.

**NMR (Evans Method).** The paramagnetic susceptibility was determined by the Evans method (Evans, 1959; Grant, 1995). The NMR spectrometer used was a Bruker Advance III 500 MHz model, and 535-PP 18 × 0.5 cm NMR tubes were used with WGS-5BL coaxial inserts. The (outer) NMR tube contained CoII/III[N₄][14] (0.0135g) dissolved in 0.8 mL deuterium oxide buffer (0.2 M sodium phosphate, pH 7.5, 0.2% in ethylenediamine diol), and the (inner) coaxial insert contained everything in the surrounding sample except the cobalt complex. 1H NMR spectra were recorded to determine the paramagnetic shifts of the ethylenediamine and HBO probe species in the sample relative to the shifts of the same species in the inner reference solution. CoII/III[N₄][14] plus cyanide solutions were prepared in a similar fashion with the addition of either 6- or 16-fold excess sodium cyanide in the sample solution as well as the reference.

The molar susceptibility of the paramagnetic species was calculated from the following equation in cgs units:

$$X_{m, cgs} = \frac{3}{4\pi} \cdot \frac{10^3}{c} \cdot \frac{\delta \nu}{\nu_0} \cdot \left( mL \cdot mol^{-1} \right)$$

(3)

where $\epsilon$ is the molar concentration, $\delta \nu$ is the difference in frequency between the sample and the probe, and $\nu$ is the operating frequency of the spectrometer in Hz. The molar susceptibility was then converted to SI units by:

$$X_{m, SI} = \frac{\sqrt{3kT X_{m, cgs} / NA \mu_p (\mu B)^2 S}}{10^4} \cdot \left( m^3 \cdot mol^{-1} \right)$$

(4)

Then the effective magnetic moment ($\mu$) of the sample was calculated from the following expression:

$$\mu = \sqrt{\frac{3kT X_{m, SI}}{NA \mu_p (\mu B)^2 S}} \cdot \left( \frac{6.355 \times 10^2}{T} \right) \cdot \frac{X_{m, SI}}{\sqrt{T}}$$

(5)

**Animal Exposures.** All animal procedures used in this study were approved by the University of Pittsburgh Institutional Animal Care and Use Committee (protocol number 16088947). Veterinary care for all animal procedures was provided by the Division of Laboratory Animal Research of the University of Pittsburgh. Six-to 10-week-old Swiss-Webster male mice (30–45g) were purchased from Taconic Biosciences, Inc. (Hudson, NY), housed four per cage, and allowed access to food and water ad libitum. These mice were allowed to adapt for 1 week prior to experiments. Solutions administered to mice were prepared by dilutions into sterilized saline in septum-sealed vials using gas-tight syringes and were given by ~0.2 mL intraperitoneal injections. A group of at least four mice were tested for each set of experiments. Efficacy was determined through assessments of either righting recovery times or pole testing (see below).

**Righting Recovery.** Righting recovery time, a time point at which the mice regained mobility following a period of cyanide-induced unconsciousness, was assessed as follows (Cambal et al., 2011). Approximately 2 minutes following cyanide challenge (5.0 mg/kg NaCN, i.p.), mice are rendered temporarily unconscious (“knocked down”). The unconscious animals were placed in the supine position in a transparent but dark-colored plastic tube (Kaytee CritterTrail, available from pet stores). The righting recovery time was recorded as the endpoint at which the mouse flipped from the supine to a prone position in the plastic tube. To avoid false positive results, the tube was rolled, after righting, to make sure the mouse could maintain the prone position.

**Pole Testing.** A modified protocol (Garrett et al., 2019) was used to study the recovery of mice following intoxication with sodium azide. Each trial consisted of placing a mouse ~20 cm from one end of the pole (60 cm in length × 1 cm in diameter) held horizontally ~15 cm above a foam pad on the bench. The other end of the pole was then slowly raised until perpendicular to the laboratory bench, and the ability of the mouse to climb the pole was assigned a score: unable to grip pole and fall off, 0; able to maintain position gripping pole but unable to climb, 1; able to climb using only forelimbs or did not reach the top, 2; and able to successfully climb to the top of the pole, 3. These animals had been trained the previous day to climb to the top of the pole repeatedly until each mouse received a score of 3 in three consecutive trials. The next day, before starting any azide challenge experiments, the readiness of the animals to climb the pole three consecutive times was verified. Following azide challenge (27 mg/kg NaN₃, i.p., +/− CoII/III[N₄][14], these tests were repeated every 10 minutes. Animals were deemed recovered (and testing stopped) when they received a score of 3 in three successive tests with no regressions to a lower score.
Tail Temperature Monitoring. A baseline tail temperature reading for each mouse was obtained by holding the mouse by its tail and placing the infrared sensor of the thermometer (BIOSEB 153 IRB IR Rodent Thermometer) at the base of the tail about a centimeter down from the fur. These tail temperatures were measured before and after the administration of sodium azide and Co(N₄)₄⁺.

Prophylactic Dose-Response. Co(N₄)₄⁺ was injected (intraperitoneally) into mice at levels of 0, 40, 50, 60, 70, or 80 μmol/kg 5 minutes before the administration (intraperitoneal) of 415 mmol/kg sodium azide (Na₃N). Co(N₄)₄⁺ was injected (intraperitoneally) into mice at levels of 0, 30, 40, 50, 60, or 70 μmol/kg 5 minutes before the administration (intraperitoneal) of 100 μmol/kg sodium cyanide (NaCN). Either the righting recovery test (for cyanide) or the pole test (for azide) was used after each prophylactic administration to determine the lowest antidotal dose giving the full (maximum) ameliorative response. In nominally lethal experimental intoxications (7.5 mg/kg NaCN, i.p.; NaCN, 1.5 × LD₅₀), the antidote (75 mmol/kg, i.p.) was administered 2 minutes before the toxicant.

Therapeutic Animal Testing. Sodium cyanide (100 μmol/kg) was administered to mice (intraperitoneally) at a sublethal level 2 minutes prior to the Co(N₄)₄⁺ antidote (50 μmol/kg, i.p.) or saline sham using righting recovery times to compare results. Mice injected with the antidote (50 μg/kg, i.p.) 5 minutes after the injection of the sodium azide (27 mg/kg = 415 mmol) were examined by pole testing.

Data Analysis. Reported values are means ±/− standard deviations (shown as error bars in figures and values in parentheses in tables) from at least three independent experiments. Statistical analyses of the data were performed using ANOVA with Tukey’s post hoc test for comparison of two groups and ANOVA with a Dunnett post hoc test for multiple comparisons. Statistical data were analyzed using KaleidaGraph (Synergy Software, Reading, PA), and a P value of <0.05 was considered statistically significant.

Results

Antidotal Effects of Co(N₄)₄⁺ on Cyanide Toxicity. Male Swiss-Webster mice, 7–8 weeks of age, were given increasing doses of Co(N₄)₄⁺ (intraperitoneally) 5 minutes prior to administration of LD₄₀ NaCN (5.0 mg/kg, 100 μmol/kg, i.p.) to determine a (prophylactic) dose-response effectiveness of the antidote toward cyanide poisoning. Male mice were used in these particular experiments due to their availability—at this time, females of suitable age and weight were not available. Over a period of more than a decade, we have compared many hundreds of male and female mice in azide and cyanide intoxication experiments (+/− antidotes) without observing any significant differences.

There was a significant reduction in righting recovery time for the cyanide-intoxicated mice following administration of 30, 40, or 50 μmol/kg Co(N₄)₄⁺ with times of 16 (±2), 10 (±1), and 5 (±0.2) minutes, respectively, compared with 27 (±2) minutes for controls given no antidote (Fig. 2, first four data sets; Supplemental Table 2). Mice that received 60 or 70 μmol/kg of Co(N₄)₄⁺ did not knock down at all (Fig. 2, final two data sets). This compares well with our previously reported results using another Schiff-base compound, cobalt 2,12-dimethyl-3,7,11,17-tetraazabicarbonyl-[11.3.1.-heptadeca-1(17)2,11,13,15-pentaenyl cation (Co(N₄)₁₁.₃.₁.), in which, for example, we observed a righting recovery of 3 (±4) minutes at 50 μmol/kg of the test antidote for the mice that knocked down (Cronican et al., 2018). It is, perhaps, worth noting that we have frequently used the righting recovery reflex in mice to assess potential antidotes to cyanide toxicity and, although the present data set contains only control animals, the recovery time obtained is within the 26–31-minute range that we have established in multiple experiments using hundreds of mice, now going back over a decade.

In nominally “sublethal” (LD₄₀ NaCN) therapeutic experiments, administering the putative antidote Co(N₄)₄⁺ (50 μmol/kg i.p.) 2 minutes after the toxicant (100 μmol/kg i.p.), both survival (100% compared with 60% for controls) and the righting recovery for the mice that knocked down (of 8) 9 (±2) minutes versus 26 (±11) minutes for cyanide-treated mice that survived, was improved (Table 1). In nominally lethal experiments (1.5 × LD₅₀ NaCN) in which the antidote had to be given prophylactically, survival was significantly improved (100% compared with 0% for controls), and none of the animals were knocked down (Table 1; Supplemental Table 1).

Antidotal Effects of Co(N₄)₄⁺ on Azide Toxicity. As Co(N₄)₄⁺ is able to ameliorate cyanide toxicity and it can be difficult to distinguish between the effects of cyanide and azide, we also determined the ability of Co(N₄)₄⁺ to antagonize azide toxicity. To determine a possible dose that can be administered for effectiveness against azide toxicity, male Swiss-Webster mice (7–12 weeks) were given 0, 40, 50, 60, 70, and 80 μmol/kg of Co(N₄)₄⁺ 5 minutes prior to a sublethal injection of 27 mg/kg (415 μmol/kg) of Na₃N. The lowest dose of Co(N₄)₄⁺ that showed the maximum therapeutic effect was determined to be 70 μmol/kg with a recovery time of 53 (±15) minutes (Fig. 3A; Supplemental Table 3), and this dose was then used to test the therapeutic effects of Co(N₄)₄⁺. Due to the less reproducible righting recovery times for azide-intoxicated mice, an alternative method using the animals’ natural pole climbing ability (Frawley et al., 2020) was employed to determine the antidotal effects of Co(N₄)₄⁺. Control mice (administered with only the sodium azide) recovered, according to the pole test assessments, at 170 (±50) minutes (Fig. 3B, solid line, no symbols; Supplemental Table 4). Mice injected with the antidote (70 μg/kg) 5 minutes after the injection of the sodium azide (27 mg/kg) had recovery times of 84 (±34) minutes as determined by the
Antidotal effectiveness of CoN₄₁₄ toward sub-lethal and lethal NaCN poisoning in Swiss-Webster mice

<table>
<thead>
<tr>
<th>Knockdown ¹</th>
<th>Control²</th>
<th>(50 µmol/kg) Co²⁺N₄₁₄</th>
<th>Control³</th>
<th>(75 µmol/kg) Co²⁺N₄₁₄</th>
</tr>
</thead>
<tbody>
<tr>
<td>Recovery (time) ²</td>
<td>38/64 (26 ± 11 min)</td>
<td>8/8 (9 ± 2 min*)</td>
<td>9/9 (N/A)</td>
<td>0/8 (N/A)</td>
</tr>
<tr>
<td>Survival</td>
<td>38/64 (60%)</td>
<td>8/8 (100%)</td>
<td>0/9 (0%)</td>
<td>8/8 (100%)</td>
</tr>
</tbody>
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¹Sublethal (LD₄₀) dose, given (intraperitoneally) 2 min before antidote.
²Lethal (1.5 × LD₄₀) dose, given (intraperitoneally) 5 min after antidote.
³No antidote given; sham controls administered same volume(s) PBS (intraperitoneally) previously shown to be equivalent.

*P < 0.0001 versus control.

TABLE 1

Antidotal effect of CoN₄₁₄ on azide intoxicated mice. (A) The dose-response of azide-intoxicated mice (7- to 8-week-old Swiss-Webster, n = 4) given (i.p.) 27 mg/kg NaN₃ (415 µmol/kg) was determined by administering CoN₄₁₄ (i.p., 40-80 µmol/kg) 5 minutes before the toxicant dose and measuring the subsequent recovery times (closed circles) using pole-test assessments (see Materials and Methods for details). Since there was no significant difference between the mean recovery times at 70 µmol/kg and 80 µmol/kg (P = 0.24), the former was taken to be a reasonable estimate for “the minimum dose with the maximum response” of CoN₄₁₄. This dose (70 µmol/kg, i.p.) was then subsequently employed in further experiments with mice to evaluate the protective effectiveness of CoN₄₁₄ toward azide intoxication. (B) CoN₄₁₄ (70 µmol/kg) was given to mice 5 minutes after the azide dose (solid line), and the ameliorative effect was determined by pole-test assessment spanning 250 minutes (filled circles) compared with controls receiving no CoN₄₁₄ antidote (solid line without symbols). At the 20-minute (post-toxicant) time point, P = 0.014, and at the 90-minute time point, P = 0.003. (C) CoN₄₁₄ (70 µmol/kg) was given to mice 5 minutes after the azide dose (solid line), and the ameliorative effect was determined by tail temperature measurements for a duration of 150 minutes (filled circles) compared with controls receiving no CoN₄₁₄ antidote (solid line without symbols). At the 90-minute (post-toxicant) time point, P = 0.023.
the emergence of a band in the near-infrared centered at 
\( \sim 1060 \text{ nm} \) (Fig. 5 inset) that is frequently observed in 
approximately octahedral Co(II) complexes (Swamy and Pola, 
2008). When the ratio of cyanide to Co(II)N4[14] was raised 
from sixfold to a 10-fold excess, the isosbestic points were not 
maintained, indicating that at least a third chromophoric 
species was present.

The addition of further excesses of cyanide (up to 100-fold 
relative to Co(II)N4[14]) resulted in marked decreases in 
absorbance of the maxima at 320, 455, and 1064 nm. The 
change in absorbance at 455 nm could be fit using an 
algorithm based on two associative equilibria (KCN1 and KCN2) 
being present, both dependent on the cyanide concentration and 
with a single intermediate (\( A \rightarrow B \rightarrow C \), see Fig. 6A). The 
two constants, corresponding to the binding of one cyanide (KCN1) 
followed by the binding of a second cyanide (KCN2), were 
determined to have values of 1100 (±40) and 16 (±3), respectively. 
However, although the first of these represents a definitive 
equilibrium constant, the second process should be thought of 
as a "pseudo-equilibrium" since it is accompanied by an oxida-
tion (see below), and there appears to be little to no back reac-
tion. The final electronic absorption spectrum of CoN4[14] at 
higher cyanide concentrations resembles spectra observed when 
cyanide is added to Co(III)N4[14] (see below).

To determine the number of cyanide ions bound to Co(II)N4 [14] under the low ratio of cyanide to cobalt complex (up to 
6:1), Job’s method was employed. A plot of the volume fraction 
of Co(II)N4[14] versus that of cyanide, called a Job’s plot, was 
carried out monitoring absorbance changes at 1064 nm. This 
wavelength appears to be associated with only the (potentially 
single) cyanide bound to the cobalt complex with no other 
spectral interference. Since isosbestic points were not 
maintained in the electronic absorption spectra of the titration of 
Co(II)N4[14] with higher amounts of cyanide, no volume frac-
tions greater than a 1:5 ratio of cobalt complex to cyanide 
were used. The plot shows that the maximum absorbance (at 
1064 nm) occurs at a 0.45 (±0.5) volume fraction of Co(II)N4[14], 
indicating a ~1:1 ratio of cyanide to cobalt complex (Fig. 6B). 
Cyanide electrode experiments, which also determined the 
amount of free cyanide in solution, further verified that roughly 
one cyanide is bound to Co(II)N4[14] (data not shown) at ratios 
less than 1:6 cobalt complex to cyanide.

EPR spectroscopy is extremely useful for delineating the 
mechanisms of ligand-metal interactions. Many Co(III)-Schiff 
base compounds are low spin with a \( d^6 \) electronic configuration 
having a ground state spin of \( S = 0 \) and, thus, exhibit no EPR 
signal. When these same compounds are reduced to Co(II), the 
additional electron produces a \( d^7 \) configuration (\( S = 1/2 \)) that 
does exhibit an EPR signal. The low-spin Co(II) complexes 
(\( S = 1/2 \)) exhibit signals near 3300 gauss that are split by
hyperfine interactions with the $^{59}$Co nucleus ($I = 7/2$). In addition, the Schiff-base complexes contain four nitrogen donor atoms ($^{14}$N, $S = 1$), which can result in superhyperfine interactions that further effect the EPR signal. The anaerobic X-band EPR spectrum of 0.85 mM Co$N_4[14]$ at 20 K in 10% glycerol sharpens the observed signals, which would be somewhat broadened in its absence (not shown). To determine the rhombic $g$-values, signal concentration, and hyperfine, as well as superhyperfine interactions, the signal was simulated using the SpinCount program (see Supplemental Table 7 for the parameters). The anaerobic addition of excess cyanide (100-fold) to the EPR sample led to almost complete loss of the signal, suggesting that the Co(II) was oxidized to Co(III). A titration of the Co$N_4[14]$ with cyanide (Fig. 7, ii–v) led initially to a broadening of the signal accompanied by small changes in the $g$-values and hyperfine interactions. When the cyanide to Co$N_4[14]$ ratio was 6:1, the signal intensity was decreased to only 20% of the initial EPR signal before any cyanide addition and diminished to only a few percent when a large excess of cyanide was added (Fig. 7, ii–v), suggesting conversion to Co(III). A cyanide-induced oxidation of the Co$N_4[14]$ is also consistent with the electronic absorption data (see discussion above regarding Figures 5 and 6).

Transition-metal-ion complexes can sometimes undergo physical changes to properties such as aggregation and spin-state upon freezing to cryogenic temperatures. To make absolutely sure that interpretation of the EPR data was not being confounded by some unexpected temperature dependence, we determined the magnetic susceptibility of Co$N_4[14]$ in the presence and absence of cyanide by the Evans method at ambient temperature (Evans, 1959; Hill and MacCarthy, 1986). This method depends upon the relative shifts in the $^1$H NMR signals of some probe species in the presence and absence of a molecular paramagnet like Co$N_4[14]$ (see Materials and Methods for details). In our measurements, there were two probe species, added ethanediol and residual HDO in the D$_2$O used. For Co$N_4[14]$, the average effective magnetic moment ($\mu_{\text{eff}}$) of 2.18 obtained at 40°C is in excellent agreement with the spin-only value of 1.8 (Bohr magnetons) and typical values (1.9–2.7) found for many octahedral low-spin Co(II) complexes with a d$^7$ configuration and one unpaired electron in the ground state with spin $S = 1/2$. Upon addition of a sixfold and 16-fold excess of cyanide to the cobalt complex, the measured effective magnetic moment values decreased, respectively, to 1.10 and 0.70 (Table 2). Co(III) has a d$^6$ configuration, and, since cyanide is a strong ligand, the resulting (octahedral) compound is mostly likely a low-spin diamagnetic complex with ground-state spin $S = 0$. Consequently, the ambient temperature magnetic data are in accord with the cryogenic EPR data, indicating reduction of Co$N_4[14]$ to a Co(III) form upon binding cyanide.

**Interaction of Cyanide with Co$\text{III}N_4[14]$.** To oxidize Co$N_4[14]$ to Co$\text{III}N_4[14]$, the reduced compound was exposed to ambient oxygen (20.95%) for several days. The resulting solution displayed an electronic absorption spectrum with a single distinct but weak peak at 540 nm (Fig. 8, solid trace) and exhibited no EPR signals (not shown) indicative of an oxidized species. The addition of 100-fold excess sodium cyanide to a solution of the Co$\text{III}N_4[14]$ compound induced changes in the absorption spectrum, but no isosbestic points could be observed indicating that there may be multiple species in solution. In addition, there appeared to be a time dependence and hyperenhancing of the signal accompanied by small changes in the $g$-values (Fig. 7, ii–v), suggesting conversion to Co(III). A cyanide-induced oxidation of the Co$N_4[14]$ is also consistent with the electronic absorption data (see discussion above regarding Figures 5 and 6).

**Fig. 7.** X-band EPR spectra of Co$\text{II}N_4[14]$ titrated with cyanide. Solutions prepared anaerobically then cryopreserved at 77 K prior to recording spectra. (i) Co$\text{II}N_4[14]$ (0.85 mM) in 50 mM phosphate buffer (pH 7.4), 10% (v/v) in glycerol, plus NaCN; (ii) 0.7 mM; (iii) 2.6 mM; (iv) 5.0 mM; and (v) 850 mM. Recording conditions: 9.8 G modulation amplitude, 63.2 $\mu$W microwave power, 20 K sample temperature.

**Table 2**

<table>
<thead>
<tr>
<th>Sample</th>
<th>$\mu_{\text{eff}}$ (Ethanediol)</th>
<th>$\mu_{\text{eff}}$ (HDO)</th>
<th>$\mu_{\text{eff}}$ (Avg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Co$\text{II}N_4[14]$: 16 NaCN</td>
<td>0.59</td>
<td>0.82</td>
<td>0.70</td>
</tr>
<tr>
<td>Co$\text{II}N_4[14]$ (2.5 mM)</td>
<td>2.17</td>
<td>2.19</td>
<td>2.18</td>
</tr>
<tr>
<td>1 Co$\text{II}N_4[14]$: 6 NaCN</td>
<td>1.08</td>
<td>1.11</td>
<td>1.10</td>
</tr>
</tbody>
</table>

$\mu_{\text{eff}}$, average effective magnetic moment.
dependence to the changes observed (Fig. 8, dashed trace, 2 minutes; dotted trace, 120 minutes). Thus, although cyanide does bind to the Co(III)-form of CoN$_4$$^{[14]}$, the length of time needed to oxidize the complex (and the complexity of the cyanide binding) probably precludes this being a significant reaction in vivo.

**Reaction of Azide with Co(II)N$_4$$^{[14]}$.** The addition of 1000-fold excess sodium azide to anaerobically prepared Co(II)N$_4$$^{[14]}$ resulted in a small shift in the visible region absorption maximum in the electronic spectrum, from 442 nm to ~450 nm (Fig. 9, solid and dashed traces, respectively), with a slight but detectable increase in intensity. These very minor changes suggest that although an aquo ligand may have been replaced by azide, the gross structural geometry around the cobalt ion was maintained, and there was probably no oxidation. Working with the difference spectrum (azide-free Co(II)N$_4$$^{[14]}$ in the reference beam, not shown)—which proved more reproducible than trying to work at the ~450 nm maximum—it was possible to optically determine a titration curve at 550 nm for the azide binding reaction (Fig. 9B, filled circles) and then, undertaking a nonlinear least-squares fit to the data assuming one azide anion bound per Co(II)N$_4$$^{[14]}$ (Fig. 9B, solid line), to estimate an association constant of ~10$^5$.

Small changes in the EPR spectrum of Co(II)N$_4$$^{[14]}$ were also evident without any obvious loss of signal intensity (Fig. 10). When $^{15}$N($S = 1/2$)-enriched sodium azide was added in excess to the Co(II)N$_4$$^{[14]}$, a distinct two-line superhyperfine was observed (Fig. 10, upper trace), indicating the presence of one azide anion associated with each Co(II) center (2S + 1 = 2 lines). If there had been more than one azide bound per Co(II), a more complicated set of superhyperfine lines is to be expected—perhaps unresolved due to overlap (as in Fig. 10, lower trace)—but not the additional two-line splitting observed. Unlike the binding of cyanide, a large excess of azide does not appear to induce any oxidation of the complex. In summary, the electronic absorption and EPR spectra together indicate that Co(II)N$_4$$^{[14]}$ can weakly bind a single azide anion.

**Discussion**

The antagonism of cyanide toxicity by CoN$_4$$^{[14]}$ (Fig. 2; Table 1) is very comparable to that which we have previously observed for another Schiff-base compound, CoN$_4$$^{[11.3.1]}$—both having measurably better antidotal characteristics in experiments with rodents, including improved efficacy, compared with the Food and Drug Administration–approved hydroxycobalamin (Lopez-Manzano et al., 2016; Cronican et al., 2018). Discovering another new candidate compound as a putative cyanide antidote is of some value since there are currently too few realistic new cyanide antidotes in preclinical development to guarantee that any of them will eventually be approved for clinical use. Being of smaller size than corrinoids and porphyrins, CoN$_4$$^{[14]}$ and CoN$_4$$^{[11.3.1]}$ have increased aqueous solubility compared with some other cobalt-containing cyanide antidotes, which translates into higher therapeautic doses per volume delivered. There are some known macrocyclic structures with an approximately planar arrange ment of four nitrogen donor ligands that are even smaller, but unlike these others, CoN$_4$$^{[14]}$ and CoN$_4$$^{[11.3.1]}$ can be purified in crystalline forms with, respectively, >80% and >60% overall yields in 2 to 3 steps from inexpensive starting materials—that is, they appear to have more promising drugability.

The present data, in particular the spectral results (Figs. 4, 6, and 7), suggest that, mechanistically, the cyanide-binding
behavior of CoN₄[14] (presented minimally in Scheme 1) appears similar to that which we have previously described for CoN₄[11.3.1.3] (Praekunatham et al., 2019). Briefly, one cyanide associates with Co(II) at the center of the macrocycle with reasonably high affinity, but note that HCN is the attacking species around neutral pH rather than the cyanide anion. A second cyanide binds trans to the first with lower affinity but accompanied by a lowering of the reduction potential of the central cobalt ion and consequent oxidation from Co(II) to Co(III). As Co(III) is famously substitution inert, the bound cyanide is kinetically stable in this dicyano form for excretion in the urine rather than being primed for systemic redistribution if it were bound to some substitution-labile center. This is an important consideration that many authors seem to miss—we have previously argued that the cyanide-binding capability of cobalt-based decapping agents does not depend on equilibrium considerations like the relative magnitudes of binding constants (Yuan et al., 2017).

The evidence provided by the in vivo studies for the CoN₄[14] complex shows that at similar ratios of complex to cyanide (Table 1), the compound is an effective cyanide ameliorating agent in mice. The in vitro studies, however, indicate that this ratio of cyanide to CoN₄[14] is not high enough to convert the compound to its dicyanoCo(III) form. This argues that the in vivo mechanism either does not depend on conversion of the antidote to a substitution-inert form or that some other modification of the mechanism is operative. Perhaps binding a single cyanide together with another strong-field ligand (such as a protein amino or imidazole group) may enable the cobalt complex to achieve a substitution-inert Co(III) form with mixed axial ligands (one cyanide plus one N-donor). This will still ameliorate cyanide poisoning as the toxicity curve is steep; simply binding some portion of the cyanide dose can reduce the active toxicant to a survivable level if the reaction is rapid enough.

Axide toxicity can be difficult to distinguish from cyanide toxicity, and so it would appear useful to have an antidote that ameliorates either both or, invarially, the clinician is likely to be confronted with a presentation where the particular toxicant has not been unambiguously identified. Similar to our findings with respect to cyanide, we have previously reported some protection using the Schiff-base complex CoN₄[11.3.1.3] (Praekunatham et al., 2020). Again, we find that CoN₄[14] is similar to CoN₄[11.3.1.3] but this time in its antagonism toward azide, in that it exhibits comparable dose-response (Fig. 3A), halves recovery time compared with controls (Fig. 3B), and essentially prevents the body temperature loss observed in mice (Fig. 3C). Unlike the case for cyanide, there is no evidence that more than one azide anion binds to CoN₄[14]. Nevertheless, although this probably means that CoN₄[14] is less potent in its antidotal capability toward azide, it could prove good enough because azide is less toxic and considerably slower acting than cyanide (Frawley et al., 2020; Praekunatham et al., 2020). Also, of course, the alternatives are presently extremely limited (Frawley et al., 2020; Tat et al., 2021).

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Data Availability

All data/data sets are included in the manuscript/supplemental material. Raw data for animal toxicity experiments (Figs. 2 and 3; Table 1) and parameters derived from fits to the EPR data (Figs. 7 and 10) are presented in the Supplemental Material.

Authorship Contributions

Participated in research design: Pearce, Garrett, Frawley, Totoni, Peterson.
Conducted experiments: Pearce, Garrett, Bae, Frawley, Totoni.
Performed data analysis: Pearce, Garrett, Bae, Frawley, Totoni.
Wrote or contributed to the writing of the manuscript: Pearce, Gar- rett, Peterson.

References


Address correspondence to: Jim Peterson, 130 D2 Soto Street, Pittsburgh, PA 15261. E-mail: jimmyg@pitt.edu; or Linda L. Pearce, 130 D2 Soto Street, Pittsburgh, PA 15261. E-mail: lip10@pitt.edu