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Methyl Mercaptan Gas: Mechanisms of Toxicity and Demonstration of the Effectiveness of Cobinamide as an Antidote in Mice and Rabbits

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Abstract

Context: Methyl mercaptan (CH_3SH) is a colorless, toxic gas with potential for occupational exposure and use as a weapon of mass destruction. Inhalation at high concentrations can result in dyspnea, hypoventilation, seizures, and death. No specific methyl mercaptan antidote exists, highlighting a critical need for such an agent. Here, we investigated the mechanism of CH_3SH toxicity, and rescue from CH_3SH poisoning by the vitamin B_{12} analog cobinamide in mammalian cells. We also developed lethal CH_3SH inhalation models in mice and rabbits, and tested the efficacy of intramuscular injection of cobinamide as a CH_3SH antidote.

Results: We found that cobinamide binds to CH_3SH (K_d = 84 μM), and improved growth of cells exposed to CH_3SH. CH_3SH reduced cellular oxygen consumption and intracellular ATP content, and activated the stress protein c-Jun N-terminal kinase (JNK); cobinamide reversed these changes. A single intramuscular injection of cobinamide (20 mg/kg) rescued 6 of 6 mice exposed to a lethal dose of CH_3SH gas, while all 6 saline-treated mice died (p = 0.0013). In rabbits exposed to CH_3SH gas, 11 of 12 animals (92%) treated with two intramuscular injections of cobinamide (50 mg/kg each) survived, while only 2 of 12 animals (17%) treated with saline survived (p = 0.001).

Conclusion: We conclude that cobinamide could potentially serve as a CH_3SH antidote.

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AUTHOR CONTRIBUTIONS

CWW, GRB, and MB secured funding. GRB, MB, and JL designed experiments and supervised the work. GP, JT, AC, JJ, DM, TB, MD, SH conducted the experiments. GP, JT, JL, and GRB analyzed the data. HHP provided advice and reagents for respirometry studies. JT, GP, and GRB wrote the manuscript with input from all authors.

DECLARATION OF INTEREST

The authors declare no declaration of interest.

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Keywords

Methyl mercaptan; cobinamide; rescue

INTRODUCTION

Methyl mercaptan (CH_3SH), also known as methanethiol and mercaptothiol, is a colorless, flammable gas. It is used in production of oil, paper, plastics, jet fuels, and pesticides [1]. It is also used as a chemical odorant to detect natural gas leaks due to its pungent smell of rotting cabbage [2]. It is produced in humans during methionine or cysteine catabolism in the mouth and colon, contributing to the smell of halitosis and flatulence, respectively [3].

Like azide, hydrogen sulfide, and cyanide, methyl mercaptan inhibits cytochrome c oxidase in complex IV of the mitochondrial electron transport chain, thereby depleting intracellular adenosine triphosphate (ATP) [4–10]. This is especially detrimental to organs with high metabolic rates, such as the brain and heart. Prolonged CH_3SH exposure can cause metabolic acidosis due to increased lactate production from anaerobic metabolism.

Although CH_3SH poisoning is rare, accidental exposures have occurred [11–15]. The Acute Exposure Guideline Level (AEGL), derived from a rat study, is to not exceed exposure to > 120 ppm for 10 min [16]. Routes of exposure include direct contact with eyes, skin, and the upper respiratory tract. Acute CH_3SH inhalation can cause hypoventilation and apnea, and in severe exposures, seizures, paralysis, myocardial infarction, coma, and/or death may ensue [11,17]. The Department of Homeland Security (DHS) lists methyl mercaptan as a potential chemical weapon of mass destruction [18]. No antidotal treatment exists for CH_3SH poisoning and all therapies are supportive [7]. A point-of-care pharmacological agent is needed for treating CH_3SH poisoning.

Sodium nitrite, sodium thiosulfate, and hydroxocobalamin are effective in treating cyanide poisoning in animals and humans, and some studies suggest hydroxocobalamin is effective against hydrogen sulfide poisoning [4,19–26]. However, it is unknown whether these drugs could be used against CH_3SH poisoning. Sodium nitrite generates methemoglobin, which scavenges cyanide, but methemoglobinemia reduces oxygen carrying capacity of blood and nitrite can cause hypotension [27–29]. Sodium thiosulfate acts against cyanide by serving as a substrate for rhodanese to convert cyanide into thiocyanate [30], but thiosulfate would unlikely be helpful against CH_3SH poisoning. Hydroxocobalamin binds cyanide and hydrogen sulfide [31,32], but it is unknown if it also binds CH_3SH, and it must be administered in large volumes via intravenous infusion.

We have demonstrated that cobinamide, a vitamin B_{12}/hydroxocobalamin analog, effectively reverses cyanide and hydrogen sulfide poisoning in cells and animal models [4,19,20,22,24–26,33,34]. Cobinamide is the penultimate molecule in the cobalamin biosynthesis pathway, lacking the dimethyl-benzimidazole ribonucleotide tail coordinated to the cobalt atom in the lower axial position (Supplemental Figure 1, [35]). Cobinamide is much more water soluble than hydroxocobalamin and is rapidly absorbed after intramuscular injection. Unlike

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intravenous infusion, intramuscular injection facilitates drug administration and is, therefore, appropriate for treating mass-casualties.

Recently, we showed that intramuscular injection of cobinamide improves survival in pigs following acute inhalation of CH_3SH [36]. This work lacked mechanistic insights, but provided impetus for further studies to investigate the molecular basis of CH_3SH toxicity and to test the antidotal efficacy of cobinamide in mammalian cells and two additional animal models, mice and rabbits.

METHODS

Materials

Methyl mercaptan sodium salt, 15% (w/w), was from Tokyo Chemical Industry, Dulbecco's modified Eagles medium (DMEM) was from Life Technologies, and antibodies against phospho-JNK and β-actin were from Cell Signaling. XF24 plates and XF assay buffer were from Agilent Technologies.

Aquohydroxocobinamide was synthesized by base hydrolysis of hydroxocobalamin (Nutrakey Industries) with purification over reversed-phase resins [37]. Only material 98% pure, as assessed by high performance liquid chromatography, was used [38]. Aquohydroxocobinamide was converted to trihistidyl-cobinamide by adding three molar equivalents of histidine (Sigma-Aldrich) to an aqueous solution of aquohydroxo-cobinamide. 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.

Assessment of CH_3SH Binding to Cobinamide

The beneficial effect of cobinamide against methyl mercaptan poisoning we observed previously in pigs could be secondary to cobinamide binding, and thereby neutralizing methyl mercaptan, analogous to cobinamide binding to and scavenging cyanide and hydrogen sulfide [4,33]. Binding of ligands to the cobalt center of cobinamide leads to spectral changes in the UV-visible range. We recorded the UV-visible spectrum of cobinamide on a Kontron 960 spectrophotometer in the absence and presence of variable concentrations of CH_3SH. Binding curves were generated at a concentration of 25 μM cobinamide.

Tissue Culture

COS-7 African green monkey kidney cells and human foreskin fibroblasts were obtained from the American Type Culture Collection (ATCC). They were grown in DMEM medium supplemented with 10% fetal bovine serum (FBS) purchased from Sigma-Aldrich.

Cell Counting

Cells were seeded at 25,000 cells per/well in a 12-well plate and cultured overnight. They were then exposed to 250 μM CH_3SH for 30 min. After CH_3SH was removed, the cells were washed with phosphate-buffered saline (PBS), and then treated with 25 μM cobinamide

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for 2.5 h. The cells were washed with PBS and incubated in drug-free medium for 48 h, at which time they were counted using a Bio-Rad TC20 Automated Cell Counter.

Measurement of Rates of Cellular Oxygen Consumption

Cells were grown in XF24 plates at 40,000 cells/well and cultured overnight in DMEM medium. Approximately one hour prior to experimentation, DMEM medium was removed, and cells were calibrated in unbuffered XF assay medium. Rates of cellular oxygen consumption were measured using a Seahorse XF24 Extracellular Flux Analyzer (Agilent Technologies) [4]. CH_3SH and cobinamide were made in assay medium and administered to the cells through automated injection ports to final concentrations of 500 μM and 10 μM, respectively.

Measurement of Cellular ATP

Cells were grown in 96 well plates overnight in DMEM medium lacking glucose, but supplemented with 25 mM galactose (as a carbon source) and 10% dialyzed FBS. The medium was removed and replaced with fresh DMEM medium. The cells were then incubated for 3 h with 1 mM CH_3SH, added both at zero time and again after 90 min due to the volatility of CH_3SH. Cobinamide was added to a final concentration of 1.5 mM, either at zero time or after 90 min, i.e., at the time of the second CH_3SH addition. The cells were extracted *in situ,* and ATP was measured using the Cell-Titer Glo 2 kit (Promega) according to the manufacturer's instructions [4].

Measurement of JNK Activation

Cells were incubated with 2.5 mM CH_3SH for 3 h in the absence or presence of 0.5 mM cobinamide. As in the experiments when measuring ATP, the CH_3SH was added twice, at zero time and after 90 min. At the end of the incubation, the cells were extracted *in situ* in a hot sodium dodecyl sulfate (SDS)-based buffer, and proteins were separated by SDS-PAGE. Proteins were transferred to nitrocellulose membranes, and phospho-JNK was identified by immunoblotting. β-actin was used a loading control. Quantification of protein bands was done with ImageJ software [4].

Exposure of Mice to CH_3SH Gas

C57BL/6 male mice (Jackson Laboratory) were exposed to CH_3SH gas in a custom-made air-tight chamber we have used for exposing mice to hydrogen cyanide and hydrogen sulfide gas [4,34]. Mice were anesthetized by injecting isoflurane into the chamber to a final concentration of 2%. Methyl mercaptan gas was then generated by injecting methyl mercaptan sodium salt into a beaker containing 10 N sulfuric acid (H_2SO_4); the calculated CH_3SH concentration was 1,400 ppm. After 15 min of CH_3SH exposure, mice were removed from the chamber and injected intramuscularly with saline, trihistidyl-cobinamide (20 mg/kg), or hydroxocobalamin (27 mg/kg). They were placed back in the chamber and re-exposed to CH_3SH gas for an additional 25 min. This simulates a real-life scenario of exposure to CH_3SH gas in an enclosed space, with 15 min required for emergency medical personnel to arrive at a disaster scene, and 25 min to treat and evacuate subjects from the

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contaminated space. Mice were monitored for survival over 120 min. At study's conclusion, surviving animals were euthanized to minimize discomfort and distress.

Exposure of Rabbits to CH_3SH Gas

Specific-pathogen-free New Zealand White rabbits (Western Oregon Rabbit Supply) weighing between 3.5 and 4.5 kg were sedated with an intramuscular injection of 50 mg/kg ketamine HCl (Ketaject, Phoenix Pharmaceutical Inc.) and 5 mg/kg xylazine (Anased, Lloyd Laboratories). They were then anesthetized with 12.5 mg/kg ketamine HCl and 0.5 mg/kg xylazine, delivered intravenously via the marginal ear vein. Animals were intubated and administered 1.5% isoflurane throughout the experiment. Respiratory rate, end-tidal CO_2, and breathing pattern were monitored using a Respironics NM3 respiratory profile monitor (Philips Healthcare, Royal Philips Electronics). A pulse oximeter probe (Masimo) was placed on the rabbit's cheek to measure heart rate and arterial oxygen saturation [39]. Once an animal's breathing pattern had stabilized, a catheter was placed in the right femoral artery to monitor blood pressure (BioPac MP100, Biopac Systems Inc) and acquire blood samples. Mean arterial pressure was calculated from the systolic and diastolic blood pressures.

A circuit was designed to ensure safe and proper delivery of CH_3SH gas to the rabbits (Supplemental Figure 2). CH_3SH gas was produced continuously for 45 min by acidifying methyl mercaptan sodium salt with 3 N hydrochloric acid (HCl), and its concentration was measured using a digital meter (RKI GX-6000, RKI instruments) at 5, 10, 15, and 20 min after start of exposure. We aimed for a methyl mercaptan concentration that resulted in >80% lethality in saline-treated animals. Animals experiencing severe hypotension, defined as a 30 mm Hg decrease from baseline mean arterial pressure for 2 min, were euthanized via an intravenous injection of Euthasol (Virbac AH, Inc.). Animals were monitored for 60 min; survivors were euthanized at the study's completion to minimize distress and discomfort.

Animals in the treatment group received a 1 cc intramuscular injection of 50 mg/kg cobinamide after the first apneic episode (within 5 min of CH_3SH exposure) and a second 1 cc intramuscular injection of 50 mg/kg cobinamide 15 min after the first injection. Apnea was defined as the cessation of breathing for roughly 30 seconds. Control animals received 0.9% saline each time. Each group contained six male and six female animals.

Animal Study Approval

Mouse and rabbit 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 Committees (IACUC) at the Universities of California, San Diego and Irvine, respectively.

Safety Features

In all experiments, personal protective equipment consisted of a lab coat, eye goggles, gloves, and closed-toe shoes. Due to the volatility of CH_3SH gas, an aqueous solution of sodium methyl mercaptan was added directly to culture medium in a certified tissue culture hood in the cell studies.

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The air-tight chamber used for exposing mice to CH_3SH gas was inside a chemical fume hood with tempered glass, which provided protection against accidental exposure due to a gas leak or explosion. A blast shield was placed between the chamber and tempered glass to provide an additional layer of protection. The fume hood was certified by the UCSD Environmental Health and Safety (EH&S) Division. In the rabbit studies, the reaction flask used to generate methyl mercaptan gas was covered in a high-density polyethylene protective netting to protect personnel from glass shards in the event of an explosion. The flask was placed in a 5-inch deep polyethylene bucket to contain any broken glass, leakage, and/or spillage. The exposure circuit (Supplemental Figure 2) was in a chemical fume hood. To detect leaks, one CH_3SH meter was inside the circuit and a second meter was outside the circuit. The circuit and flow hood were certified by UC Irvine EH&S.

Statistics

Statistical tests were completed using GraphPad Prism Statistics Software Version 8.4 (GraphPad Prism). Data for cell-based assays are presented as the mean ± SD; a one-way ANOVA with either Dunnett or Tukey correction was used for data analysis. For the mouse and rabbit studies, statistical significance was detected using a Grehan-Breslow-Wilcoxon test. In rabbit studies, a one-way ANOVA was used to evaluate parameter changes over time. This was done using a mixed-effects model to allow for missing data at time-points where animals were deceased. A p-value <0.05 was deemed significant.

RESULTS

Mechanism of Methyl Mercaptan Toxicity and Reversal by Cobinamide

We assessed if cobinamide binds CH_3SH by adding increasing concentrations of CH_3SH and monitoring the UV-visible spectrum of cobinamide. We observed progressive changes in the cobinamide spectrum as increasing amounts of CH_3SH were added to a 25 μM cobinamide solution (Figure 1A). Changes at 300 nm, 349 nm, 460 nm, and 525 nm were especially pronounced, and we estimated a K_d of 84 μM based on the absorbance changes at 349 nm (Figure 1A, inset). Although this indicates a relatively modest affinity of cobinamide for CH_3SH, the value may be sufficiently high to explain at least part of cobinamide's mechanism of action in neutralizing CH_3SH.

To study the mechanism of CH_3SH toxicity, we first assessed the effect of CH_3SH on cell growth. We exposed COS-7 cells to CH_3SH for 30 min, removed the CH_3SH, and then treated the cells with cobinamide for 2.5 h. We found that CH_3SH decreased COS-7 cell growth by ~40%, and that cobinamide yielded a moderate, yet significant, rescue of cells (Figure 1B). Since CH_3SH inhibits mitochondrial cytochrome C oxidase, we next assessed its effect on oxygen consumption, since mitochondria account for the vast majority of cellular oxygen use. We found that 500 μM CH_3SH rapidly reduced the oxygen consumption rate of COS-7 cells by ~40%, and that oxygen consumption remained below baseline for the duration of the study (Figure 1C). Adding 10 μM Cbi 20 min after the initial CH_3SH exposure fully restored oxygen consumption rates (Figure 1C). Consistent with inhibiting mitochondrial respiration, treating both COS-7 cells and human foreskin fibroblasts with 1 mM CH_3SH for 3 h reduced intracellular ATP content by ~50% in both cell types (Figures

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1D and 1E). Due to the volatile nature of CH_3SH, it was added twice during the exposure period. Having cobinamide present during the full 3 h of CH_3SH exposure returned the ATP concentration to baseline values in both cell types (Figures 1D and 1E), while adding cobinamide during the last 90 min led to almost complete rescue of ATP in COS-7 cells (Figure 1D). Methyl mercaptan has been shown to increase markers of oxidative stress in rats [40]. We, therefore, assessed phosphorylation of JNK as a readout of oxidative stress, and found that CH_3SH increased phospho-JNK by ~2.5-fold in COS-7 cells, and that cobinamide returned phospho-JNK to baseline values (Figure 1F).

Cobinamide Rescues Mice and Rabbits from Methyl Mercaptan Poisoning

Mice were exposed for a total of 40 min to CH_3SH, with injection of test agent 15 min after exposure onset. All mice injected with saline died over a narrow time range, whereas all mice injected intramuscularly with 20 mg/kg cobinamide lived (Figure 2A). For comparison, we tested hydroxocobalamin at a dose equivalent to that of cobinamide (27 mg/kg hydroxocobalamin) and found that only one animal survived (Figure 2A). Thus, cobinamide rescues mice from CH_3SH poisoning, and it is considerably more effective than hydroxocobalamin.

Rabbits were exposed to CH_3SH gas via inhalation for a total of 45 min. Animals became apneic, on average, within 5 min after exposure onset. At this point, half of the rabbits received 50 mg/kg cobinamide via intramuscular injection and the other half received saline. A second intramuscular injection of 50 mg/kg cobinamide (or saline) was administered 15 min after the initial injection. The average survival time was 39.2 ± 4.1 min for the control group, with 2 of 12 animals surviving (17%), while 11 of 12 cobinamide-treated animals (92%) survived the full 60 minutes of the experiment (Figure 2B; $p = 0.001$).

The Effects of Methyl Mercaptan on Rabbit Cardiopulmonary Functions

In the rabbits, we measured several cardiopulmonary parameters over the time course of the study. The mean arterial pressure increased following CH_3SH exposure, but after ~20 min, it fell progressively in the saline-treated animals (Figure 3A); the graph does not reflect the full extent of the fall, because only surviving animals are shown, which by definition maintained a reasonable blood pressure. Blood pressure remained elevated in the cobinamide-treated animals until the end of the experiment, when it returned to baseline (Figure 3A; $p < 0.02$ for difference between saline- and cobinamide-treated groups). Despite the change in blood pressure, heart rate in both groups remained relatively stable during the study (Figure 3B). Arterial oxygen saturation fell in both groups initially; it then stabilized in the cobinamide-treated animals but continued a slow progressive fall in the saline-treated animals (Figure 3C). Both groups of animals showed a progressive decrease in respiratory rate, that began to increase towards normal at the end of the experiment (Figure 3D). End-tidal CO_2, the maximum concentration of CO_2 at the end of an exhaled breath, increased in both groups but was significantly higher in the control animals (Figure 3E, $p < 0.001$), possibly reflecting the decreased ventilation and any contribution from increased anaerobic metabolism. Correspondingly, minute ventilation decreased in both groups, with cobinamide-treated animals returning to baseline at the end of the study (Figure 3F).

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DISCUSSION

Although rare, methyl mercaptan poisoning may occur in specific high-risk work environments such as oil refineries and pulp mills, and has resulted in severe illness and death [11–15]. Furthermore, CH_3SH could be used in chemical warfare or as a terrorist weapon [18]. Therefore, a need exists for an antidote against CH_3SH poisoning that can be administered easily, especially in a mass-casualty event.

The vitamin B_{12} analog cobinamide administered by intramuscular injection has shown promising efficacy in preclinical studies against hydrogen cyanide and hydrogen sulfide in three different mammalian models and against methyl mercaptan in pigs [4,19,20,22,24–26,33,34,36]. In this study, we explored the molecular basis of CH_3SH cellular toxicity and test whether cobinamide could rescue mammals other than pigs from acute CH_3SH poisoning.

We showed that CH_3SH exposure decreased cell growth, oxygen consumption rate, and intracellular ATP, while increasing JNK phosphorylation in mammalian cells; the latter is a marker of oxidative stress. These biochemical and cellular alterations are consistent with methyl mercaptan inhibition of mitochondrial cytochrome c oxidase, further suggesting this is the major mechanism of methyl mercaptan toxicity. Importantly, all of the CH_3SH-induced changes were reversed by cobinamide, likely through a combination of directly binding methyl mercaptan and reducing oxidative stress.

At the organismal level, intramuscular delivery of cobinamide provided full recovery for mice exposed to a lethal dose of CH_3SH gas and rescued 11 of 12 rabbits exposed to a CH_3SH dose that was lethal in 10 of 12 saline-treated rabbits. Combined with the previous results in pigs, the data indicate that cobinamide administered by intramuscular injection can rescue three different mammalian species from inhalation of a lethal dose of CH_3SH gas [41].

The moderate hypertension observed in the rabbits early during methyl mercaptan exposure is consistent with reports that methyl mercaptan leads to elevated blood pressure, albeit by an unknown mechanism [13,42]. As CH_3SH exposure continued, control animals experienced a drop in mean arterial pressure. In contrast, cobinamide-treated animals maintained a mildly elevated blood pressure throughout the study. Cobinamide can scavenge nitric oxide, which could lead to vasoconstriction and mild hypertension [43]. Indeed, we have seen a transient increase in blood pressure of rabbits exposed to hydrogen sulfide and rescued with cobinamide [20].

All animals, both the mice and rabbits, experienced apnea, but the mechanism is unknown. Hydrogen sulfide has been reported to cause central apnea when delivered at high concentrations by affecting medullary respiratory neurons [44]. CH_3SH could act similarly. The respiratory rate never fully returned to normal in cobinamide-treated animals, even though all but one cobinamide-treated animal survived. This is in contrast to pigs, where cobinamide restored a normal breathing pattern in animals exposed to a lethal dose of CH_3SH [36]. This difference is likely due to differences in the study design. Both the mice and rabbits remained exposed to CH_3SH, even after the antidote was administered.

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This was done to replicate a scenario of mass casualties where victims may be treated while still exposed to CH_3SH gas, due to difficulty evacuating them from the contaminated area. Continuous CH_3SH exposure for 45 min could have overwhelmed animals' pulmonary response. Yet, that cobinamide still rescued the mice and rabbits, demonstrates its efficacy as a methyl mercaptan antidote.

LIMITATIONS

Tissue culture clearly does not recapitulate organismal complexity. However, the purpose of the *in vitro* studies was to determine mechanisms of CH_3SH toxicity and how cobinamide rescues cells, since cultured cells are well-suited for mechanistic studies.

Survival was the main outcome in the animal studies. Thus, a relatively small number of animals were needed, but this could have masked differences in physiological parameters.

Both IACUCS overseeing these studies required animals to be euthanized at the end of the experiment. Thus, we could have missed delayed sequelae from the methyl mercaptan poisoning.

CONCLUSIONS

Methyl mercaptan toxicity is likely due to inhibiting mitochondrial respiration and inducing oxidative damage, both of which were reversed by cobinamide. Intramuscular cobinamide injection increased survival in mice and rabbits exposed to lethal doses of methyl mercaptan. Cobinamide may be a viable methyl mercaptan antidote.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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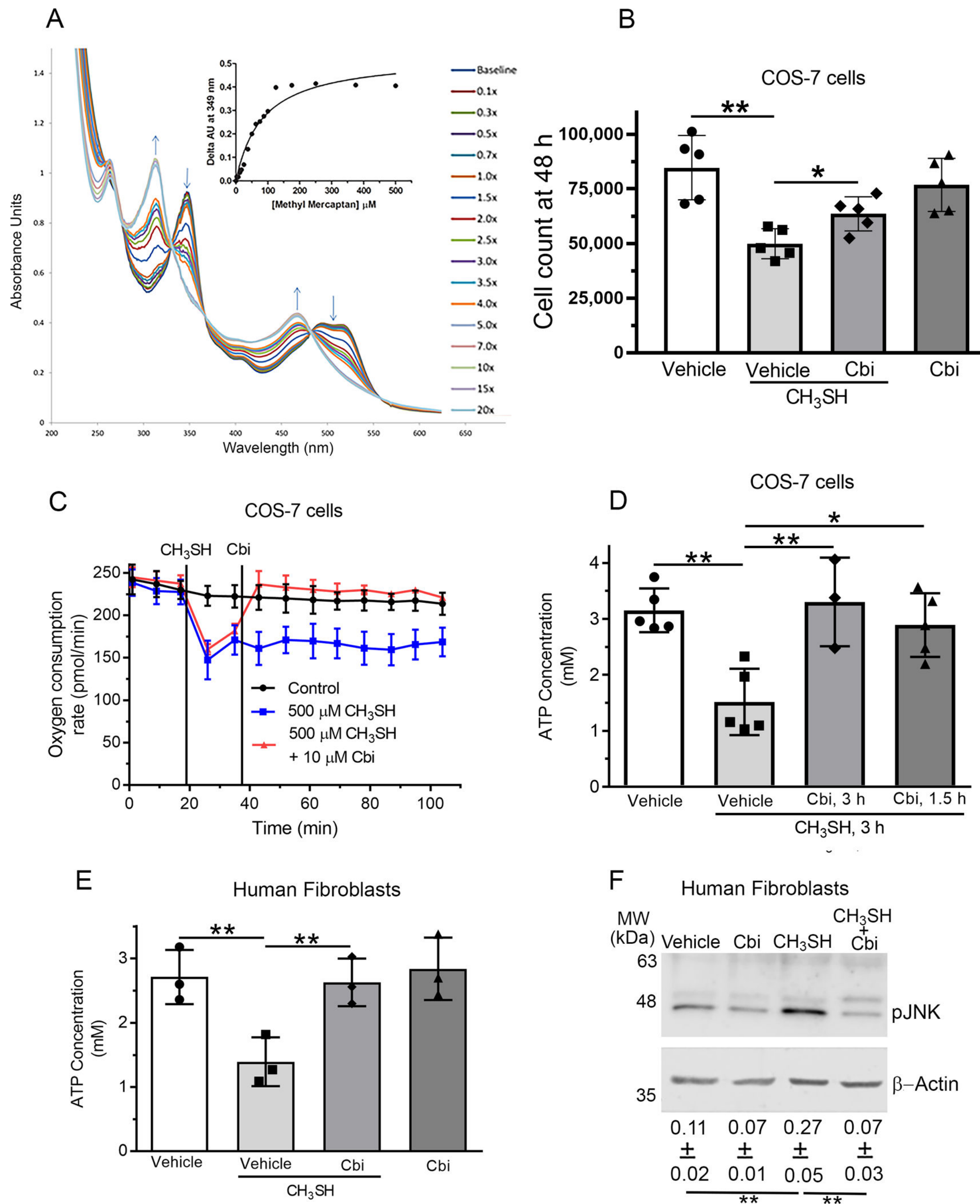

Figure 1. Cobinamide Binds to Methyl Mercaptan and Rescues Cells from Methyl Mercaptan Toxicity

A. Methyl mercaptan was added to a cobinamide solution at final concentrations ranging from 0.1 to 20x the cobinamide concentration. The ultraviolet-visible spectrum was recorded from 200 to 625 nm. Arrows indicate the direction of change with increasing concentrations of methyl mercaptan. The inset is a plot of the change in absorbance at 349 nm versus the methyl mercaptan concentration. **B.** COS-7 cells were treated with vehicle (clear bar) or 250 μM CH_3SH for 0.5 h, and then incubated for 2.5 h in the absence (light grey bar) or presence

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of 25 μM cobinamide (medium grey bar). Some cells received cobinamide only (dark grey bar). The number of cells was counted after 48 h of incubation. **C.** Oxygen consumption rates were measured in COS-7 cells. At the time indicated by the first vertical line, cells received 500 μM CH_3SH or assay buffer, with the CH_3SH-exposed cells subsequently receiving 10 μM cobinamide or assay buffer (second vertical line). **D, E.** COS-7 cells (D) and human foreskin fibroblasts (E) were incubated for 3 h, receiving 1 mM CH_3SH at zero time and again after 1.5 h. For the COS-7 cells, 1.5 mM cobinamide was added either at zero time (3 h) or at 1.5 h, and for the human fibroblasts 1.5 mM cobinamide was added only at zero time. Cells were extracted *in situ* and ATP was measured in the extracts. **F.** Human foreskin fibroblasts were treated with 2.5 mM CH_3SH for 3 h in the absence or presence of 0.5 mM cobinamide. CH_3SH was added twice, once at zero time and again at 1.5 h. Phospho-JNK (p-JNK) was analyzed in cell extracts via immunoblotting. p-JNK bands were normalized to β-actin bands, and the numbers below the blot are the mean density ± SD of three independent experiments. With the exception of Panel A, the data are the mean ± SD of three experiments performed in triplicate. The data in Panels B, D, E, and F were analyzed by one-way ANOVA, with * and ** indicating $p < 0.05$ and 0.01, respectively, for the indicated comparisons. Cbi = cobinamide.

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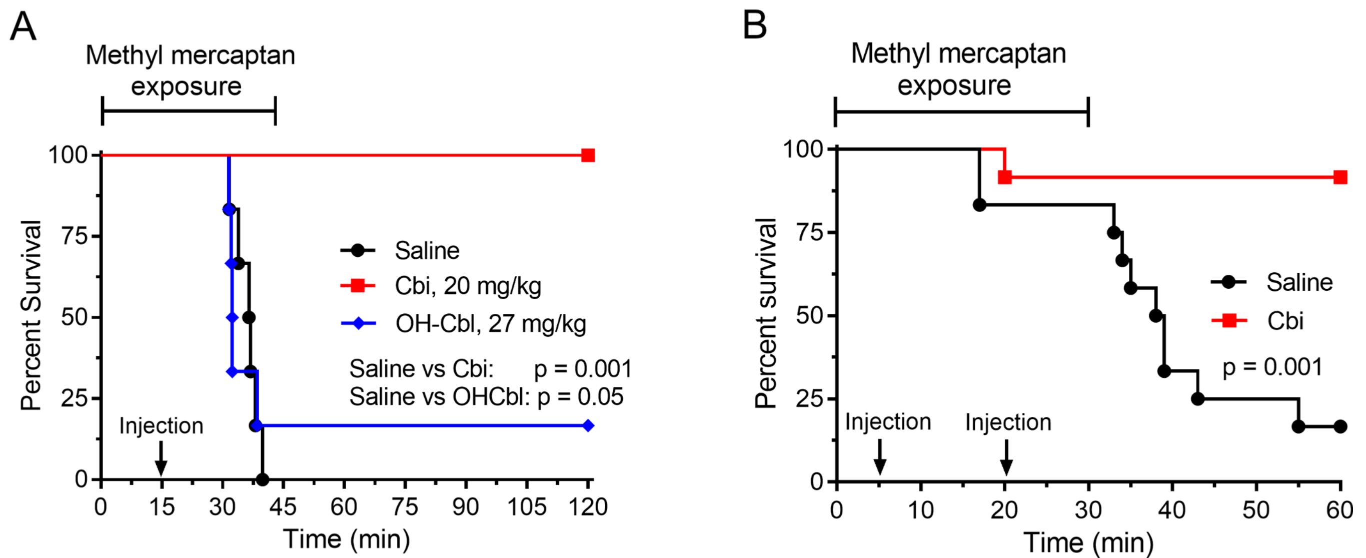

Figure 2. Cobinamide Rescues Mice and Rabbits Exposed to CH_3SH Gas.

A. Mice were exposed to 1,400 ppm CH_3SH gas for 15 min in a gas-tight chamber, and then removed from the chamber and injected intramuscularly with the indicated agents and doses. They were returned to the chamber, for an additional 25 min, with survivors removed from the chamber and observed for 80 min. N = six mice per condition. The difference between saline- and cobinamide-treated animals was significant by a log rank Mantel-Cox test. **B.** Rabbits—males and females—were exposed to approximately 5,000–8,500 ppm CH_3SH for 30 min. A treatment group received 50 mg/kg cobinamide intramuscularly at the onset of apnea, which, on average, occurred 5 min following CH_3SH exposure. An additional cobinamide injection of 50 mg/kg was administered 15 min after the first injection. Control animals received saline at both injections. All animals were monitored for 60 min. N = 12 rabbits per condition. Gehan-Breslow-Wilcoxon test indicates significant difference in survival between saline- and cobinamide- treated animals. Cbi = cobinamide; OHCbl = hydroxocobalamin.

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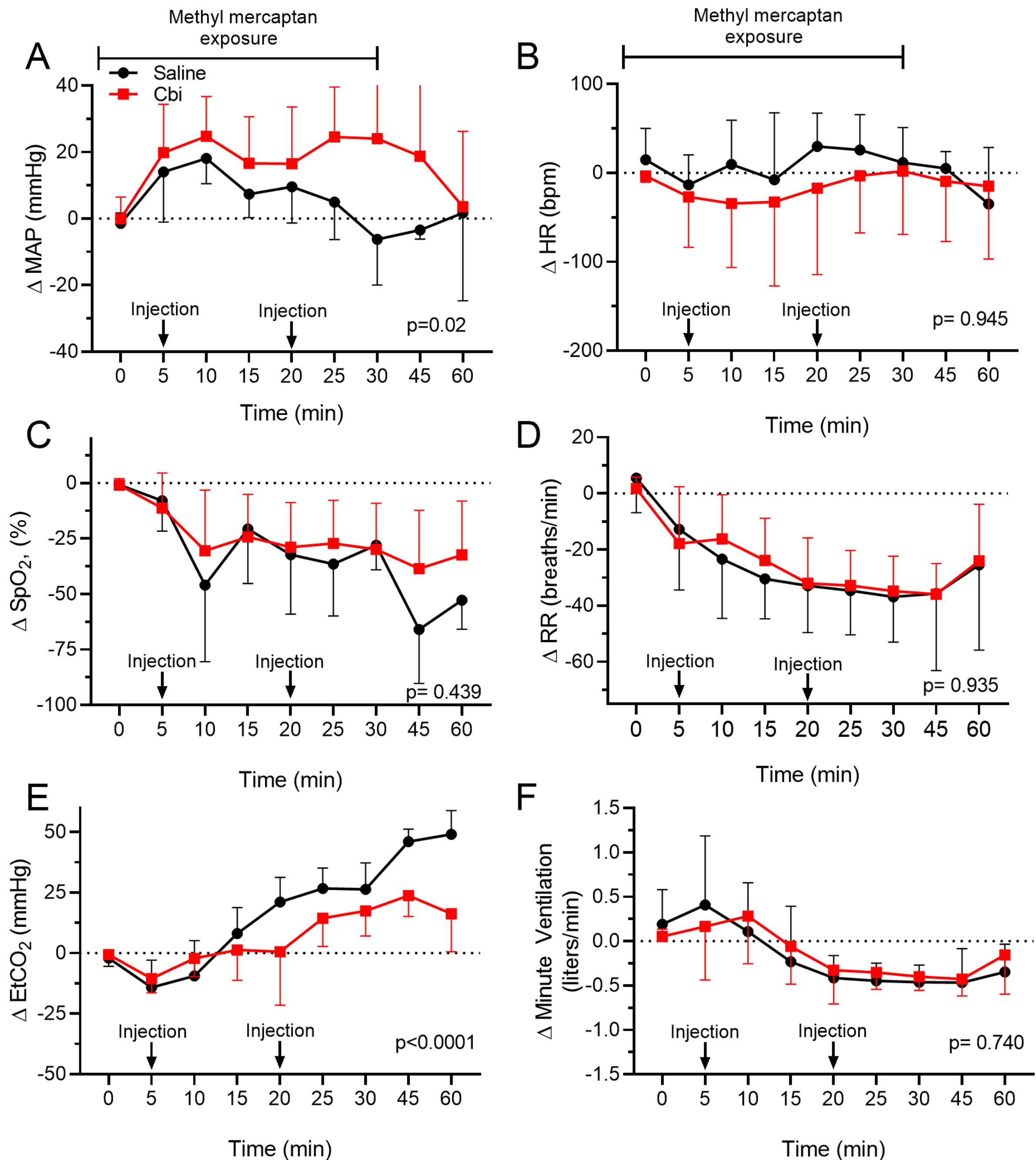

Figure 3. Cardiopulmonary Parameters in Rabbits Exposed to CH_3SH Gas.

Cardiopulmonary parameters were measured in the rabbits described in Figure 2B. **A.** Mean arterial blood pressure was calculated from systolic and diastolic blood pressure. **B, C.** Heart rate (B) and oxygen saturation (C) were recorded using a pulse oximeter. **D, E, F.** Respiratory rate (D), end-tidal CO_2 (E), and minute-ventilation (F) were recorded using an NM3 respiratory monitor. Absolute changes in cardiopulmonary functions were recorded every 5 min. N = twelve rabbits per condition. The data were analyzed by two-way ANOVA. Mixed effects model accounted for missing values, since not all rabbits survived the full 60-min study. Data at each time point are the mean ± SD. P 0.05 was considered

statistically significant. Cbi = cobinamide; MAP = mean arterial pressure; HR = heart rate; SpO_2 = oxygen saturation; RR = respiratory rate; $EtCO_2$ = end-tidal CO_2; MV = minute ventilation.

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