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The vitamin B_{12} analog cobinamide ameliorates azide toxicity in cells, *Drosophila melanogaster*, and mice

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Abstract

Context: The azide anion (N_3-) is highly toxic. It exists most commonly as sodium azide, which is used widely and is readily available, raising the potential for occupational incidents and use as a weapon of mass destruction. Azide-poisoned patients present with vomiting, seizures, hypotension, metabolic acidosis, and coma; death can occur. No specific azide antidote exists, with treatment being solely supportive. Azide inhibits mitochondrial cytochrome c oxidase and is likely oxidized to nitric oxide *in vivo*. Cytochrome c oxidase inhibition depletes intracellular adenosine triphosphate and increases oxidative stress, while increased nitric oxide causes hypotension and exacerbates oxidative damage. Here, we tested whether the cobalamin (vitamin B_{12}) analog cobinamide, a strong and versatile antioxidant that also neutralizes nitric oxide, can reverse azide toxicity in mammalian cells, *Drosophila melanogaster*, and mice.

Results: We found cobinamide bound azide with a moderate affinity (K_a 2.87 x 10^5 M^{-1}). Yet, cobinamide improved growth, increased intracellular adenosine triphosphate, and reduced apoptosis and malondialdehyde, a marker of oxidative stress, in azide-exposed cells. Cobinamide rescued Drosophila *melanogaster* and mice from lethal exposures to azide and was more effective than hydroxo-cobalamin. Azide likely generated nitric oxide in the mice, as evidenced by increased serum nitrite and nitrate and reduced blood pressure and peripheral body temperature in the animals; the reduced temperature was likely due to reflex vasoconstriction in response to the hypotension. Cobinamide improved recovery of both blood pressure and body temperature.

Conclusion: We conclude cobinamide likely acted by neutralizing both oxidative stress and nitric oxide, and that it should be given further consideration as an azide antidote.

Keywords

Sodium azide; azide poisoning; oxidative stress; survival treatment

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AUTHOR CONTRIBUTIONS

Conceptualization, Funding Acquisition, Supervision: GRB; Investigation: JT, SCC, CDL, SR, MJI, BK, AC, HK, and RBP; Data Acquisition and Formal Analysis: JT and GRB. Writing: JT and GRB with input from co-authors.

DECLARATION OF INTEREST STATEMENT

The authors report there are no competing interests to declare.

INTRODUCTION

The azide (N_3-) anion is available in a variety of forms, most commonly as sodium azide (NaN_3). Azide is used in several industries with over 1,000 tons of NaN_3 produced annually [1]. Azide is highly toxic, with the human lethal dose of NaN_3 estimated to be ~700 mg or ~10 mg/kg [2]. Fortunately, azide poisoning is rare with 156 reported cases worldwide between the years 2000 and 2020 [3]. However, this infrequency may prove detrimental in a mass casualty event, such as an industrial incident or terrorist attack, as most medical personnel will not have encountered an azide-poisoned patient and therefore not be well-informed about azide toxicity and treatment options. A major terrorist attack is possible, since azide may be purchased through online retailers, and it has been used in several planned and executed attacks [4, 5, 6, 7, 8, 9]. On several occasions, azide was used to poison communal beverages, leading to high casualties and highlighting the potential of azide as a terrorist weapon [10, 11, 12, 13, 14]. Moreover, azide is a common suicidal agent, especially among laboratory workers, likely due to its common presence in research laboratories [2, 3, 15].

Symptoms occur within minutes of azide exposure, ranging from dizziness, nausea, vomiting, and restlessness at low doses, to seizures, hypotension, metabolic acidosis, coma, and respiratory failure at high doses. Like carbon monoxide, cyanide, hydrogen sulfide, and methyl mercaptan, azide inhibits cytochrome c oxidase in complex IV of the mitochondrial electron transport chain, reducing adenosine triphosphate (ATP) production [16, 17, 18, 19]. This results in a compensatory increase in glycolysis with attendant metabolic acidosis. Inhibiting complex IV increases mitochondrial electron leakage, thereby increasing superoxide anion ($O_2\cdot^-$) generation. Consistent with this mechanism, brain, lung, and heart tissues taken from azide-poisoned rats showed significant increases in malondialdehyde concentrations, a marker of lipid peroxidation [20]. Through a poorly understood mechanism, azide appears to be converted to nitric oxide (NO) [21, 22, 23], which likely underlies the profound hypotension that can occur in azide-poisoned victims.

No specific antidote is available for azide poisoning and treatment is solely supportive [3]. Due to mechanistic similarities between azide and cyanide, azide poisoning victims have been given cyanide antidotes such as sodium thiosulfate, amyl nitrite, sodium nitrite, or hydroxocobalamin [3, 12, 24, 25, 26]. Sodium thiosulfate detoxifies cyanide by converting it to thiocyanate, but no evidence exists that sodium thiosulfate reacts with azide. Amyl nitrite and sodium nitrite generate methemoglobin, which has a high affinity for cyanide, but binds azide only weakly [27, 28]; nitrite might also exacerbate azide-induced hypotension by virtue of its reduction to nitric oxide. Hydroxocobalamin is a rational treatment for azide poisoning because it binds both azide and NO, and thereby could potentially neutralize both species [29, 30, 31]. However, hydroxocobalamin has low water solubility, necessitating administration in relatively large volumes via intravenous infusion. It, therefore, does not lend itself well to pre-hospital use.

Cobinamide is the penultimate precursor in the biosynthesis of cobalamin, lacking a dimethylbenzimidazole ribonucleotide group coordinated to the lower axial position of the cobalt atom (Supplemental Figures S1A,B, [32]). Absence of the dimethylbenzimidazole

ribonucleotide group imparts several chemical differences between cobinamide and cobalamin: (i) cobinamide has two, rather than one, ligand binding site; (ii) cobinamide has higher affinity for ligands [33]; (iii) cobinamide is more easily oxidized and reduced [34]; (iv) cobinamide is more water soluble, providing the potential for intramuscular administration in the field; and (v) cobinamide reacts more readily with NO and $O_2\cdot^-$ [30, 34, 35]. Since hydroxocobalamin binds azide, this suggested to us that cobinamide would also bind azide, and thus could serve as an azide scavenger. Moreover, the strong antioxidant and NO neutralizing properties of cobinamide further suggested it could serve as a sodium azide antidote.

MATERIALS AND METHODS

Materials

Aquohydroxocobinamide was synthesized by base hydrolysis of hydroxocobalamin (Nutrakey Industries, Qingdao, China) with purification over reversed-phase resins [36]. Histidyl-cobinamide was generated by adding two molar equivalents of histidine (Sigma-Aldrich, St. Louis, MO) to an aqueous solution of aquohydroxocobinamide. The histidyl ligand was added to increase cobinamide absorption after intramuscular injection. The term “cobinamide” is used generically in the text without reference to the axial ligands. All experiments were performed with cobinamide in the +3 oxidation state.

Assessment of Azide Binding to Cobinamide and Cobalamin

Binding of ligands to the cobalt center of cobinamide or cobalamin leads to spectral changes in the UV-visible range. We recorded the UV-visible spectra of aquohydroxocobinamide and hydroxocobalamin on a Kontron 960 double-beam spectrophotometer in the absence and presence of varying concentrations of sodium azide. Spectra were recorded after incubating 25 μM aquohydroxocobinamide or 25 μM hydroxocobalamin with indicated amounts of sodium azide for 5 min at room temperature in 20 mM sodium phosphate buffer, pH 7.4.

Tissue Culture

A549 type II human alveolar adenocarcinoma cells and COS-7 monkey kidney cells were obtained from the American Type Culture Collection. They were grown in Dulbecco’s Modified Eagles medium supplemented with 25 mM glucose and 10% fetal bovine serum (FBS).

Cell Survival Studies

Approximately 5 x 10^4 A549 or COS-7 cells were seeded in six-well plates overnight. Cells were then treated with sodium azide at the indicated concentrations for 48 hours in the presence or absence of 25 μM aquohydroxocobinamide or 25 μM hydroxocobalamin. After treatment, cells were washed with phosphate-buffered saline (PBS) and counted using a hemocytometer.

To assess clonogenic survival, A549 cells were seeded overnight at a low density (~100 cells per six-well plate). The cells were then treated for 24 hours with NaN_3 at the indicated concentrations in the presence or absence of 25 μM aquohydroxocobinamide. The cells

were washed with PBS and incubated in drug-free medium for 15 days. To evaluate colony number, cells were first washed in PBS and then stained with 0.25% w/v crystal violet in 25% methanol overnight. Following removal of the stain, the plates were air-dried and colonies were counted by visual inspection.

Measuring Intracellular ATP Content

A549 and COS-7 cells were seeded in 24-well plates in standard growth medium and then incubated for 24 hours in glucose-free medium supplemented with 25 mM galactose (as a carbon source) and 10% FBS. The cells were then treated for 24 hours with 0.1 mM NaN_3 in the presence or absence of 25 μM aquohydroxocobinamide. ATP was measured using the Cell-Titer Glo 2.0 kit (Promega, Madison WI). Briefly, cells were lysed *in situ* and ATP was measured in the lysates using the firefly luciferase-luciferin system. Luminescence was recorded using a Tecan Infinite™ 200 Microplate Reader. The amount of ATP was normalized to 100,000 cells.

Measuring Lipid Peroxidation Products

A549 cells in 10-cm plates were treated with indicated amount of NaN_3 with or without 25 μM aquohydroxocobinamide for 24 hours. The cells were harvested using trypsin/EDTA, counted with a hemocytometer, and washed with PBS. The cells were lysed in H_2O and extracted in 3% trichloroacetic acid/33% thiobarbituric acid. The extracts were boiled for 45 minutes and lipids were extracted in butanol. Malondialdehyde, a product of lipid peroxidation, reacts with thiobarbituric acid to generate a pink-colored species that was measured spectrofluorometrically at 530_{ex} and 590_{em}. The amount of malondialdehyde in samples was determined from standard curves (Cayman Chemical Company), and recovery of malondialdehyde in spiked samples was greater than 90%.

Detecting Cleaved Caspase-3

A549 cells were plated on glass coverslips and then treated with indicated amounts of azide in the presence or absence of 25 μM cobinamide for 24 hours. The cells were fixed in 3.7% paraformaldehyde, permeabilized with 1% Triton-X-100, and incubated with a cleaved caspase-3-specific antibody (1:100 dilution) (Cell Signaling) overnight at 4°C. After incubation with a fluorescein isothiocyanate-conjugated secondary antibody (1:100 dilution) (Jackson ImmunoResearch, West Grove, PA), nuclei were stained using Hoechst 33342 dye. Immunofluorescence images were recorded using a Keyence BZ-X700 fluorescence microscope.

Drosophila Melanogaster Poisoning Models

Drosophila melanogaster were raised on 400 μM aquohydroxocobinamide or 400 μM hydroxocobalamin for at least one week to preload the animals with the drugs. We included animals of both sexes and various ages.

To simulate azide inhalation, hydrazoic acid (HN_3), which is volatile at room temperature, was generated by adding 100 μL of a 100 mM NaN_3 solution to a 1 cm^2 piece of Whatman paper that had been previously saturated with 1 N hydrochloric acid and then air-dried. The Whatman paper was immediately transferred to vials containing *D. melanogaster* and the

number of animals falling motionless over 2 minutes was recorded. The animals were then transferred to new vials and were monitored for recovery over 3 days.

To simulate azide ingestion, gauze soaked in 100 mM NaN_3 or sodium phosphate was placed into empty vials, with the gauze as the only source of water. *D. melanogaster* were transferred to the vials and their survival was recorded over 8 hours.

Mouse Poisoning Models

Male and female C57BL/6J mice aged 3-21 months were purchased from Jackson Laboratory (Bar Harbor, ME); using both sexes and varying-aged mice simulated a heterogenous human population, as would be present in a mass casualty event. The experiments were conducted according to the National Academies of Sciences, Engineering, and Medicine Institute for Laboratory Animal Research Guide to the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of California, San Diego.

In survival studies, mice were anesthetized with 2% isoflurane and then given 30 mg/kg NaN_3 by intraperitoneal injection as a 100-μL bolus from a 1 mL plastic syringe. This dose was determined empirically to be ≥75% lethal by exposing four groups of mice, 4-6 animals per group, to varying amounts of NaN_3 ranging from 25 mg/kg to 33 mg/kg. Two minutes after the NaN_3 injection, the mice were given an intramuscular injection of 350 mg/kg histidylcobinamide, 450 mg/kg hydroxocobalamin, or water vehicle into the hind quadricep. The animals were removed from isoflurane and monitored for survival over 3 hours. Survivors were returned to the vivarium. The cobinamide dose is the same as that used to rescue mice from hydrogen sulfide poisoning [18], and corresponds to 28.4 mg/kg when converted to a human equivalent dose using body surface area correction; the cobalamin dose corresponds to the cobinamide dose on a molar basis.

To evaluate the effect of sodium azide on blood pressure, the systolic and diastolic blood pressure was measured by tail-cuff plethysmography (Kent Scientific Corporation CODA system, Torrington, CT). The occlusion cuff was placed 10 mm from the base of the tail and the volume pressure recording cuff was placed 10 mm distal to the occlusion cuff. Mice were then given an intraperitoneal injection of water or 20 mg/kg NaN_3 as a 100-μL bolus. Blood pressure was measured every 5 minutes for 20 minutes, at which time the mice were given one intraperitoneal injection of either 350 mg/kg histidylcobinamide or water vehicle as a 100-μL bolus. The blood pressure was measured every 5 minutes over a 15-minute period and then at 60, 120, and 180 minutes after the NaN_3 injection.

The same treatment protocol as described for measuring blood pressure was applied to measuring tail vein temperature. Measurements were taken at indicated times using a laser thermometer. To assure temperature was taken at the same location each time, a spot was marked 20 mm from the base of the tail. Room temperature was also recorded during the duration of the tail temperature assessment.

Measurement of Serum Nitrite and Nitrate

Mice were given an intraperitoneal injection of 20 mg/kg NaN_3 (or water vehicle) as a 100-μL bolus, and then euthanized via carbon dioxide asphyxiation at indicated times. Blood was collected immediately via cardiac puncture and centrifuged to remove cells. Nitrite and nitrate were measured in the serum using the Griess reagent-based assay. However, azide interferes with the assay [37]; to circumvent this problem, azide was eliminated from samples by first converting it to hydrazoic acid, which was then removed by evaporation. Briefly, serum was deproteinated using centrifugal concentrators with a 5,000 Dalton molecular weight cut-off. The samples were then acidified using HCl, incubated for 2 hours at 37°C, and neutralized with NaOH and 50 mM Tris pH 7.4. Sample volumes were equalized, and the samples were incubated with nitrate reductase (Active Motif, Carlsbad, CA) to reduce nitrate to nitrite. Griess reagent (Sigma-Aldrich, St. Louis, MO) was then added to each sample, and after a 20 min incubation period, absorbance was measured at 540 nm in a microplate reader. The amount of nitrite and nitrate in samples was determined from standard curves, and the recovery of nitrite and nitrate in spiked samples was greater than 90%.

Statistics—Statistical tests were performed with GraphPad Prism Statistics Software Version 9.3.0 (GraphPad Prism, Carlsbad, CA). Data are presented as the mean (or the mean normalized to controls set at 100%) ± SEM. Both one-way and two-way ANOVAs with Šadák correction were used to detect differences in cell-based assays. In the *D. melanogaster* studies and in the mouse studies where we measured serum nitrite and nitrate concentrations, blood pressure, and peripheral body temperature, differences among conditions were assessed using a two-way ANOVA with Šadák correction. In the mouse survival studies, a log-rank test was used to assess differences. A P-value <0.05 was considered significant.

RESULTS

Cobinamide Binds Azide and Protects Cultured Mammalian Cells from Azide Toxicity

Since cobalamin binds azide [31], we postulated that cobinamide would also bind azide. We added increasing concentrations of NaN_3 from 6.25 to 400 μM, to a 25 μM aquohydroxocobinamide solution and found a progressive change in the UV-visible spectrum of cobinamide (Figure 1A). From these data, we estimated a K_a of azide binding to cobinamide of $2.87 \times 10^5\ M^{-1}$ based on the absorbance change at 515 nm (Figure 1A, **inset**). For comparison, we found a K_a of azide binding to hydroxocobalamin of $2.3 \times 10^5\ M^{-1}$, which is similar to the published value of $7.2 \times 10^4\ M^{-1}$ ([31] and Figure 1B, **inset**). Although cobinamide had a slightly higher affinity for azide than cobalamin, both binding affinities are rather modest and will be discussed later.

To determine if cobinamide reduced azide toxicity in cells, we tested cobinamide in COS-7 and A549 cells exposed to NaN_3 . We found that 25 μM aquohydroxocobinamide increased the IC_{50} of NaN_3 by about 3.4-fold in A549 cells (Figure 1C), but 25 μM hydroxocobalamin had essentially no effect on reducing azide toxicity in these cells (Figure 1D). A549 cells were derived from a patient with a lung adenocarcinoma and can generate clones from

single cells, allowing us to query the effect of azide on clonal growth. We found that 0.1 mM and 1 mM NaN_3 significantly reduced A549 cell clonal growth, and that 25 μM aquohydroxocobinamide improved growth at both NaN_3 concentrations (Figures 1E,F). Similar to A549 cells, 25 μM aquohydroxocobinamide increased the IC_{50} of NaN_3 by about 2.3-fold in COS-7 cells, but 25 μM hydroxocobalamin led to only marginal reduction in azide toxicity (Supplemental Figures S2A,B). Since cobinamide and cobalamin had a similar K_a for azide and yet only cobinamide reduced azide toxicity, this suggests cobinamide was ameliorating azide toxicity through mechanism(s) other than binding azide. We addressed this issue in subsequent experiments and we will consider it further in the Discussion.

Mechanisms of Azide Toxicity; Rescue by Cobinamide

The effect of azide on cellular metabolites has received little attention. Since azide inhibits mitochondrial cytochrome c oxidase, we predicted azide would reduce the intracellular concentration of ATP. We found exposing COS-7 and A549 cells for 24 h to 0.1 mM NaN_3 (near the IC_{50} for both cell types) markedly decreased the amount of intracellular ATP in both cell types (Figures 2A,B). Aquohydroxocobinamide significantly increased intracellular ATP in the azide-treated cells (Figures 2A,B).

Inhibition of cytochrome c oxidase increases electron leakage from the electron transport chain, and this can result in increased generation of superoxide and increased oxidative stress. We assessed intracellular malondialdehyde as a readout for lipid oxidation and found that while 0.1 mM NaN_3 marginally increased malondialdehyde in A549 cells, 1 mM NaN_3 significantly increased the malondialdehyde concentration (Figure 2C). Consistent with cobinamide being a strong antioxidant [34], we found that it significantly reduced the increase in malondialdehyde by 1 mM NaN_3 (Figure 2C).

The combination of ATP depletion and increased oxidative stress could increase cellular apoptosis. We found NaN_3 caused a dose-dependent increase in apoptosis as assessed by measuring cleaved caspase-3 positive cells (Figure 2**F**). Cobinamide significantly reversed apoptosis induced by 1 mM NaN_3.

Cobinamide Rescues *D. melanogaster* and Mice from Azide Toxicity

We next tested whether cobinamide could rescue an intact animal from azide toxicity. In the initial studies, we used *D. melanogaster*, which are increasingly used in drug discovery due to their low cost, rapid life cycle, and wide availability of genetic variants. We tested two common modes of azide poisoning—inhalation and ingestion [3]. In both models, we pre-administered aquohydroxocobinamide or hydroxocobalamin to the animals for one week by adding the drugs to their food.

To simulate inhalation toxicity, *D. melanogaster* were exposed to hydrazoic acid (HN_3), which was generated in a tightly-capped vial by spotting NaN_3 on acidified filter paper. The calculated HN_3 concentration in the vial was 1500 ppm. All animals became motionless within 2 minutes following HN_3 exposure, regardless of whether they had been pre-treated with cobinamide or cobalamin (Figure 3A). This rapid state of immobility recapitulated the “knock-down” and unconscious state experienced by humans exposed to

concentrated HN_3 [38]. The animals were then moved to vials containing standard food and monitored over 3 days. *Drosophila melanogaster* raised on both aquohydroxocobinamide and hydroxocobalamin showed better recovery than control animals, with cobinamide being superior to cobalamin (Figure 3A).

To simulate oral poisoning, which is the main route of azide exposure in humans [3], *D. melanogaster* were placed in vials containing a gauze soaked in 100 mM NaN_3 or sodium phosphate as their only source of water. The animals were monitored for survival over 8 hours. Animals that had received hydroxocobalamin showed a marginal improvement in survival, whereas animals that had received aquohydroxocobinamide showed a major improvement in survival (Figure 3B).

We next tested whether cobinamide could improve survival in mice that received a lethal dose of NaN_3 administered by intraperitoneal injection. We administered histidylcobinamide or hydroxocobalamin by intramuscular injection, the preferred route for administering drug in a pre-hospital setting, as in a large casualty event. We found that cobinamide-treated mice, both males and females, showed significantly better survival than their control counterparts (Figure 3C). Although cobalamin-treated mice showed some improvement in survival compared to control mice, the difference between the two groups was not significant and rescue by cobinamide was significantly different than that by cobalamin.

Therapeutic Effect of Cobinamide on Blood Pressure and Peripheral Body Temperature

Sodium azide has been shown to increase nitric oxide generation *in vivo* through an undefined mechanism [21, 22, 23]. Nitric oxide is rapidly oxidized to nitrite and nitrate and the latter can be used as a proxy for nitric oxide concentration. We found that serum nitrite and nitrate concentrations rose rapidly and remained elevated for 3 hours following NaN_3 injection into both male and female mice (Figure 4A). We were unable to measure serum nitrite and nitrate concentrations in mice receiving cobinamide, which we will consider further in the Discussion.

Since nitric oxide is a potent vasodilator, we assessed changes in blood pressure in mice following a sub-lethal dose of azide. Within 5 min of receiving an intraperitoneal injection of NaN_3, the mean arterial pressure decreased by 30-40 mm Hg (Figure 4B). At 20 min after azide exposure, comparable to the amount of time required for emergency medical personnel to reach the scene of a poisoning event, mice received either water or 300 mg/kg histidylcobinamide by intraperitoneal injection. Although blood pressure began to rise in both conditions, recovery was more rapid among animals that received cobinamide compared to those that received vehicle (water). Mice given vehicle or cobinamide alone had a steady mean arterial pressure throughout the study (Supplemental Figure S3A).

Peripheral vasoconstriction is a compensatory mechanism to increase blood flow to visceral organs and the brain under conditions of hypotension; this can lead to peripheral hypothermia. In alignment with the decreased blood pressure after NaN_3 injection, tail temperature dropped, albeit not as rapidly as the fall in blood pressure (Figure 4C). In contrast to the mean arterial pressure which reached its nadir within 5 minutes post azide exposure, tail temperature reached its nadir 20 minutes post-exposure. Room temperature

remained steady throughout the experiment. Following injection of either the water vehicle or cobinamide, the tail temperature remained low for ~40 minutes, and then increased at a significantly faster rate in cobinamide- than vehicle-treated animals. Treatment with water or cobinamide alone did not affect tail temperature (Supplemental Figure S3B).

DISCUSSION

Due to its widespread production and ease of accessibility [1, 9], NaN_3 is a highly hazardous chemical. The Department of Homeland Security lists azide as a Chemical of Interest because it could cause mass casualties if high amounts were released in an industrial incident or terrorist attack [14]. Although poisoning is rare, the infrequency of reported cases may mean most medical personnel will not possess sufficient knowledge to properly treat azide-poisoned victims. This poor knowledge in conjunction with the absence of an azide-specific antidote may have contributed to varied success in treating patients. Thus, an urgent need exists for an azide antidote that can be administered easily, especially in a mass-casualty event.

The mechanism of azide-induced toxicity is not understood fully but is likely due to the combination of cytochrome c oxidase inhibition and increased nitric oxide production [21, 22, 23]. We found that sodium azide decreased cell growth and intracellular ATP content, and increased apoptosis and oxidative stress in mammalian cells. The decrease in ATP and increase in apoptosis and oxidative stress were likely from cytochrome c oxidase inhibition, with an attendant increase in electron leakage from the mitochondrial electron transport chain. Electron leakage decreases the mitochondrial membrane potential, thereby decreasing ATP synthase activity and increasing O_2^- and hydrogen peroxide production. These reactive oxygen species lead to apoptosis through mitochondrial release of cytochrome c and apoptosis-inducing factor [39], and to oxidative stress by oxidizing DNA, lipids, and proteins. Thus, within cultured cells, all of the toxicity of azide could be explained by cytochrome c oxidase inhibition. However, this seems not to be the case in mice, where the observed hypotension was more likely from increased NO production as assessed by a marked increase in serum nitrite and nitrate concentrations. Overall, our data support important roles for both cytochrome c oxidase inhibition and increased NO production as mechanisms of azide toxicity.

Cobinamide is well suited as an azide antidote for the following three reasons. First, cobinamide is a potent and versatile antioxidant, serving as both a superoxide dismutase mimetic and a catalase mimetic, thereby neutralizing O_2^- and hydrogen peroxide, respectively [34]. Cobinamide also neutralizes peroxynitrite, a strong oxidizing species that is generated by the diffusion-limited reaction of O_2^- and NO [34]. Second, cobinamide oxidizes NO to nitrite and then the resulting reduced form of cobinamide binds NO; thus, one cobinamide molecule can neutralize two NO molecules [30]. And third, we now report that cobinamide binds azide, and thus could potentially neutralize azide directly. However, the affinity of cobinamide for azide is relatively modest, and thus this mechanism would likely play a role only when azide is present at relatively high concentrations.

Although no drug is approved for azide poisoning, hydroxocobalamin has been used and it is a reasonable treatment due to its binding of azide and its antioxidative properties [26, 31, 34]. We found that cobinamide was superior to cobalamin in rescuing cells, *D. melanogaster*, and mice from azide exposure. Thus, cobinamide would appear to be favoured over cobalamin for treating azide poisoning. We did not compare cobinamide to cobalamin in the mouse physiological experiments because we wanted to minimize the use of mammals.

Although the rate of azide conversion to NO in an animal is unknown, it likely is fast since the blood pressure in mice dropped by ~50% within 5 min of azide exposure. Consistent with a swift *in vivo* conversion of azide to NO, we found that the serum concentration of nitrite and nitrate increased rapidly in the mice. Within 15 minutes of an intramuscular injection of cobinamide, the blood pressure of the mice began to rise, likely due to cobinamide's neutralization of NO. We were unable to determine if cobinamide reduced nitrite and nitrate in azide-poisoned animals because the red color of cobinamide interfered with the spectrophotometric measurement of nitrite and nitrate. Due to the small volume of blood available from a mouse, we were unable to successfully remove cobinamide from the serum.

Other agents have shown some benefit as azide antidotes, including the azide scavenger $Co(II)N_4$[11.3.1] [40, 41] and the antioxidant quercetin [20]. Thus, scavenging azide and neutralizing reactive oxygen species are reasonable approaches for antagonizing azide's toxic effects. However, cobinamide could prove superior to these other agents due to its multiple effects, including neutralizing nitric oxide.

Limitations

In the mouse survival studies, we were required by our IACUC to anesthetize the animals while they received the sodium azide, and we used 2% isoflurane. In addition to the concern that prolonged anesthesia exposure may not reflect a real-life poisoning scenario, isoflurane can cause hypotension, and it seemed possible that azide-generated nitric oxide plus isoflurane exposure could cause severe and irreversible hypotension [42, 43]. We, therefore, limited the duration of isoflurane exposure, but this also limited our ability to test delayed administration of cobinamide, because we wanted to inject cobinamide while the animals were still anesthetized, both for ease of experimental manipulation and to minimize pain from the intramuscular injection. While the results show cobinamide can counteract azide toxicity in mice, administrating cobinamide within two minutes of azide exposure is not clinically possible or relevant. Future studies in larger mammalian species administering cobinamide at later times after azide exposure will better test the efficacy of cobinamide as an azide antidote.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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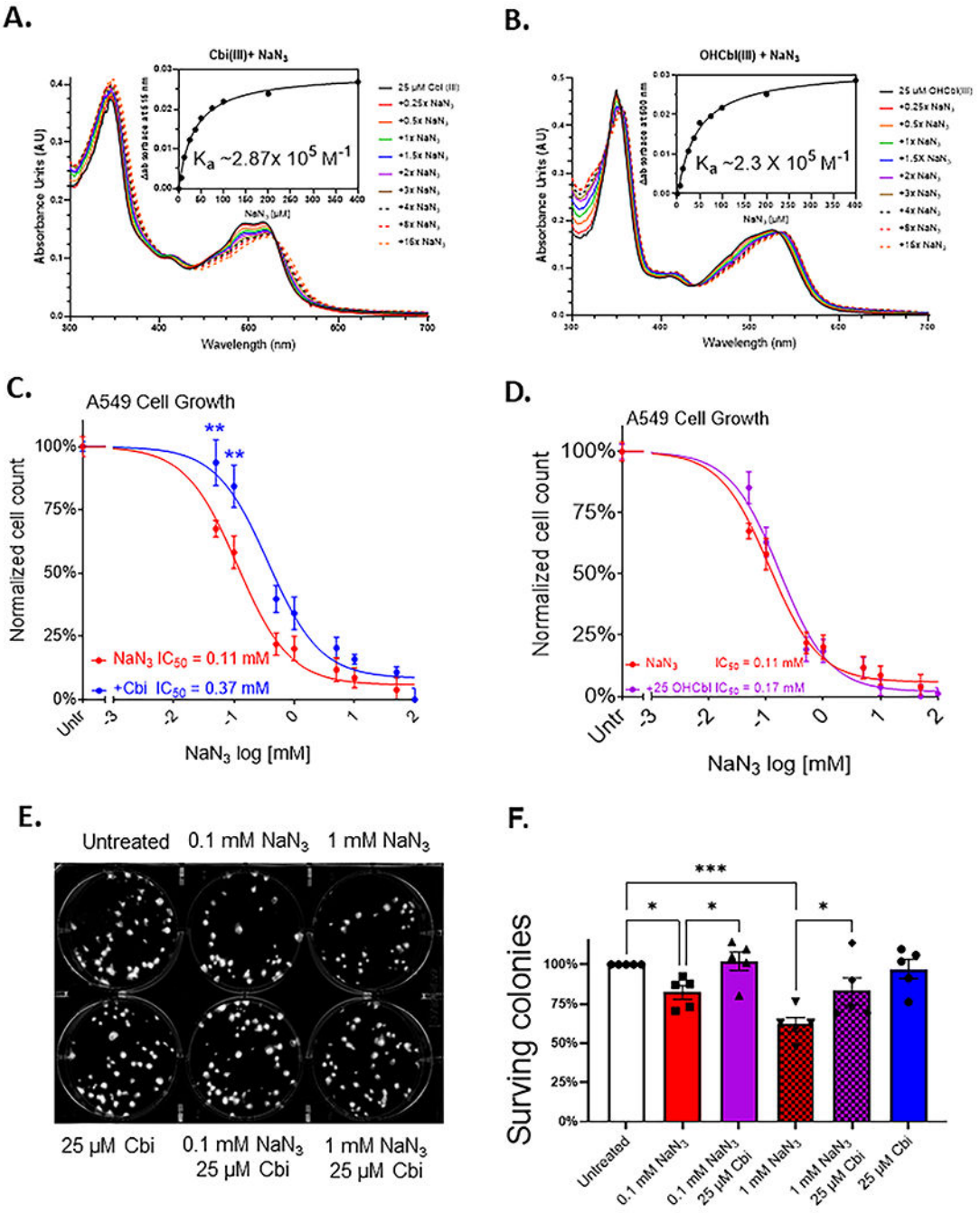

Figure 1. Cobinamide binds azide and rescues cells from azide toxicity.

(**A,B**) Sodium azide was added to a 25 µM aquohydroxocobinamide (A) or 25 M hydroxocobalamin (B) solution at final concentrations ranging from 0.25x to 16x of the cobinamide/cobalamin concentration. UV-visible spectra were recorded from 300 to 700 nm. The insets are a plot of the change in the absorbance of cobinamide/cobalamin at 515 nm versus the NaN_3 concentration. (**C, D**) A549 cells were treated with varying concentrations of NaN_3 (50 µM to 100 mM) for 48 hours in the absence or presence of 25 µM aquohydroxcobinamide (**C**) or 25 µM hydroxocobalamin (**D**); the cells were

then harvested and counted. Data are normalized to controls set at 100% and represent the mean ± SEM of four independent experiments done in duplicate. The comparison of azide exposure alone versus azide plus aquohydroxocobinamide or hydroxocobalamin was analyzed by two-way ANOVA. (**E**) A549 cells were seeded overnight at a low density, and then treated with the indicated drugs for 24 hours. The cells were washed with PBS, released in drug-free medium for 15 days, and then stained with 0.25% w/v crystal violet and counted. (**F**) Quantification of the data in (**E**). Data are normalized to controls set at 100% and represent the mean ± SEM of six independent experiments; statistical analysis was done by one-way ANOVA. *P≤0.05, **P≤0.01, and ***P<0.001. Cbi = cobinamide; NaN_3 = sodium azide; IC_{50} = azide concentration where 50% inhibition occurs; Untr = untreated; K_a = binding affinity.

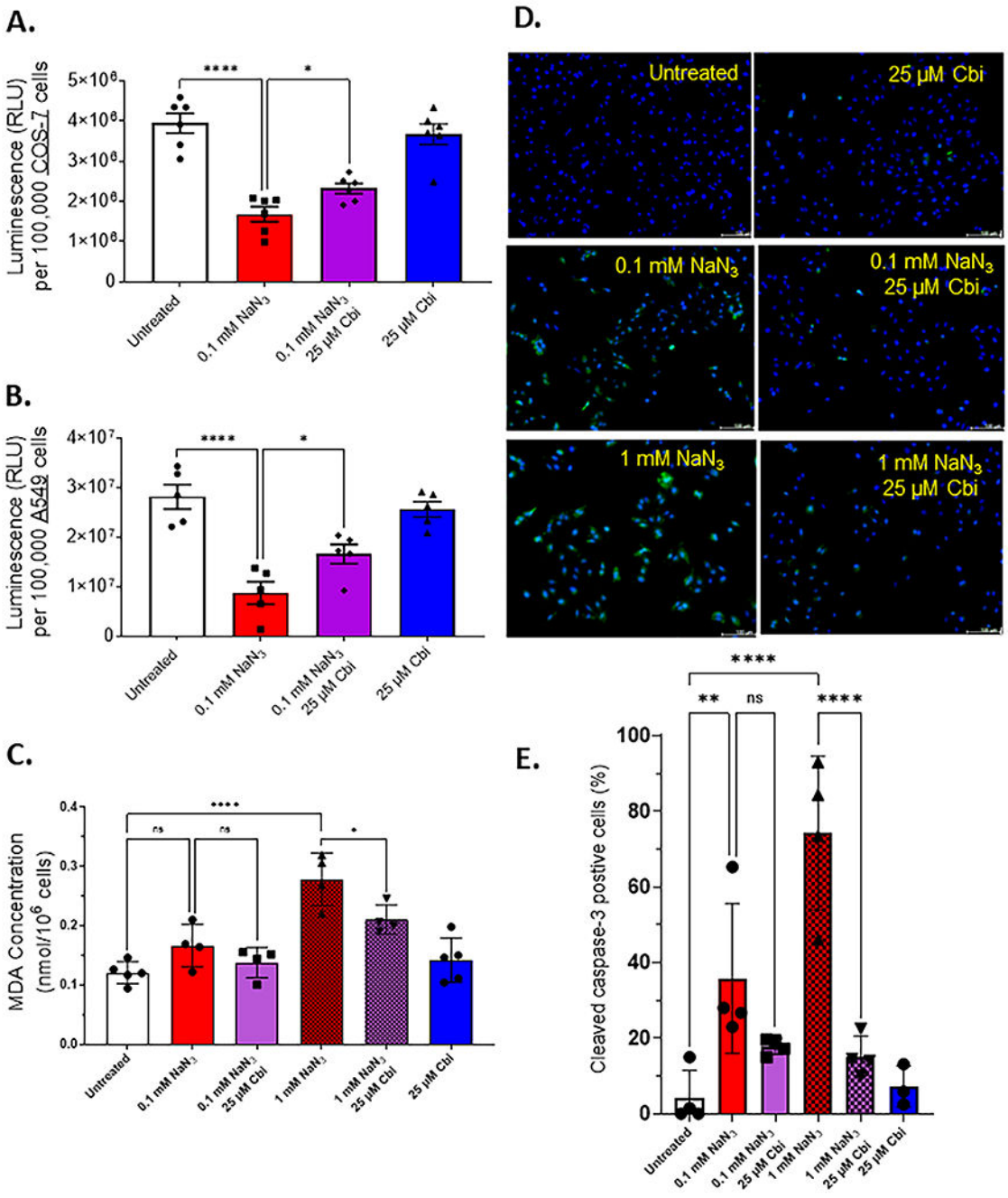

Figure 2. Mechanisms of azide toxicity; reversal by cobinamide.

(**A, B**) COS-7 cells (**A**) or A549 cells (**B**) were incubated in glucose-free medium containing 25 mM galactose for 24 hours. The next day, cells were exposed for 24 hours to 0.1 mM NaN_3 with or without 25 μM aquohydroxocobinamide. The cells were extracted *in situ* and ATP was measured in the extracts. Data represent the mean ± SEM of six (**A**) and five independent experiments (**B**), respectively. (**C**) A549 cells were incubated for 24 hours with 0.1 mM and 1 mM NaN_3, in the absence or presence of 25 μM aquohydroxocobinamide and the intracellular malondialdehyde (MDA) content was measured 24 hours later. Data

represent the mean ± SEM of five independent experiments and were analyzed by one-way ANOVA. (**D, E**) A549 cells were incubated for 24 hours with 0.1 mM and 1 mM NaN_3, in the absence or presence of 25 μM aquohydroxocobinamide and induction of apoptosis based on caspase-3 cleavage was measured by immunofluorescence. (**D**) Fluorescence microscopy images of cleaved caspase-3 positive cells (green); Hoeschst stain (blue) indicates the DNA of cells. Images were taken at 20x magnification and each scale bar represents 100 μm. (**E**) Quantification of the data in (**D**); the data are the mean ± SEM of three to four independent experiments. All experiments were analyzed by one-way ANOVA. *P≤0.05, **P<0.01, and ****P≤0.0001. Cbi = cobinamide; NaN_3 = sodium azide; ns = non-significant.

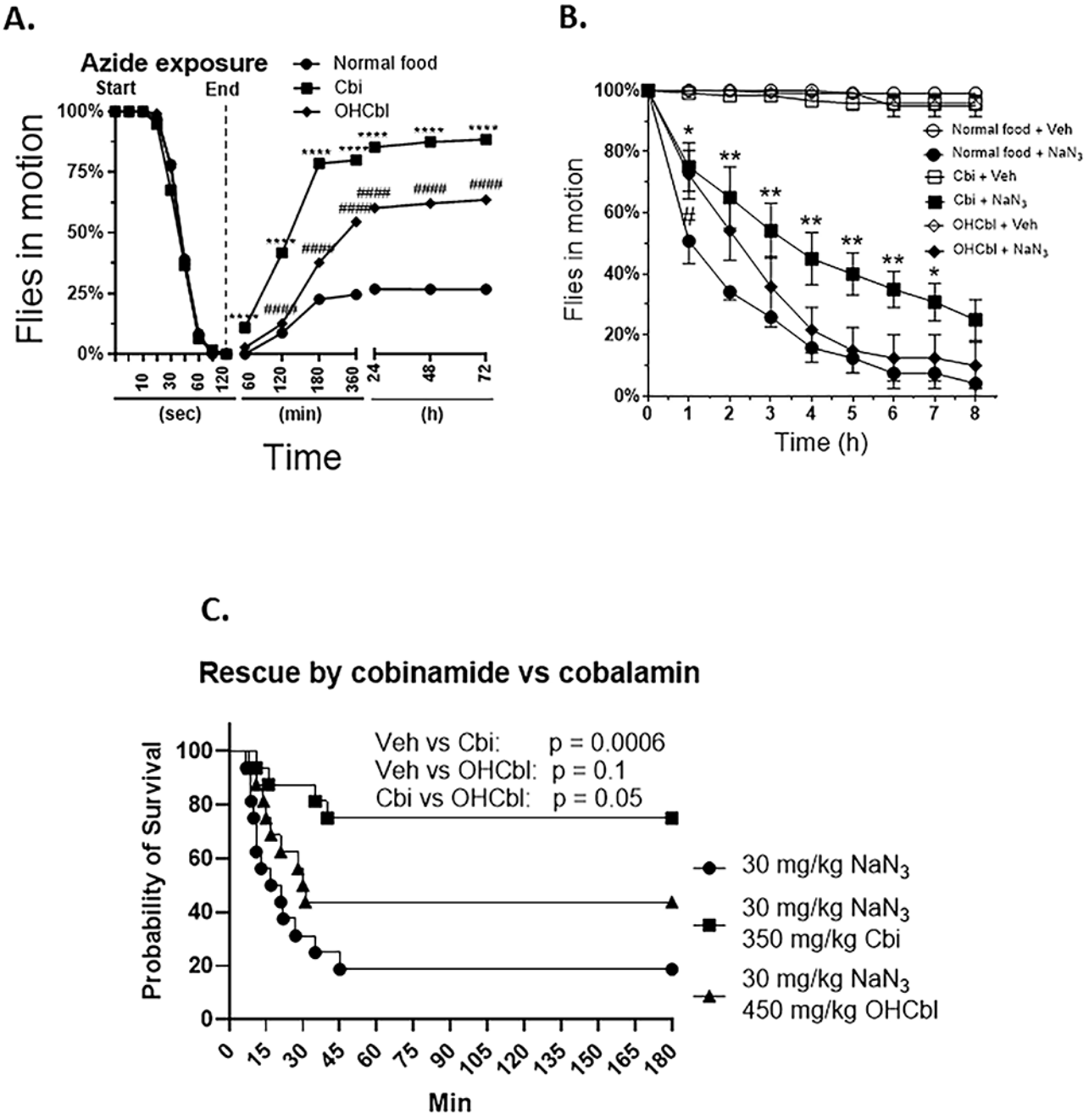

Figure 3. Cobinamide protects animals from acute azide poisoning.

(**A**) *D. melanogaster* were exposed to ~ 1500 ppm hydrazoic acid gas. After 2 min, when all the animals had fallen motionless, they were removed from the hydrazoic acid and monitored every 60 minutes for 4 hours and then every 24 hours until 72 hours post exposure; recovery was defined as the ability to walk or fly. (**B**) *D. melanogaster* were placed in vials containing gauze that had been saturated with solutions of either 100 mM NaN_3 or sodium phosphate as the only source of water. Survival, as determined by the ability to walk or fly, was determined every hour for 8 hours. In both studies, some of

the animals had been fed food containing 400 μM aquohydroxocobinamide or 400 μM hydroxocobalamin for one week prior to the experiment. Data represent the mean ± SEM of seven independent experiments with 15 animals per condition in each experiment. We compared the cobinamide- and cobalamin-treated animals to control animals by two-away ANOVA; asterisks and pound symbols are for comparison of cobinamide and cobalamin, respectively, to untreated animals. (**C**) Mice of both sexes (8 males and 8 females) and variable age (4–15 months old) received an intraperitoneal injection of 30 mg/kg NaN_3 . Two minutes later, the animals received an intramuscular injection of 350 mg/kg of histidylcobinamide, 450 mg/kg hydroxocobalamin, (or water). The difference between the cobinamide- and cobalamin-treated animals compared to water-treated animals was analyzed by a by log-rank sum test; * and $^{\#}P \leq 0.05$, **$P \leq 0.01$, and **** and $^{\#\#\#\#}P \leq 0.0001$. Cbi = cobinamide; OHCbl = cobalamin; NaN_3 = sodium azide; Veh, vehicle.

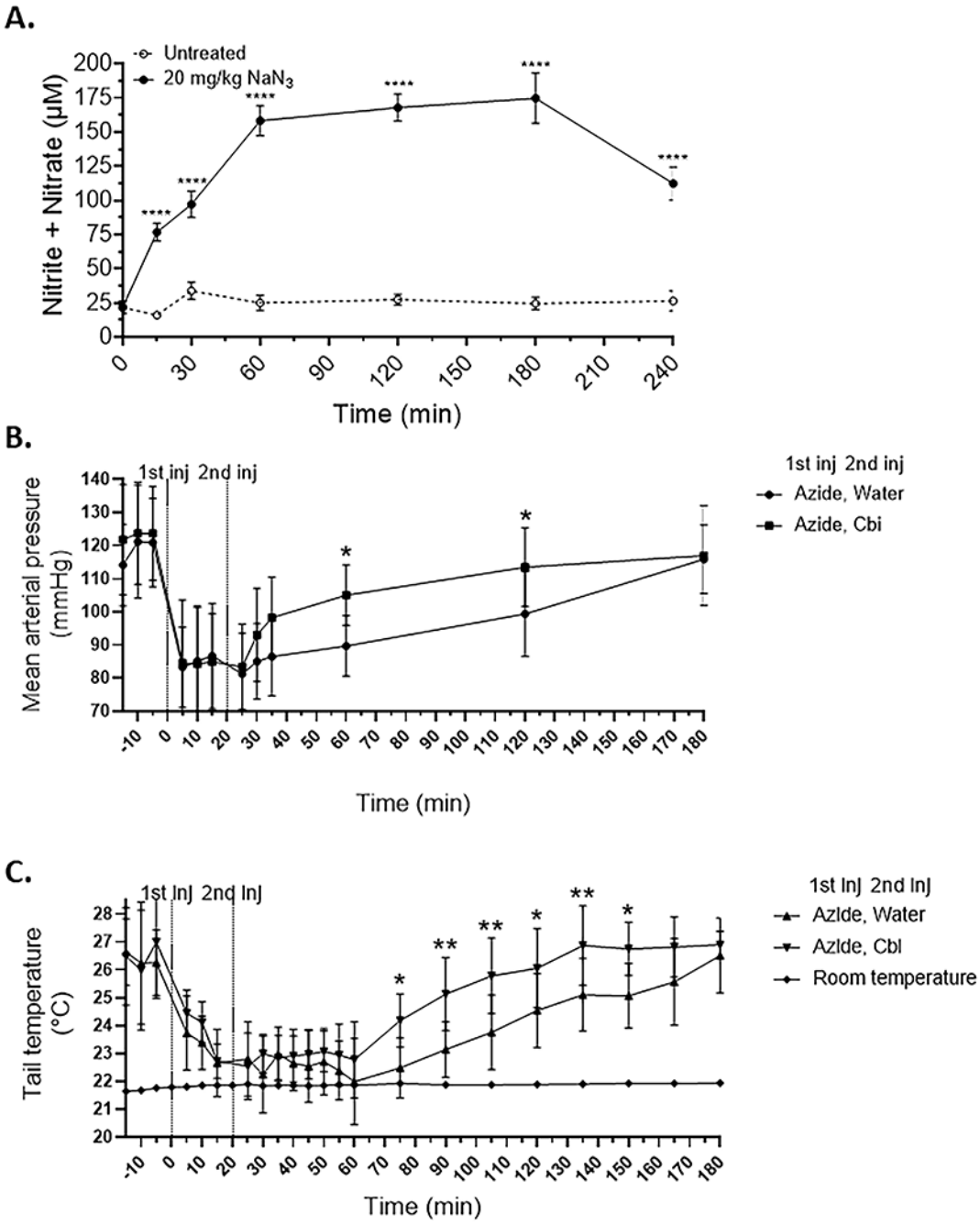

Fig 4. Therapeutic effect of cobinamide on blood pressure and peripheral body temperature following azide exposure.

(**A**) Mice of both sexes received an intraperitoneal injection of 20 mg/kg NaN_3 or water. At the indicated times, animals were euthanized by carbon dioxide asphyxiation, and blood was collected via cardiac puncture. Serum nitrite and nitrate concentrations were measured using a modified Griess-reagent method. Data at each time point represent the mean ± SEM of 6–8 mice. (**B**, **C**) Male and female mice received an intraperitoneal injection of 20 mg/kg NaN_3 at t = 0 min followed by an intraperitoneal injection of 300 mg/kg

histidylcobinamide (or water) 20 min later (t = 20 min). (**B**) Blood pressure was assessed via tail cuff plethysmography and the data represent the mean ± SEM of 8 mice (**C**). Tail temperature was assessed via a non-contact laser thermometer and the data represent the mean ± SEM of 7 mice. The data were analyzed by two-way ANOVA; *P≤0.05, **P≤0.01, and ****P≤0.0001. Cbi = cobinamide; Inj = injection; NaN_3 = sodium azide.