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Cyanide Scavenging by a Cobalt Schiff-Base Macrocycle: A Cost-Effective Alternative to Corrinoids

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Abstract

The complex of cobalt(II) with the ligand 2,12-dimethyl-3,7,11,17-tetraazabicyclo-[11.3.1]heptadeca-1(17)-2,11,13,15-pentaene ($\text{CoN}_4[11.3.1]$) has been shown to bind two molecules of cyanide in a cooperative fashion with an association constant of $2.7 (\pm 0.2) \times 10^5$. *In vivo*, irrespective of whether it is initially administered as the Co(II) or Co(III) cation, EPR spectroscopic measurements on blood samples show that at physiological levels of reductant (principally ascorbate) $\text{CoN}_4[11.3.1]$ becomes quantitatively reduced to the Co(II) form. However, following addition of sodium cyanide, a dicyano Co(III) species is formed, both in blood and in buffered aqueous solution at neutral pH. In keeping with other cobalt-containing cyanide-scavenging macrocycles like cobinamide and cobalt(III) *meso*-tetra(4-*N*-methylpyridyl)-porphine, we found that $\text{CoN}_4[11.3.1]$ exhibits rapid oxygen turnover in the presence of the physiological reductant ascorbate. This behavior could potentially render $\text{CoN}_4[11.3.1]$ cytotoxic and/or interfere with evaluations of the antidotal capability of the complex toward cyanide through respirometric measurements, particularly since cyanide rapidly inhibits this process, adding further complexity. A sublethal mouse model was used to assess the effectiveness of $\text{CoN}_4[11.3.1]$ as a potential cyanide antidote. The administration of $\text{CoN}_4[11.3.1]$ prophylactically to sodium cyanide-intoxicated mice resulted in the time required for the surviving animals to recover from “knockdown” (unconsciousness) being significantly decreased (3 ± 2 min) compared to that of the controls (22 ± 5 min). All observations are consistent with the demonstrated antidotal activity of $\text{CoN}_4[11.3.1]$ operating through a cyanide-scavenging mechanism, which is associated with a $\text{Co(II)} \rightarrow \text{Co(III)}$ oxidation of the cation. To test for postintoxication neuromuscular sequelae, the ability of mice to remain in position on a rotating cylinder (RotaRod test) was assessed during and after recovery. While intoxicated animals given $\text{CoN}_4[11.3.1]$ did recover ~30 min more quickly than controls given only toxicant, there were no indications of longer-term problems in either group, as determined by continuing the RotaRod testing up to 24 h after the intoxications and routine behavioral observations for a further week.

Graphical Abstract

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Notes

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INTRODUCTION

Compounds that lower the circulating level of free cyanide in the bloodstream have been shown to be effective antidotes to cyanide poisoning.^{1–6} (Hydroxo)cobalamin (Cbl) is currently the only FDA-approved cyanide-binding coordination compound available as an antidote for emergency and clinical uses. Cbl is far from ideal, however, requiring manipulation prior to use because it is not storable in a usable form and because rapid delivery of large volumes intravenously is needed as each Cbl molecule binds only a single cyanide anion. The immediate biological precursor, cobinamide (Cbi), should be a better antidote with improved cyanide-binding characteristics (two cyanides per Cbi) and is currently under development.^{7–9} However, both of these compounds are expensive to produce, with Cbi being significantly more so than Cbl, and cheaper alternatives are clearly desirable. We have very recently shown that a Co(III)-containing water-soluble porphyrin, exhibiting slightly better cyanide-binding capability than Cbi, functions as a cyanide antidote in mice.^{10,11} Consequently, it appears that many compounds in which cobalt(III) is surrounded by a square-planar arrangement of nitrogen donors with two labile ligands in the axial positions are good candidate cyanide antidotes. Some known metalloporphyrins, including the one we have studied, are certainly easier to synthesize and therefore cheaper to produce than corrinoids like Cbl and Cbi. Unfortunately, when the counterions required for isolation are included, these metalloporphyrins can have formula weights in excess of 1000 Da, with the large size severely limiting their molar solubility. The syntheses of numerous smaller complexes of Co(III) having a similar surrounding ligand geometry have been reported, and many more are accessible by trivial derivatization of compounds reported in the existing inorganic literature. Macrocyclic ligands of the Schiff-base variety containing Co(III) coordinated by approximately four square-planar nitrogen donors can be synthesized in a single step, employing the metal ion as a template, and then purified by recrystallization.^{12,13} These compounds are of significantly lower formula weight than corrinoids or metalloporphyrins and, because the Co(III) ion is retained by a chelate ring, are themselves expected to be essentially nontoxic at the levels required to be efficacious in treating cyanide intoxication.

In fact, upon examining a number of cobalt macrocycles, we found that the previously synthesized¹⁴ cobalt complex of Schiff-base compound 2,12-dimethyl-3,7,11,17-tetraazabicyclo-[11.3.1]heptadeca-1(17)2,11,13,15-pentaene ($\text{CoN}_4[11.3.1]$; see Figure 1 inset) gives good results when used as an antidote in a sublethal cyanide intoxication assay carried out in mice. The following study shows the effectiveness of $\text{CoN}_4[11.3.1]$ in mice

and suggests that the physiologically important form of this potential antidote for cyanide-binding capability is its reduced Co(II) complex.

EXPERIMENTAL SECTION

Chemicals

All nongaseous reagents were ACS grade, or better, used without further purification, and, unless stated to the contrary, were purchased from Fisher or Sigma-Aldrich. Argon and nitrogen gases were purchased from Matheson Incorporated and used without further purification. Sodium cyanide solutions were prepared in septum-sealed vials with minimized headspaces immediately prior to use, and volumetric transfers were made with gastight syringes.

Synthesis of Co(II) N_4 [11.3.1]

Cobalt(II) 2,12-dimethyl-3,7,11,17-tetraazabicyclo-[11.3.1]-heptadeca-1(17)2,11,13,15-pentaene dibromide (Co(II) N_4 [11.3.1]) was synthesized under argon according to the method of Long and Busch¹⁴ as modified by Lacy et al.¹⁵ CoBr₂ (1.35g, 6.17 mmol) and 2,6-diacetylpyridine (1.00 g, 6.13 mmol) were dissolved in 20 mL of deoxygenated EtOH and 0.5 mL of water. The addition of deoxygenated 3,3'-diaminodipropylamine (0.857 mL, 6.13 mmol) was carried out, under argon, slowly over the course of several minutes. Upon the addition of the amine, the blue-green solution of CoBr₂ and 2,6-diacetylpyridine turned dark red. Glacial acetic acid (1 μ L) was then added to the solution, and this was heated at 50 °C for 12 h with stirring. The resulting solution was transferred to a glovebox (purified Ar, <0.5 ppm O₂, ~4 ppm H₂O) and filtered through a fritted-glass funnel after cooling to room temperature. The resulting purple solid was washed with diethyl ether and dried over P₂O₅ for at least 24 h. Anal. Calcd for C₁₅H₂₂N₄Br₂Co (Atlantic Microlab): 37.76% C, 4.65% H, 11.74% N, 33.50% Br. Found: 37.66% C, 4.67% H, 11.62% N, 33.16% Br. Mass spectra were obtained using a Finnegan LCQ quadrupole field ion trap mass spectrometer equipped with an electrospray ionization source (ESI-MS). Sample solutions were diluted in a solvent consisting of a 50:50 mixture of acetonitrile (HPLC grade, Fisher) and Milli-Q water plus 1% acetic acid (Optima LC/MS, Fisher). Prior to diluting, the concentrated samples and solvent were each filtered through a syringe filter (0.45 μ m PTFE). Analytical solutions were prepared the day of analysis and consisted of 100 μ L of concentrated sample and 900 μ L of solvent. The oxidized compound, cobalt(III) 2,12-dimethyl-3,7,11,17-tetraazabicyclo-[11.3.1]-heptadeca-1(17)2,11,13,15-pentaene tribromide (Co(III)- N_4 [11.3.1]), was prepared by starting with CoBr₃ in an otherwise similar procedure. These preparations contained variable amounts of residual water, as judged by elemental analyses, with the presence of the target macrocyclic complex being determined by mass spectrometry. Cobalt(III) complexes are often reduced on the probe during mass spectral analyses, and since $M_r = 317.3$, we anticipated a m/z of 158.6 ($M_r/2+$) for Co(II) N_4 [11.3.1], analogous to the result that we previously obtained with a cobalt porphyrin.¹⁰ However, the observed m/z of 315.2, obtained irrespective of whether the Co(II) or Co(III) compound was introduced on the probe, indicates that the cobalt has been reduced to Co(I) in this case (formally at least) with elimination of H₂. Oxidation of the secondary nitrogen in the target structure to an imine is a known reaction¹⁵ that appears to have occurred during ionization in

the mass spectrometer. The synthesis was carried out under rigorously anaerobic conditions, and hydrogen analysis (4.67% found) does not fit the structure with an oxidized secondary nitrogen (4.24% calculated for $C_{15}H_{20}N_4Br_2Co$) compared to the target structure (4.65% calculated for $C_{15}H_{22}N_4Br_2Co$). The previous authors, whose preparation we followed, had verified by 1H NMR spectroscopy that the secondary amine moiety was preserved in the Co(II) form and showed by crystallography that it was a planar imine in the formally Co(I) form,¹⁵ in complete agreement with our present elemental analysis and mass spectral data.

Titration

For determinations of cyanide-binding equilibria, all solutions were deoxygenated and maintained anaerobically, under argon, in capped (septum-sealed, with head volumes minimized) vessels. All transfers were made with gastight syringes and careful mixing to ensure the dissolution of cyanide into the solution. Small aliquots of concentrated cyanide solutions (dissolved in water at pH 10) were titrated into larger volumes of relatively dilute solutions of Co(II)N₄[11.3.1] (freshly made phosphate-buffered saline (PBS), pH 7.4) to maintain neutrality at 25 °C. Electronic absorption measurements were made using Shimadzu UV-1650PC and UV-2501PC spectrophotometers. Binding constants were determined by the construction of Hill plots, where *Y* (representing the proportion of sites occupied by cyanide or the fractional saturation) was plotted against the concentration of the free cyanide concentrations. Potentiometric determinations of free cyanide were carried out with a cyanide-ion-selective electrode (Orion Ionplus Sure-Flow Combination, Thermo Scientific). Before measuring samples, the electrode was calibrated with freshly made sodium cyanide standard solutions. Prior to each measurement, standards and samples were all stabilized with alkaline ionic strength adjustor solution (Orion, Thermo Scientific). Binding constants were determined by the construction of Hill and Scatchard Plots.

Animals, Exposure, and Blood Collection

All animal procedures were approved by the University of Pittsburgh Institutional Animal Care and Use Committee (protocol number 13092637). Veterinary care was provided by the Division of Laboratory Animal Research of the University of Pittsburgh. Male Swiss-Webster (CFW) mice weighing 35–40 g (6–7 weeks old) were purchased from Taconic, Hudson, NY, housed four per cage, and allowed access to food and water *ad libitum*. Experiments commenced after the animals were allowed to adapt to their new environment for 1 week. All animals were randomly assigned to experimental groups of predetermined size. All solutions were prepared by dilutions into sterilized saline in septa-sealed vials using gastight syringes and administered through ~0.1 mL intraperitoneal (ip) injections. In general, a group of at least 6 mice was tested for each experimental point. At the end of exposures and tests, mice were euthanized with CO₂. For collection of blood samples, mice were first euthanized in an atmosphere of CO₂ and blood was then drawn by cardiac puncture into a 1 mL syringe. The blood was expelled into the bottom of a quartz EPR tube and then quickly frozen by immersion in liquid nitrogen. This entire process could comfortably be completed in 2 min. The cryogenically preserved sample was stored in liquid nitrogen and subsequently transferred to the EPR spectrometer without ever having been thawed. For urine collection, mice were placed on a plastic surface and urine was collected using a 1 mL syringe approximately 40 min after ip injection of compound. The urine was

then expelled into the bottom of a quartz EPR tube and frozen by immersion in liquid nitrogen.

Righting-Recovery Testing

Following intoxications, the duration of time required for recovery of righting ability in mice was determined on the basis of some of the recommendations of Crankshaw et al.¹⁶ regarding their measurement of the righting reflex but adopting a simpler procedure. Following the administration (ip) of toxicant (5 mg/kg NaCN), mice were placed in a transparent but dark green-colored plastic tube (Kaytee CritterTrail, available from pet stores) in the supine position. The duration from the time of toxicant injection until the mouse flipped from the supine to prone position in the plastic tube was taken as the end point (righting-recovery time).

RotaRod Testing

To assess motor skill learning and recovery following intoxication, we used an accelerating RotaRod (Coulbourn Instruments, Whitehall, PA), a rotating cylindrical apparatus (4 to 40 r.p.m.) on which the mice were placed. The animals were evaluated for three trials per time point on three consecutive days, with a resting time of 30 s between each trial. An individual trial was considered to have ended when the mouse either fell off or remained on the rotating cylinder for 60 s. Latency to fall and highest speed reached were recorded for each trial. Mice were trained for eight trials on the first day by placing them on an accelerating RotaRod for 60 s, during which time the rotation rate was varied linearly from 4 to 22 rpm. On the day of the toxicity experiments (day 2), animals were tested for a single set of three trials, with the same parameters as those during training to establish baseline performance before any treatment was given. Trained animals were tested at 15 min intervals after toxicant administration for a period of 2.5 h and also at 24 h by placing them on an accelerating RotaRod. Motor performance was assessed by determining the duration before the animal fell off the apparatus, using the mean duration of three trials for each mouse at each experimental time point. Data was analyzed using two-way ANOVA over the 2.5 h testing window to determine main effect of treatment and time, with Tukey's multiple-comparison test to determine the differences between groups.

Electron Paramagnetic Resonance (EPR) Spectroscopy

X-band (9 GHz) EPR spectra were recorded on a Bruker ELEXSYS-II E500 spectrometer equipped with an Oxford Instruments ESR 910 flow cryostat for ultra-low-temperature measurements. Access to this instrument and the software (SpinCount) used to analyze the EPR spectra were provided by Professor Michael Hendrich, Carnegie Mellon University. Quantification of EPR signals was performed by simulating the spectra using known (or determined) parameters for each sample in question. Simulations employed a least-squares fitting method to match the line shape and signal intensity of a selected spectrum. Simulated spectra were expressed in terms of an absolute intensity scale, which could then be related to sample concentration through comparison with a Cu^{II}(EDTA) spin standard of known concentration.

Oxygraph Experiments

An Oxygraph O2k polarographic instrument (Oroboros), equipped with a Clark-type oxygen electrode for high-resolution respirometry and a fluorescence detector, was used to simultaneously measure oxygen fluxes and hydrogen peroxide levels (determined with Amplex Red/HRP). Cobalt compounds were dissolved into phosphate buffer (50 mM, pH 7.4), loaded into the oxygraph chamber, and equilibrated (sealed from the atmosphere) at 37 °C for 10 min. Subsequently, ascorbate and/or cyanide was added by gastight syringe to the sealed oxygraph chambers. Data analysis was carried out with DatLab software provided by Oroboros.

Data Analysis

Statistical data was analyzed using either Kaleida-graph or GraphPad Prism 6 software. A *p*-value 0.05 was considered statistically significant.

RESULTS

Binding of Cyanide to Co(II)N₄[11.3.1]

The addition of Co(III)N₄[11.3.1] to mouse blood results in the quantitative conversion of the compound to the Co(II) form, as judged by EPR spectroscopy (see below). In fact, most cobalt complexes exhibit reduction potentials that allow them to be easily reduced in the bloodstream,^{17,18} and, indeed, we previously showed that a similar reduction also occurs for Co(III)TMPyP, another potential cyanide antidote. Therefore, we directed our attention to the cyanide-binding capabilities of reduced cobalt macrocycle Co(II)N₄[11.3.1].

Cyanide at varying concentrations was anaerobically added to Co(II)N₄[11.3.1] solutions (in PBS, pH 7.4) in gastight vials with limited headspace, and the amount of free cyanide was determined using the cyanide-selective electrode after changing the pH to 11 (see Experimental Section). By plotting the amount of free cyanide versus the fractional saturation of Co(II)N₄[11.3.1] (Figure 1), we determined that two molecules of cyanide were bound by each Co(II)N₄[11.3.1]. Using a nonlinear least-squares fit of the data to the fractional saturation of Co(II)N₄[11.3.1]CN₂ versus free cyanide, we determined that the data was best fit to two equilibria, with $\alpha_H = 2$, suggesting that the second cyanide has a higher affinity than the first when binding to the complex. However, the potentiometric titrations using the cyanide-selective electrode were designed to work at pH > 11 in order to detect the cyanide anion without losing HCN ($pK_a = 9.2$) to the atmosphere. Therefore, although we can presume that Co(II)N₄[11.3.1] also axially binds two cyanides at pH 7.4, the alkaline environment of this method cannot lead to binding constants representative of physiological conditions.

In order to obtain the overall equilibrium constant for the binding of the two cyanides to Co(II)N₄[11.3.1], we carried out titrations of the compound with cyanide and monitored the changes in the electronic absorption spectra at physiological pH (PBS, pH 7.4). A solution of Co(II)N₄[11.3.1] in a sealed cuvette with little to no headspace was monitored spectrophotometrically while adding known amounts of sodium cyanide solution (pH 10; see Experimental Section) with a gastight syringe. The electronic absorption spectra

obtained during the titration of Co(II)N₄[11.3.1] with cyanide at 25 °C display well-maintained isosbestic points (Figure 2A), indicating the presence of only two species, the second of which was the dicyano adduct. As we wish to report a physiologically relevant reaction, an effective formation constant, K'_β , can be defined at pH 7.4 as

$$K'_\beta = \frac{[\text{Co(II)N}_4[11.31](\text{CN})_2]}{[\text{Co(II)N}_4[11.3.1]][\text{HCN}]^2}$$

where the hydrogen ion concentration is ignored. An additional complication occurs because 98% of the cyanide free in solution is protonated, but the form bound to Co(II)N₄[11.3.1] is most likely the anionic form. From the spectra (at 540 nm), the fraction of the Co(II)N₄[11.3.1](CN)₂ per total cobalt macrocycle (fractional saturation, Y) was determined; hence, the free cyanide concentration (protonated plus unprotonated) could be calculated. In Figure 2B, free cyanide is plotted versus fractional saturation (Y), and the data was fit using a nonlinear regression and the Hill equation (where L represents the free total cyanide in solution):

$$Y = \frac{[L^{\alpha_H} / (K'_\beta)^{\alpha_H}]}{[1 + L^{\alpha_H} / (K'_\beta)^{\alpha_H}]}$$

Using $\alpha_H = 2$ gave the best fits, confirming the cooperativity of the binding, and K'_β was found to be $2.7 (\pm 0.1) \times 10^5$.

Antidotal Activity of CoN₄[11.3.1] in Mice

Given that the cyanide-binding constant for the reduced form of CoN₄[11.3.1] at physiological pH and temperature was found to be favorable, we investigated the effectiveness of the compound against cyanide toxicity in mice. Swiss-Webster mice (7–8 weeks of age) administered NaCN in saline (5.0 mg/kg ip) are typically rendered unconscious within about 2 min. The animals that subsequently survived (28 of 35 in total) remained “knocked down” for around 22 min, during which time they could be placed on their backs, returning themselves to a prone position upon regaining consciousness. This period indicates the time required for the existing detoxification activities (e.g., rhodanese and 3-mercaptopyruvate sulfurtransferase, plus basal nitric oxide synthase activity that produces the NO required to liberate bound cyanide from cytochrome *c* oxidase).^{19,20} To examine the prophylactic antidotal activity of CoN₄[11.3.1] and determine an effective dose range for later ameliorative testing, mice were given various doses of CoN₄[11.3.1] 5 min before the toxicant (Figure 3A). Mice that received a 70, 56, 52, 44, 39, or 30 $\mu\text{mol/kg}$ dose of Co(II)N₄[11.3.1] before cyanide intoxication had improved survival (100% in nearly all groups) compared to that of mice receiving no treatment. In addition, mice receiving 44 μmol of Co(II)N₄[11.3.1] and greater showed a significant decrease in righting-recovery time, with the maximal effect starting at the 52 μmol dose (3 ± 3 min) (Figure 3A). A subset of the mice were given similar doses of the oxidized form (as prepared) of the antidote,

Co(III)N₄[11.3.1], with no detectable difference in the ameliorative effect (data not shown). Clearly, in this experimental mouse model, CoN₄[11.3.1] exhibits impressive prophylactic antidotal activity toward cyanide intoxication when administered at or above a 1:2 cobalt/cyanide ratio (~50 μ mol/100 μ mol CoN₄[11.3.1]/NaCN, consistent with the titration results demonstrating that CoN₄[11.3.1] can bind two cyanide ligands.

Using the dose of 52 μ mol/kg of Co(II)N₄[11.3.1], we next investigated the therapeutic antidotal activity toward cyanide (Figure 3B). Mice received CoN₄[11.3.1] either 1, 2, or 5 min after the cyanide dose. At 1 min after the toxicant dose, most mice are feeling the effects of cyanide, evident from behavioral changes, but are not yet knocked down. Animals that succumbed did so within 3 min, and by 5 min, all mice that received a cyanide dose were knocked down. When mice received CoN₄[11.3.1] 1 min after a cyanide injection, only 57% experienced knockdown, whereas in the case of mice receiving the antidote 2 or 5 min after cyanide, 100% were knocked down. Mice that received CoN₄[11.3.1] at 1 or 2 min after cyanide had significantly shorter righting-recovery times (3 ± 3 and 13 ± 6 min, respectively, as shown in Figure 3B) than mice that did not receive any antidote. Consistent with CoN₄[11.3.1] acting as a scavenger of free HCN in the blood, rather than any tendency to decorporate HCN already diffused into the other tissues, mice receiving CoN₄[11.3.1] 5 min after cyanide injections had a righting-recovery time that was indistinguishable from that of mice given cyanide alone (Figure 3B). At this juncture, we compared this behavior with that of a better developed candidate cyanide antidote and found that cobinamide had no effect on righting-recovery time when given 5 min after cyanide injections (Figure 3B).

Recovery of Neuromuscular Function Following Acute Cyanide Intoxication

Some reports in the occupational medicine literature contain anecdotal evidence suggesting that there may be neurological dysfunction in humans following acute exposures to HCN.^{21,22} To address this possibility experimentally, we employed an approach based on the RotaRod device, which is primarily a determinant of neuromuscular coordination but can also assess muscle strength, learning capability, and memory. The mice were trained 24 h before the intoxication procedures, and baseline performance was established at 1 h prior to administration of toxicant. Following toxicant injections (ip), mice were then tested every 15 min, up to 2.5 h, and subsequently at 24 h. During the training period and the pre-ip testing there was no difference in performance between groups, but there was a significant difference as measured by two-way ANOVA in RotaRod performance of mice during the post-ip recovery period (Figure 4). Mice that received only 5 mg/kg NaCN (closed circle) had significantly impaired performance until 90 min after exposure compared to that of mice receiving saline (open triangle) ($p < 0.05$, Tukey's multiple-comparisons test). Interestingly, mice that received 50 μ mol/kg prophylactic CoN₄[11.3.1] and 5 mg/kg NaCN 5 min later (closed diamond) were barely (not significantly) different from mice that received just CoN₄[11.3.1] (closed diamond) during the post-ip recovery period, but they did demonstrate improved performance during the first 30 min of recovery compared to that of cyanide-only mice (closed circle) ($p < 0.05$, Tukey's multiple-comparisons test). These data show that all of the animals have essentially recovered neuromuscular coordination at 2 h after administration of NaCN, irrespective of whether CoN₄[11.3.1] was given, but prophylactic administration of the antidote did speed the recovery. We also note that in both the cyanide

alone and cyanide plus CoN₄[11.3.1] cases there is no indication of persistent impairment of neuromuscular coordination, as judged by Rotarod testing at 24 h, at which time point all groups performed similarly (Figure 4). In fact, we maintained a subset of these animals (from all groups, approximately two dozen in total) for at least 1 week following the exposures, and while we did not submit them to further RotaRod testing, their behavior together in the cages and when placed individually in open fields continued to appear normal. That is, we did not observe anything similar to the delayed Parkinson-like symptoms documented in some clinical reports that can start to become apparent after several days in humans.²³

EPR Spectroscopy

The details of the mechanisms of cyanide binding to cobalt complexes can be better understood through the application of EPR spectroscopy. Co(III) complexes have an electronic configuration of d⁶ and are low-spin ($S = 0$) when coordinated to strong-field ligands; therefore, they exhibit no EPR signal. However, similarly, ligated Co(II) complexes (d⁷) are also often low spin, but they are EPR active ($S = 1/2$) and, in the case of Co(II)N₄[11.3.1] which has an axis of symmetry with $g_{\parallel} < g_{\perp}$, exhibit signals near 3300 G. The observed hyperfine in these signals results from the interaction of the unpaired electron with the ⁵⁹Co nucleus ($I = 7/2$). Reduction of Co(III)N₄[11.3.1] by sodium ascorbate in 50 mM phosphate buffer, pH 7.4, 10% (v/v) glycerol (glassing agent) resulted in an X-band EPR spectrum with visible (if weak) hyperfine and g-crossover of 3085 G (not shown). An identical spectrum was obtained following dissolution of Co(II)N₄[11.3.1] in the same 10% glycerol/phosphate buffer (Figure 5A-i). The addition of cyanide (10-fold excess) results in a modest but readily observable shift of the g value to 3097 G (Figure 5A-ii). This signal in the 10% glycerol medium persisted if the sample was maintained at ambient temperature for up to ~10 min before freezing. Similar EPR signals were observed for Co(II)N₄[11.3.1] in glycerol-free phosphate buffer, but the hyperfine due to the ⁵⁹Co nucleus was significantly broadened (not observable) in the absence of the glassing agent (Figure 5A-iii). When excess cyanide was added to Co(II)N₄[11.3.1] in phosphate buffer, the EPR signal (Figure 5A-iv) disappeared more rapidly than with glycerol present. Importantly, the presence of oxygen in samples of the Co(II)N₄[11.3.1]–cyanide complex had little effect on the rate of disappearance of the EPR signal (data not shown).

Upon the anaerobic addition of one molecule of cyanide per Co(II)N₄[11.3.1] and allowing 4 min reaction time before freezing the sample, the resulting EPR signal (Figure 5B-i) was essentially identical to that observed before (Figure 5A-iv). Upon the addition of a second equivalent of cyanide, the monocyano-Co(II)N₄[11.3.1] signal decreased in intensity from 1 to ~0.2 mM (Figure 5B-ii) and the addition of excess cyanide rapidly resulted in the disappearance of the EPR signal (Figure 5B-iii). This data strongly suggests that binding of the second cyanide to monocyano-Co(II)N₄[11.3.1] induces the cobalt to oxidize; thus, it is no longer EPR active.

Mouse Blood/Urine EPR

EPR spectroscopy represents a convenient method for assessing the biological reactions of CoN₄[11.3.1] in blood, since the majority hemoglobin (Hb) species, deoxyHb and oxyHb,

are EPR silent. Thus, while the absorption characteristics of hemoglobin derivatives in blood tend to obscure the electronic absorption spectra of other species, like CoN_4 [11.3.1], the EPR spectrum of the reduced form is readily observable. Following the addition of Co(III)-N_4 [11.3.1] to freshly drawn mouse blood, the appearance of an EPR signal at ~ 3000 G indicated reduction to Co(II)-N_4 [11.3.1] (Figure 5C-i). In addition to blood samples, mouse urine was collected ~ 40 min after the administration of Co(III)-N_4 [11.3.1] (26 mg/kg ip; $n = 3$) and rapidly frozen in liquid nitrogen. The EPR signals observed, peak at ~ 2900 G, is very slightly shifted compared to the EPR signals observed in mouse blood (Figure 5C-iii) or in buffer (Figure 5A-i,B-i), which may be due to either the ionic strength or the urea present in the urine. Upon addition of cyanide (5 mg/kg ip) 2 min after the injection of CoN_4 [11.3.1] (26 mg/kg, ip), samples ($n = 4$) of mouse blood were obtained by cardiac puncture following CO_2 euthanasia and frozen for subsequent EPR measurements. In these samples, only very weak EPR signals attributable to Co(II)-N_4 [11.3.1] could be observed (Figure 5C-ii), again indicating that the cobalt complex was oxidized upon reaction with cyanide. EPR samples obtained from mouse urine in similar experiments (5 mg/kg NaCN, followed by 26 mg/kg Co(II)-N_4 [11.3.1] at 2 min post cyanide, with urine collected at ~ 40 min post cyanide) also exhibited little or no Co(II) signal (Figure 5C-iv), again indicating the oxidation of Co(II)-N_4 [11.3.1] to a Co(III) form in the presence of cyanide.

Oxygraph Measurements

The tendency of cobalt complexes to activate/react with molecular oxygen has been known for a long time.^{24–27} Since inhibition of cytochrome *c* oxidase, the terminal oxygen acceptor in the mitochondrial electron-transport chain, is the primary toxic action of cyanide, it behooves us to ascertain the extent to which CoN_4 [11.3.1] exhibits this behavior. After all, from a clinical perspective, there might not be much point in alleviating cytochrome *c* oxidase inhibition if the cyanide scavenger involved also successfully outcompetes the enzyme for available oxygen. Co(III)-N_4 [11.3.1] (30 μM) was introduced into an Oxygraph (O2k) chamber, and after a several minute incubation at 37 °C (when the oxygen flux had stabilized), excess sodium ascorbate was added. There followed a rapid and dramatic drop in the oxygen concentration from 245 μM to ~ 0 in a matter of seconds (Figure 6, broken trace A). This result is highly reproducible and cannot be simple binding of oxygen to reduced Co(II)-N_4 [11.3.1] as the initial concentration of oxygen was ~ 245 μM and the concentration of Co(II)-N_4 [11.3.1] was roughly one-eighth of that. Therefore, we are observing the catalytic reduction of oxygen to water, a well-known reaction reported for several metal ion catalysts (e.g., copper and iron).^{28,29} The accepted mechanism^{30,31} for this type of oxygen turnover, using copper as the example, is shown in eqs 1–4, where AA represents ascorbic acid and DHAA stands for dehydroascorbic acid:

When cyanide (2 equiv) was quickly added to the Co(III)-N₄[11.3.1]/ascorbate reaction mixture, the oxygen turnover was essentially halted (Figure 6, solid trace B). Using the Amplex Red/HRP coupled reaction to detect the presence of hydrogen peroxide fluorometrically (simultaneously measured with oxygen concentration/flux using the O2k system) showed that very little hydrogen peroxide (1–2 μM) was generated during the reaction (data not shown). The last reaction of the known catalytic cycle (i.e., reaction 4, above) is known to be rapid; thus, it is reasonable to observe very little H₂O₂ production. Using catalytic amounts (30 μM) of either Cbi or CoTMPyP in the presence of excess ascorbate resulted in very similar behavior (not shown) to that observed for Co(II)N₄[11.3.1] (Figure 6). From a practical experimental point of view, the fact that these compounds all consume oxygen means that it is difficult to assay the oxygen turnover capability of cytochrome *c* oxidase (restoration of respiratory activity) in their presence. Ironically, while these cobalt compounds will deplete oxygen in the presence of reductant, this activity is inhibited in the presence of cyanide (Figure 6, solid trace B). Therefore, if used as cyanide-decorporating agents, the antidotal cyanide-scavenging reaction will tend to counteract the undesirable oxygen consumption activity, provided that cyanide remains in excess.

DISCUSSION

Potential New Class of Inexpensive Cyanide-Scavenging Compounds

Many four-to-five coordinate cobalt compounds were synthesized over 40 years ago, partly in an effort to make reversible oxygen carriers.^{12,13,25,27} While these compounds mainly failed in their ability to reversibly bind oxygen, they have been shown to be effective catalysts for the oxidation of a variety of small molecules including oxygen.^{15,25,27,32} Potentially, most similar complexes of cobalt with tetradentate ligands containing strong donors like nitrogen should also have the capacity to additionally bind two molecules of cyanide and thus function as cyanide scavengers, as we have recently observed in the case of a water-soluble cobalt porphyrin (CoTMPyP).^{10,11,33} Consequently, it is not altogether surprisingly that we now find the Schiff-base macrocyclic complex CoN₄[11.3.1] binds two cyanide anions (Figures 1 and 2) and is an efficacious cyanide antidote when given to mice both before and after the administration of an LD₄₀ dose of NaCN (5.0 mg/kg ip) (Figures 3 and 4). We have also shown that following recovery from acute effects there are no obvious short-term neuromuscular sequelae resulting from either the toxicant or the toxicant plus antidote (Figure 3), an encouraging result. Importantly, in head-to-head comparisons, using identical doses of antidote (on a molar basis) Cbi performed no better than CoN₄[11.3.1] in these assays (see Figure 3). From a practical (cost-effective) perspective, this is a highly significant observation as, based upon chemical company catalog prices of the starting materials, Cbi is 3 orders of magnitude more expensive than CoN₄[11.3.1]. Moreover, the

synthesis of $\text{CoN}_4[11.3.1]$ ($M_r = 317$ for the cation) is a one-step procedure, with the required product being crystallized from the reaction mixture at up to 60% yield,^{14,15,34} which are attractive characteristics from a manufacturing point of view. The better aqueous molar solubility due to the smaller size of $\text{CoN}_4[11.3.1]$ (and other similar macrocyclic compounds) compared to that of corrinoids and porphyrins is an additionally advantageous property, suggesting that this relatively simple Schiff-base complex is a representative of a remarkably promising new class of cyanide-decorporating agents.

Redox-Dependent Mechanism of *in Vivo* Cyanide Scavenging

The reduction of $\text{Co(III)N}_4[11.3.1]$ is facile and easily carried out with sodium ascorbate. The presence of an EPR signal with characteristics corresponding to the reduced $\text{Co(II)N}_4[11.3.1]$ form in mouse blood (Figure 5C), where the ascorbate concentration is near $60 \mu\text{M}$, lends further support to the argument that the reduced form must be prevalent under physiological circumstances. Therefore, even if the complex is given as $\text{Co(III)N}_4[11.3.1]$, it is to be anticipated that it will become reduced to $\text{Co(II)N}_4[11.3.1]$ *in vivo*, as we have recently shown to be the case for the metalloporphyrin CoTMPyP system.^{11,33} Therefore, if $\text{CoN}_4[11.3.1]$ is to be used as an antidote to cyanide toxicity, then its ability to bind cyanide in the reduced form is of paramount importance. The current results (Figures 2A and 5), however, lead us to conclude that cyanide rapidly catalyzes the conversion of the reduced complex to the oxidized form, $\text{Co(III)N}_4[11.3.1]\text{-(CN}^-\text{)}_2$. Again, this is reminiscent of CoTMPyP , where the oxidizing equivalents appear to be provided by protons with evolution of hydrogen.^{11,33} Titrations using a cyanide-selective electrode (Figure 1) and a spectrophotometric approach (Figure 2B) show that two cyanide molecules bind per $\text{Co(II)N}_4[11.3.1]$ cation and that the overall binding constant is $2.7 (\pm 0.2) \times 10^5$. In summary, as a cyanide-scavenging system, $\text{CoN}_4[11.3.1]$ appears to function via a redox-dependent mechanism, which takes advantage of (i) the typically facile nature of ligand substitution by Co(II) compared to that by Co(III) ^{35,36} coupled to (ii) the improved stability of anion binding to a trivalent metal ion and (iii) the relatively substitution inert behavior of Co(III) complexes, which prevents the reversal of the process and release of any cyanide once bound.

Application of $\text{CoN}_4[11.3.1]$ to Two-Component Antidotal Combinations

In the vast majority of cases (>99%), mice that do not survive the single-shot administration of toxicant NaCN that we employ (see Experimental Section) succumb within about 3 min.^{10,11,37,38} Thus, there cannot be much doubt that by this time point the toxicant has already become systemically distributed, passing into the tissues from the bloodstream, where it is able to inhibit cytochrome *c* oxidase (mitochondrial complex IV). Consequently, it is disappointing, but not especially surprising, that neither $\text{CoN}_4[11.3.1]$ nor Cbi given 5 min after the toxicant dose had any antidotal effect (Figure 3B). There is considerably less ambiguity involved in interpreting the results of these sublethal (righting recovery) intoxication assessments than some other experimental paradigms. Importantly, the animals are conscious, avoiding the known interfering complications of anesthesia.^{38,39} Most significantly, however, if there is a truly therapeutic effect of some putative antidote, then this can be routinely demonstrated up to at least 20 min following the toxicant dose; there is no experimental/practical necessity requiring that the antidote be given prophylactically or

coadministered with the toxicant. So, for instance, it was possible to demonstrate unambiguously by this method that sodium nitrite given *20 min after* a sodium cyanide dose clearly accelerated the recovery of righting ability in mice compared to that of control animals receiving only the toxicant.³⁷

At this juncture, we should also introduce a cautionary note regarding cell-based assays of respiratory recovery in the presence of CoN₄[11.3.1] and other cobalt-containing cyanide scavengers. We show that introduction of CoN₄[11.3.1] to a respirometer in the presence of reductant (ascorbate) in the complete absence of any isolated mitochondria, cultured cells, or minced tissue results in the rapid consumption of oxygen (Figure 6). Therefore, in any cell-based experiment where respiration has been inhibited by cyanide, an increase in oxygen consumption following the introduction of a cobalt-containing agent such as CoN₄[11.3.1], CoTMPyP, or one of the corrinoids cannot be interpreted as restoration of respiration through decorporation of cyanide. Catalytic consumption of oxygen by the cobalt-containing agent is much more likely for the additional reason that these cations are all too large and highly charged to enter the cytochrome *c* oxidase active site where they might influence cyanide decorporation.

The only agent of which we are aware that can actively displace cyanide from the active site of cytochrome *c* oxidase is NO.^{19,20} In our opinion, claims in the literature concerning the ability of other antidotal species to reverse cyanide inhibition of cytochrome *c* oxidase are based on data compromised by the ambiguities delineated above and/or failure to take into account the NO synthase activities in the systems under study. It follows, therefore, that in developing an antidotal protocol we suggest a combined approach where initially an NO donor (e.g., sodium nitrite) is given to sustain mitochondrial respiration,^{19,20,37,38} which is to be followed by a decorporating agent (e.g., CoN₄[11.3.1]) to bind any released cyanide in excretable form(s); this should prove to be better than giving either type of antidote alone. It is probably necessary that the antidotal action of the decorporating agent occurs in the bloodstream, since there are potentially toxic consequences (e.g., oxygen consumption for one) of the cobalt compounds being taken up by cells. Since it quickly appears in the urine of mice, CoN₄[11.3.1] seems to adequately fulfill this criterion.

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ABBREVIATIONS

CoN ₄ [11.3.1]	cobalt(II/III) 2,12-dimethyl-3,7,11,17-tetraaza-bicyclo-[11.3.1]-heptadeca-1(17)2,11,13,15-pentaenyl cation(s)
Cbi	cobinamide

Cbl	cobalamin
CoTMPyP	[[4,4',4'',4'''-porphyrin tetrayltetrakis(1-methylpyridiniumato)]-(2-)]cobalt(III) penta iodide
EDTA	ethylenediamine-tetraacetate

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Figure 1.

Cyanide electrode titration data. Main panel: Small aliquots of concentrated sodium cyanide solutions (dissolved in water at pH 11) were titrated into larger volumes of Co(II)N₄[11.3.1] (0.1 mM, freshly made in PBS, pH 7.4) at 25 °C. Potentiometric determinations of free cyanide were carried out with a cyanide-ion-selective electrode (Orion Ionplus Sure-Flow Combination, Thermo Scientific). Before measuring the samples, the electrode was calibrated with freshly made sodium cyanide standard solutions (10⁻⁵ to 10⁻² M). Prior to each measurement, standards and samples were all stabilized with alkaline ionic strength adjustor solution (Orion, Thermo Scientific). Potentiometric measurements for the additions of cyanide from 0.0 to 0.6 mM were recorded. The dashed line represents a nonlinear least-squares simulation of the data using the Hill equation with $\alpha_H = 2$. The error bars represent the standard deviation. Inset: Structure of Co(II)N₄[11.3.1].^{12,15}

Figure 2.

Titration of $\text{Co(II)N}_4[11.3.1]$ with cyanide at pH 7.4 and 25 °C. Small aliquots of sodium cyanide solution (pH 10) were titrated into a solution of $\text{Co(II)N}_4[11.3.1]$ (0.3 mM in PBS, pH 7.4) using gastight syringes and a 1.00 cm path length septum-sealed cuvette (see Experimental Section for further details). (A) Electronic absorption spectra of $\text{Co(II)N}_4[11.3.1]$ titrated with NaCN. Initial $\text{Co(II)N}_4[11.3.1]$ (solid trace); subsequent absorbance changes observed during the addition of cyanide to concentrations of 0.1, 0.2, 0.3, 0.4, and 0.5 mM (dotted traces); final spectrum of $\text{Co(II)N}_4[11.3.1](\text{CN})_2$ (bold solid trace) after the addition of cyanide to 0.6 mM. (B) Titration of 0.3 mM $\text{Co(II)N}_4[11.3.1]$ with cyanide following the absorbance changes at 540 nm. The dashed line represents a nonlinear least-squares fit to the data using the Hill equation with $\alpha_H = 2$.

Figure 3.

Dose and time response of administered CoN₄[11.3.1] ameliorating NaCN toxicity in male mice. (A) Dose response. To establish the effective range of CoN₄[11.3.1], mice (male Swiss-Webster, 7–8 weeks of age) received either 70, 56, 52, 44, 39, 37, or 30 μmol/kg of Co(II)N₄[11.3.1] in saline (ip) 5 min before receiving 0.1 mmol/kg NaCN in saline (ip), and durations until righting recovery were recorded. A range of doses from 70 to 44 μmol/kg of Co(II)N₄[11.3.1] were found to decrease righting-recovery time significantly compared to that for mice that did not receive any antidote. 5.0 mg/kg NaCN only ($n = 28$); 70 μmol/kg Co(II)-N₄[11.3.1] ($n = 6$); 56 μmol/kg Co(II)N₄[11.3.1] ($n = 5$); 52 μmol/kg Co(II)N₄[11.3.1] ($n = 13$); 44 μmol/kg Co(II)N₄[11.3.1] ($n = 5$); 39 μmol/kg Co(II)N₄[11.3.1] ($n = 4$); 37 μmol/kg Co(II)N₄[11.3.1] ($n = 4$); and 30 μmol/kg Co(II)N₄[11.3.1] ($n = 6$). (B) Time response. To establish the post-toxicant therapeutic window for CoN₄[11.3.1], mice (male Swiss-Webster, 7–8 weeks of age) received 52 μmol/kg Co(II)N₄[11.3.1] in saline (ip) either 5 min before or 1, 2, or 5 min after receiving 0.1 mmol/kg NaCN in saline (ip), and durations until righting recovery were recorded. For comparison, a group in which 50 μmol/kg cobinamide (Cbi) was given (ip) 5 min after NaCN injection is also included. 5.0 mg/kg NaCN only ($n = 28$); CoN₄[11.3.1] was given at –5 min ($n = 13$); +1 min ($n = 6$); +2 min ($n = 6$); +5 min ($n = 6$); or Cbi + 5 min ($n = 3$). Error bars represent standard deviation. ****, $p < 0.0001$; ***, $p < 0.001$; *, $p < 0.05$ compared to NaCN (only) controls, as determined by one-way ANOVA in GraphPad Prism.

Figure 4.

RotaRod testing of neuromuscular coordination following NaCN/CoN₄[11.3.1] exposures in male Swiss-Webster mice. Mice were trained on the RotaRod 24 h before any injections, and baseline performance was obtained 1 h before injections (pre-ip). Mice were tested every 15 min after injections for 2.5 h and again at 24 h to assess recovery. Comparison of performance for injections of 100 μ mol/kg NaCN (closed circle); 52 μ mol/kg CoN₄[11.3.1] (open square); 52 μ mol/kg CoN₄[11.3.1] given 5 min prior to 100 μ mol/kg NaCN (closed diamond); and saline control (open triangle). Numbers of animals (in parentheses) used in each set of experiments are as follows: NaCN (9), CoN₄[11.3.1] (7), CoN₄[11.3.1] + NaCN (7), and saline (5).

Figure 5.

X-band EPR spectra of $\text{Co(II)N}_4[11.3.1]$ in buffer, mouse blood, and mouse urine at 20 K. (A) Samples of $\text{Co(II)N}_4[11.3.1]$ (1 mM) were prepared anaerobically (room temperature): (i) in 50 mM potassium phosphate buffer (PB), pH 7.4, with 10% (v/v) glycerol, (ii) excess NaCN (20 mM) was added to $\text{Co(II)N}_4[11.3.1]$ in PB/glycerol, (iii) in 50 mM PB alone, or (iv) 1 equiv of NaCN was added to $\text{Co(II)N}_4[11.3.1]$ in PB alone. These samples were rapidly frozen in liquid nitrogen for subsequent recording of spectra. (B) Samples of $\text{Co(II)N}_4[11.3.1]$ (1 mM) were prepared anaerobically in 50 mM PB (i) with 1 equiv of NaCN, (ii) plus 2 equiv of NaCN, or (iii) plus excess sodium cyanide (20 mM). Samples were frozen by immersion in liquid nitrogen after allowing 4 min reaction time. (C) Samples of mouse blood were obtained by heart puncture. (i) $\text{Co(II)N}_4[11.3.1]$ was added to 1 mM or (ii) $\text{Co(II)N}_4[11.3.1]$ was added to 1 mM and then excess sodium cyanide was added to 20 mM. (iii) Mouse urine was collected ~30 min after the administration of $\text{Co(II)N}_4[11.3.1]$ (26 mg/kg, ip) or (iv) $\text{Co(II)N}_4[11.3.1]$ (26 mg/kg, ip) was added followed by NaCN (5 mg/kg, ip) 5 min later, and the urine was collected after another 30 min. Samples were rapidly frozen in liquid nitrogen for subsequent recording of spectra. EPR conditions: 9.8 G modulation amplitude, 63.2 μW microwave power.

Figure 6.

Oxygen consumption by $\text{CoN}_4[11.3.1]$ in the presence of excess ascorbate. Samples of $30 \mu\text{M}$ $\text{Co(III)N}_4[11.3.1]$ in 50 mM phosphate buffer ($\text{pH } 7.4$) were introduced into Oxygraph chambers (O2k, Oroboros) and equilibrated for several minutes at 37°C . Sodium ascorbate (final concentration of 1 mM) was added using a gastight syringe (traces A and B). In trace B, sodium cyanide was added ($60 \mu\text{M}$, final concentration). The addition of ascorbate (only) to the Oxygraph chamber showed no significant oxygen consumption (data not shown). Traces A and B are representative of several traces ($n = 4$) for each condition.