Title
Development of sodium tetrathionate as a cyanide and methanethiol antidote

Journal
Clinical Toxicology, 60(3)

ISSN
1556-3650

Authors
Chan, Adriano
Lee, Jangwoen
Bhadra, Subrata
et al.

Publication Date
2022-03-04

DOI
10.1080/15563650.2021.1953517

Peer reviewed

HHS Public Access

Author manuscript

Clin Toxicol (Phila). Author manuscript; available in PMC 2023 March 01.

Published in final edited form as:

Clin Toxicol (Phila). 2022 March ; 60(3): 332–341. doi:10.1080/15563650.2021.1953517.

Development of sodium tetrathionate as a cyanide and methanethiol antidote

Adriano Chan[a], **Jangwoen Lee**[b], **Subrata Bhadra**[c], **Nesta Bortey-Sam**[d], **Tara B. Hendry-Hofer**[e], **Vikhyat S. Bebarta**[e], **Sari B. Mahon**[b], **Matthew Brenner**[b], **Brian Logue**[c], **Renate B. Pilz**[a], **Gerry R. Boss**[a]

[a]Department of Medicine, University of California, San Diego, La Jolla, CA, USA

[b]Beckman Laser Institute, University of California, Irvine, Irvine, CA, USA

[c]Department of Chemistry and Biochemistry, South Dakota State University, Brookings, SD, USA

[d]Graduate School of Public Health, University of Pittsburgh, Pittsburgh, PA, USA

[e]Department of Emergency Medicine, University of Colorado, Aurora, CO, USA

Abstract

Context: Hydrogen cyanide and methanethiol are two toxic gases that inhibit mitochondrial cytochrome *c* oxidase. Cyanide is generated in structural fires and methanethiol is released by decaying organic matter. Current treatments for cyanide exposure do not lend themselves to treatment in the field and no treatment exists for methanethiol poisoning. Sodium tetrathionate (tetrathionate), a product of thiosulfate oxidation, could potentially serve as a cyanide antidote, and, based on its chemical structure, we hypothesized it could react with methanethiol.

Results: We show that tetrathionate, unlike thiosulfate, reacts directly with cyanide *in vitro* under physiological conditions, and based on rabbit studies where we monitor cyanide poisoning in real-time, tetrathionate likely reacts directly with cyanide *in vivo*. We found that tetrathionate administered by intramuscular injection rescues >80% of juvenile, young adult, and old adult mice from exposure to inhaled hydrogen cyanide gas that is >80% lethal. Tetrathionate also rescued young adult rabbits from intravenously administered sodium cyanide. Tetrathionate was reasonably well-tolerated by mice and rats, yielding a therapeutic index of ~5 in juvenile and young adult mice, and ~3.3 in old adult mice; it was non-mutagenic in Chinese Hamster ovary cells and by the Ames bacterial test. We found by gas chromatography-mass spectrometry that both tetrathionate and thiosulfate react with methanethiol to generate dimethyldisulfide, but that tetrathionate was much more effective than thiosulfate at recovering intracellular ATP in COS-7 cells and rescuing mice from a lethal exposure to methanethiol gas.

Conclusion: We conclude that tetrathionate has the potential to be an effective antidote against cyanide and methanethiol poisoning.

CONTACT Gerry R. Boss gboss@health.ucsd.edu Department of Medicine, University of California, San Diego, La Jolla, 92093-0652, CA, USA.

Disclosure statement

The authors report no conflict of interest.

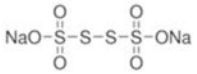
Keywords

Cyanide; methanethiol; mice; rabbits; sodium tetrathionate; sodium thiosulfate

Introduction

Cyanide is a well-known toxic chemical. It is generated as hydrogen cyanide gas in structural fires and is a major contributor to death by smoke inhalation [1]. It is used in a variety of industries, with over three billion pounds of cyanide salts produced annually worldwide [2]. It has the potential to be released by terrorists and is considered a high-priority chemical threat by the Center for Disease Control. Currently approved treatments for cyanide poisoning in the United States are hydroxocobalamin and the combination of sodium nitrite and sodium thiosulfate. Both treatments must be given intravenously over 10–15 min, which would not be practical in the setting of a major fire, industrial accident, or terrorist attack. A treatment is needed that can be given quickly, for example by intramuscular injection using an autoinjector. This requires that the drug is sufficiently potent and soluble that it can be administered in a small volume.

Sodium tetrathionate, shown at the end of this sentence, is highly water-soluble. One molecule of tetrathionate can potentially detoxify two molecules of cyanide [3,4]. At a



. high, non-physiological pH, tetrathionate can react directly with cyanide via a reaction known as “cyanolysis” to generate thiocyanate and sodium thiosulfate (Equation 1) [3]. The resulting thiosulfate can serve as a substrate for rhodanese converting a second molecule of cyanide to thiocyanate (Equation 2).

$$Na_2S_4O_6 + CN^- \rightarrow SCN^- + {SO_4}^{-2} + Na_2S_2O_3 \quad (1)$$

$$Na_2S_2O_3 + CN^- \xrightarrow{\text{Rhodanese}} SCN^- + {SO_3}^{-2} \quad (2)$$

where $Na_2S_4O_6$ is sodium tetrathionate, CN^- is cyanide, SCN^- is thiocyanate, $SO_4{}^{-2}$ is sulfate, $Na_2S_2O_3$ is sodium thiosulfate, and $SO_3{}^{-2}$ is sulfite. Consistent with these reactions, tetrathionate has been shown to rescue young adult animals from cyanide poisoning, with several investigators showing that on a molar basis tetrathionate was 1.5- to 3.3-fold more potent than thiosulfate [5–10]. The latter data suggest that one mole of tetrathionate can indeed neutralize two moles of cyanide, but it has never been demonstrated that tetrathionate reacts directly with cyanide, either *in vitro* under physiological conditions or *in vivo*. The demonstration of a direct reaction of tetrathionate with cyanide *in vivo* is complicated by the fact tetrathionate is reduced to thiosulfate *in vivo* [11]. In the previous studies evaluating tetrathionate as a cyanide antidote, a cyanide salt was injected into animals, and most of the studies lacked appropriate control groups. We recently showed that tetrathionate administered by intramuscular injection rescued adolescent pigs from cyanide poisoning, but

again, the cyanide was administered parenterally and animals of only one age were studied [12].

In addition to reacting directly with cyanide, we hypothesized that tetrathionate could also react with methanethiol, another toxic chemical that like cyanide, inhibits cytochrome c oxidase of the mitochondrial electron transport chain [13–16]. Methanethiol, also known as methyl mercaptan and mercaptothiol, is a catabolic product of methionine and is generated during the decay of organic matter [17]. It is present in sewage treatment plants, wood pulp mills, oil and natural gas processing plants, and factories that produce pesticides, poultry feed, and jet fuel [18,19]. Seven deaths related to methanethiol exposure have occurred from occupational exposures over the last 10 years, and like cyanide, methanethiol is listed as a high priority chemical threat by the US Agency for Toxic Substances and Disease Registry [20,21]. But, unlike cyanide, no specific antidote exists for methanethiol.

For a drug to be useful against toxic gases in mass casualty scenarios, it needs to be effective against inhaled gases in people of all ages, and it needs to have an acceptable safety profile. We now show that tetrathionate: (1) reacts directly with cyanide at physiological pH; (2) reverses cyanide toxicity more rapidly than sodium thiosulfate in a highly monitored rabbit model, suggesting that tetrathionate reacts directly with cyanide *in vivo*; (3) rescues juvenile, young adult, and old adult mice from a lethal dose of inhaled hydrogen cyanide gas, and rescues rabbits from a lethal dose of intravenous cyanide; (4) has a therapeutic index towards cyanide of 3–5 in mice, and has no genetic toxicity; and (5) reacts directly with methanethiol, restores intracellular ATP in cultured cells exposed to methanethiol, and rescues mice from inhaled methanethiol gas.

Materials and methods

Materials

Sodium tetrathionate dihydrate, sodium thiosulfate pentahydrate, and potassium and sodium cyanide were purchased from Sigma-Aldrich, and sodium methanethiolate, the sodium salt of methanethiol ($NaSCH_3$, 95% pure), was purchased from Acros Organics. A thermal desorption tube filled with Tenax® TA adsorptive material (Gerstel Inc.) was conditioned for 8 h at 315 °C under 68 psi before being used for the first time. Purified water (18 MΩ-cm resistivity) was generated using a Water PRO PS system.

Measurement of tetrathionate and thiosulfate reaction with cyanide in vitro

The reaction of tetrathionate or thiosulfate with cyanide generates thiocyanate, which can be measured spectrophoto-metrically by conversion to ferric thiocyanate, a colored product [3]. Sodium tetrathionate or sodium thiosulfate were incubated with potassium cyanide either under conditions of excess cyanide at a high pH (Figure 1(A)) or a limiting cyanide concentration at physiological pH (Figure 1(B)). In Figure 1(A) experiments, cyanide was adjusted to a concentration five times higher than the concentration of sodium tetrathionate or sodium thiosulfate, which ranged from 50 μM to 5 mM; these experiments were conducted in 50 mM sodium phosphate buffer, pH 11.0. In Figure 1(B) experiments, the cyanide concentration was maintained at 100 μM, the sodium tetrathionate and sodium

thiosulfate concentration was 1 mM, and the pH of the sodium phosphate buffer was 7.4; these experiments were conducted in filled 1.5 ml screw-capped microcentrifuge tubes to minimize loss of HCN gas into the headspace above the sample. In both sets of experiments, samples were incubated at 37 °C for either 15 min (Figure 1(A)) or the indicated times (Figure 1(B)). At the end of the incubation period, a sample aliquot was mixed with an equal volume of ferric nitrate reagent [3], and absorbance of the resulting ferric thiocyanate product was measured at 460 nm.

Assessment of reversal of cyanide poisoning by continuous-wave near-infrared spectroscopy

We used a non-lethal rabbit model of cyanide poisoning to follow the animal's degree of cyanide poisoning in real-time [22]. We studied 4–6 month old male New Zealand white rabbits (Western Oregon Rabbit Supply) weighing 3.5–4.5 kg. The rabbits were anesthetized with a 1:1 mix of ketamine and xylazine, and mechanically ventilated with 100% oxygen at a respiratory rate of 20–22 breaths per minute and a tidal volume of 60 cc. After a 30 min equilibration period, the animals were injected intravenously for 55 min with sodium cyanide at a rate of 0.04 mg/kg/min (Figure 1(C)). When the cyanide infusion was stopped, the animals were injected into the right triceps longus muscle with 150 μl of either 2 M sodium tetrathionate or 2 M sodium thiosulfate. Sixty minutes later, they were euthanized by an intravenous injection of 1.0 mL Euthasol (Vibrac AH, Inc, Fort Worth, TX, USA). Throughout the experiment, tissue oxy- and deoxy-hemoglobin and total hemoglobin were monitored by continuous-wave near-infrared spectroscopy (CWNIRS) [21,22]. A custom-designed CWNIRS fiber optic probe with a broadband light source and detector was placed on the animals' head, and the intensity of reflected light was measured every second at five specific wavelengths in the near-infrared region (732, 758, 805, 840, and 880 nm) using a CCD spectrometer. Relative changes of oxy-, deoxy-, and total hemoglobin were calculated using a modified Beer-Lamberts' law. All data were processed with MATLAB (Mathworks, MA) software.

Lethal mouse model of cyanide poisoning

Male C57/BL6 mice were exposed to hydrogen cyanide gas as described previously (Figure 2(A)) [23,24]. Briefly, mice were placed into a 2.4 liter sealed chamber, anesthetized by injecting isoflurane to a final concentration of 2%, and then HCN gas was generated in the chamber by injecting potassium cyanide into a stirred beaker containing 10 M H_2SO_4. After 15 min of HCN exposure, the mice were removed from the chamber briefly and injected intramuscularly with saline or sodium tetra-thionate. They were then placed back into the chamber and exposed to HCN gas for an additional 25 min, for a total of 40 min of HCN exposure. Mice that were still alive at 90 min were considered survivors, and all surviving mice remained alive and well when followed for two weeks. This model simulates a real-life exposure to cyanide gas in an enclosed space, such as a subway or train station, where about 15 min would be required for emergency medical personnel to arrive at a disaster scene and another 25 min required to treat and evacuate victims. We performed the experiments on juvenile mice (4 week old), young adult mice (15–20 week old), and old adult mice (14–18 month old). We used an HCN concentration that resulted in ≥80% lethality in saline-treated

animals, i.e., 427, 584, and 427 ppm HCN for the 4 week old, 15–20 week old, and 14–18 month old mice, respectively.

Lethal rabbit model of cyanide poisoning

Male New Zealand white rabbits received a continuous intra-venous infusion of sodium cyanide (0.08 mg/kg/min) as shown in Figure 2(B) and described previously [22]. When the mean arterial blood pressure measured by an intra-arterial catheter decreased to <70% of baseline, the animals were injected intramuscularly with saline or sodium tetrathionate. The cyanide infusion was continued for an additional 30 min, and the animals were then observed for 60 min more. Survival was the primary outcome; animals with a mean arterial blood pressure of <30 mm Hg were euthanized.

Assessment of sodium tetrathionate toxicity and genetic mutagenesis

Toxicity studies—Mice and rats received an intraperitoneal injection of sodium tetrathionate, and they were then observed for four days (rats) or two weeks (mice). The rats had daily complete blood counts and chemistry panels performed in a certified veterinary medicine laboratory; the chemistry panel included serum electrolytes, renal function tests, and liver function tests.

Mutagenesis studies—We performed screening non-GLP mutagenicity studies, conducting the chromosomal aberration test in Chinese hamster ovary cells, and the Ames bacterial mutagenesis assay. As screening studies, we tested only two *Salmonella typhimurium* LT2 strains in the Ames test (strains TA98 and TA100); these two strains are generally the most sensitive to mutation.

Chromosomal aberration studies.: Cells were exposed to sodium tetrathionate for 3–21 h at concentrations ranging from 40.8 mM to 0.49 mM in the absence or presence of a rat S9 metabolic activation mixture. Cells were arrested in mitosis using 0.5 μM colchicine, and at least 100 cells per condition were scored for evidence of chromosomal aberrations. Methyl methanesulfonate and cyclophosphamide were used as positive controls.

Ames bacterial mutagenesis assay.: *Salmonella typhimurium* strains TA98 and TA10, were exposed to sodium tetrathionate for 48 h at concentrations ranging from 0.28 to 22 mM in the absence or presence of a rat S9 metabolic activation mixture. Revertant colonies were scored using an automated colony counter. Sodium azide and 2-nitrofluorene were used as positive controls for strains TA100 and TA98, respectively.

Measurement of sodium tetrathionate and sodium thiosulfate reaction with sodium methanethiolate

Aqueous solutions of 500 μM sodium tetrathionate or 500 μM sodium thiosulfate were mixed with 500 μM sodium methanethiolate at ratios of 1:3, 1:2, and 1:1 (*v:v*). The mixtures were incubated in a 20 mL headspace vial and analyzed by dynamic headspace-gas chromatography-mass spectrometry using a multiple positioning system samplers. Electron ionization coupled with a quadrupole mass filter in scan mode was used to identify reaction products.

Cell culture and measurement of ATP

COS-7 cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum. Twenty-four hours before measuring ATP, they were switched to DMEM without glucose, supplemented with 25 mM galactose and 10% fetal bovine serum that had been dialyzed against normal saline. Sodium methanethiolate was added at the start of an experiment to a final concentration of 1 mM and again after 1.5 h; it was added twice because, at physiological pH, it is converted to methanethiol, which is volatile and evaporates from the medium. Sodium tetrathionate and sodium thiosulfate were added at the indicated concentrations at 1.5 h, i.e., at the same time as the second sodium methanethiolate addition. After 3 h, the cells were extracted *in situ*, and ATP was measured in the extracts using the Cell Titer-Glo 2 kit (Promega Corp.). Protein was measured by the Bradford method [25], and the data are expressed as nmol/mg protein, using an ATP standard curve generated with every assay.

Exposure of mice to methanethiol gas

Male C57/BL6 mice (15–20 week old) were exposed to methanethiol gas. The experiment was analogous to exposure to hydrogen cyanide gas, except sodium methanethiolate was injected into the beaker containing H_2SO_4, leading to a final methanethiol concentration of 1400 ppm. As with the hydrogen cyanide exposure, the mice were removed from the chamber after 15 min of methanethiol exposure, injected intramuscularly with the test article, and placed back into the chamber for an additional 25 min. Mice that were alive at 90 min were considered survivors, and they all remained alive and well when followed for two weeks.

Animal study approval and number of animals used

All experiments were conducted according to the National Academies of Sciences, Engineering, and Medicine Institute for Laboratory Animal Research (ILAR) Guide to the Care and Use of Laboratory Animals, and were approved by the local Institutional Animal Care and Use Committee (IACUC) accredited *via* the American Association for Accreditation of Laboratory Animal Care (AAALAC).

In the mouse survival studies, six animals were used in each group, in the rabbit survival studies, at least 10 animals were used in each group, and in the rabbit reversal of cyanide poisoning experiments, three animals were used in each group. These numbers were established by *a priori* power analyses to achieve a p-value of <0.05 between groups: for the mouse survival studies we assumed 100% lethality in saline-treated animals and >80% survival in the treated animals and for the rabbit survival studies we assumed >80% lethality in saline-treated animals and >80% survival in treated animals. For the rabbit reversal studies, we wanted to assess the rate of recovery from cyanide poisoning in tetrathionate-treated and thiosulfate-treated animals, but we were not necessarily interested in achieving statistical significance.

Statistics and data analysis

Data were graphed and analyzed in Prism 7.04 (GraphPad, San Diego, CA, USA). Survival curves were compared by a Mantel-Cox log-rank test, and two or more conditions were compared by a one-way ANOVA. A p-value <0.05 was considered significant.

Results

Tetrathionate reacts directly with cyanide in vitro under physiological conditions and reverses cyanide poisoning in rabbits more rapidly than thiosulfate

In vitro studies—Previous studies of tetrathionate's reaction with cyanide were conducted at a high cyanide concentration (~25 mM), a relatively high pH (pH ~ 9), and 4 or 20 °C, because HCN's pK_a is 9.3 and its boiling point is ~22 °C. Tetrathionate reacts with the cyanide anion (CN^-), and thus conducting experiments under the above conditions will maintain the CN^- concentration at a high and relatively constant concentration throughout the experiment, allowing one to calculate the reaction rate (V_{max}). We confirmed the earlier results and showed complete conversion of tetrathionate to thiocyanate at tetrathionate concentrations from 50 mM to 5 mM (Figure 1(A); cyanide was present at five times the tetrathionate concentration; note that the amount of thiocyanate formed was identical to a thiocyanate standard). Under these same optimal conditions for reaction with cyanide, thiosulfate showed no generation of thiocyanate, consistent with previous work showing that thiosulfate reacts with CN^- only in the presence of excess cupric ions (Figure 1(A)) [3].

We next designed an experiment to allow the measurement of tetrathionate's reaction with cyanide under physiological conditions. At pH 7.4, ~99% of cyanide will be in the form of HCN, with <1% as CN^-. Thus, one would not expect as rapid or as complete a reaction of tetrathionate with cyanide as at a high pH and high cyanide concentration, but tetrathionate still reacted with cyanide, generating a linear increase in thiocyanate concentration over time (Figure 1(B)). As would be expected, thiosulfate showed no reaction with cyanide under these conditions (Figure 1(B)).

Rabbit studies—To assess if tetrathionate reacts with cyanide *in vivo*, we used a well-described non-lethal rabbit model of cyanide poisoning [23,24]. We infused sodium cyanide intravenously for 55 min, and then injected equimolar amounts of sodium tetrathionate or sodium thiosulfate intramuscularly (Figure 1(C)). We then measured the rate of return of tissue deoxy-hemoglobin towards baseline concentrations and found that tetrathionate more rapidly and fully returned tissue deoxy-hemoglobin to baseline values than thiosulfate, with 50% recovery of deoxy-hemoglobin concentration occurring at 13.5 min for tetrathionate and 21.6 min for thiosulfate (Figure 1(D)). This suggests that tetrathionate reacts directly with cyanide *in vivo*; we consider this matter further in the Discussion.

Tetrathionate is effective as a cyanide antidote in juvenile, young adult, and old adult mice, and young adult rabbits

Since cyanide exposure from a fire, a terrorist attack, or an industrial accident could affect people of all ages, we studied 4 week old, 15–20 week old, and 14–18 month old mice, corresponding to 2, 20–30, and 50–60 year old humans, respectively [26]. We chose mice,

because the age correlation between mice and humans is well-defined, and conducting these experiments in a larger species, such as rabbits would have been prohibitively expensive. In a cyanide gas inhalation model (Figure 2(A)), no saline-injected 4-week old and 15–20 week old mice lived, and <20% of saline-injected 14–18 month old mice lived (Figures 2(C–E), black circles). We found that at 108 mg/kg (0.4 mmol/kg) sodium tetrathionate >80% of 4 week old and 15–20 week old mice lived, while at a 50% higher dose of 162 mg/kg (0.6 mmol/kg) sodium tetrathionate, 100% of the 14–18 month old mice lived (Figures 2(C–E), grey squares; the difference between saline-injected animals and tetrathionate-injected animals was significant by a log-rank test). Possible reasons for the higher tetrathionate requirement in the older mice will be considered in the Discussion.

The FDA Animal Rule Pathway requires efficacy studies to be conducted in at least one non-rodent species [27]. We used a lethal rabbit model of cyanide poisoning, with survival as the measured outcome (Figure 2(B)). We administered saline or tetrathionate by intramuscular injection and found that 3 of 11 saline-injected rabbits lived, compared to survival in 7 of 10 rabbits that received 23 mg/kg (0.09 mmol/kg) sodium tetrathionate (Figure 2(F); compare black circles to grey squares; the difference between the two groups was significant by a log-rank test).

Safety profile of tetrathionate

Acute toxicity studies in mice and rats—We found that five of five mice appeared completely normal after a dose of 540 mg/kg (2 mmol/kg) sodium tetrathionate; the drug was administered by intraperitoneal injection because the volume was more than could be administered by intramuscular injection. Mice are too small to tolerate serial blood removal, and we, therefore, could not assess laboratory tests, but they remained completely healthy for two weeks post tetrathionate injection at which time they were euthanized.

Previous investigators have shown that the major toxicity of tetrathionate is from acute renal injury [11,28,29]. To assess renal toxicity of tetrathionate, we used rats, which can sustain seral blood removal. We found that both male and female rats tolerated 200 and 250 mg/kg (0.74 and 0.93 mmol/kg) sodium tetrathionate without any clinical abnormalities, and that male rats showed no change in serum blood urea nitrogen or creatinine at either dose(Table 1). The female rat that received 200 mg/kg sodium tetrathionate showed a small transient increase in the blood urea nitrogen and creatinine; the female rat that received 250 mg/kg sodium tetrathionate showed a large increase in both renal function parameters that peaked at 48 h and were returning towards baseline by 96 h (Table 1). All other laboratory tests including serum electrolytes and liver function tests were normal in both genders and at both sodium tetrathionate doses. When corrected by body surface area, doses of 200 and 250 mg/kg in rats correspond to ~400 and 500 mg/kg in mice.

Genetic toxicology studies—We tested sodium tetrathionate in the Chinese hamster ovary chromosomal aberration assay and the *Salmonella*/ microsome plate incorporation assay (Ames test). We found no evidence of mutagenicity at the maximal testable dose of 0.49 mM in the chromosomal aberration assay and 22 mM in the Ames test.

Tetrathionate and thiosulfate react directly with methanethiol, revert methanethiol cellular toxicity, and rescue mice from methanethiol poisoning

Reaction of tetrathionate and thiosulfate with methanethiol—We incubated sodium tetrathionate or sodium thiosulfate with sodium methanethiolate, and analyzed the samples by dynamic headspace gas-chromatography mass-spectrometry. We found that a 1:1 molar ratio of sodium tetrathionate or sodium thiosulfate mixed with sodium methanethiolate yielded a clearly defined dominant peak at 1.6 min, which accounted for 98% of the overall peak area on the chromatogram (Figure 3(A)). This peak was absent in samples containing only sodium tetrathionate, sodium thiosulfate, or sodium methanethiolate (Figure 3(A); for sake of simplicity, sodium methanethiolate alone is not shown). The 1.6 min peak was positively identified as dimethyldisulfide by (i) comparing the peak to the known mass spectrum of dimethyldisulfide; (ii) adding a dimethyldisulfide standard to a blank solution; and (iii) fragmentation analysis (Figure 3(B)).

Reversal of methanethiol toxicity in cells and mice—We tested if tetrathionate and thiosulfate could recover ATP in methanethiol-exposed COS-7 cells and rescue mice from methanethiol poisoning. Due to the volatility of methanethiol, we added it twice to the COS-7 cells, once at zero time and then again after 1.5 h, both times at a final concentration of 1 mM; the ATP concentration was then measured at 3 h. We added the tetrathionate and thiosulfate at 1.5 h, i.e., at the time of adding the second dose of methanethiol. We found that 1.5 mM sodium tetrathionate completely recovered the ATP concentration to that of control cells, whereas 5 mM sodium thiosulfate yielded only partial recovery of ATP (Figure 3(C); neither 1.5 nor 3 mM sodium thiosulfate had a significant effect).

We studied methanethiol poisoning in mice, using an analogous model to that of cyanide poisoning. At a calculated methanethiol concentration of 1400 ppm, all saline-injected animals died, whereas 5 of 6 animals that received 162 mg/kg (0.6 mmol/kg) sodium tetrathionate lived (Figure 3(D)). Sodium thiosulfate at 186 mg/kg (0.75 mmol/kg) rescued 1 of 6 animals, while 372 mg/kg (1.5 mmol/kg) sodium thiosulfate yielded the same recovery as 162 mg/kg sodium tetrathionate, i.e., 5 of 6 animals lived (Figure 3(D)).

Discussion

In this study, we show that sodium tetrathionate is effective against cyanide and methanethiol poisoning in cultured cells and animals. This makes tetrathionate a potentially important therapeutic because it is active against two high-priority threat agents. Three notable aspects of this study are that: (i) we used inhaled models of cyanide and methanethiol exposure, (ii) we tested animals of multiple ages in the cyanide experiments, and (iii) we followed the animals for at least two weeks after exposure to the chemical agent. Most studies of cyanide exposure inject cyanide solutions intravenously, intraperitoneally, or subcutaneously, use animals of only one age, and euthanize the animals within 1–3 h after cyanide exposure. Inhalation of hydrogen cyanide gas in a fire or methanethiol gas in an industrial accident is the most likely mode of exposure to these agents, and, in the case of a fire, people of multiple ages will be exposed. Thus, it is critical to show that a cyanide or methanethiol antidote works against inhalation of the gases and that it is effective in animals

of varying ages. It is also important to show that acute reversal of toxicity by an antidote translates to long-term survival without evident clinical sequelae.

The finding that 14–18 month old mice required about 50% more tetrathionate to achieve full rescue from cyanide toxicity than younger mice underscores the necessity of testing antidotes in animals of varying ages. We can think of two possible reasons for an increased tetrathionate requirement in older mice. First, aged mice may have decreased blood perfusion of skeletal muscle, which would impair tetrathionate absorption, since we administered the drug by intramuscular injection. And second, although tetrathionate appears to react directly with cyanide *in vivo* (discussed further below), some of the tetrathionate's neutralizing capacity towards cyanide is almost certainly from conversion to thiosulfate; to neutralize cyanide, thiosulfate requires the enzyme rhodanese, which may decrease in activity with age. Thus, more tetrathionate would be required for direct reaction with cyanide.

Tetrathionate is known to react directly with cyanide *in vitro* under non-physiological conditions, and we now show that it reacts readily with cyanide under physiological conditions. Thiosulfate can react with cyanide, but the reaction requires a catalyst, such as cupric ions [3]. Consistent with these latter data, we found no evidence of thiosulfate reaction with cyanide in the absence of a catalyst, either under non-physiological or physiological conditions.

Tetrathionate is reduced to thiosulfate *in vivo*, with differing rates of reduction among species [11]. In rabbits, about 50% of an intravenously administered dose of tetrathionate is reduced to thiosulfate over 30 min [11]. Because the direct reaction of tetrathionate with cyanide and the enzymatic reaction of thiosulfate with cyanide *via* rhodanese both yield thiocyanate, it would be difficult to determine whether tetra-thionate reacts directly with cyanide *in vivo* or indirectly *via* reduction to thiosulfate. Thus, we conducted the rabbit cyanide reversal experiments, which suggest that tetrathionate reacts directly with cyanide *in vivo*: if tetrathionate reversed cyanide poisoning only by being converted to thiosulfate, then tetrathionate's rate of reversal would have been slower than that of thiosulfate, assuming one mole of tetrathionate yields two moles of thiosulfate, and that 30 min is required for half of the tetrathionate to be reduced to thiosulfate. However, tetrathionate reversed cyanide toxicity almost twice as fast as an equimolar amount of thiosulfate, pointing to a direct reaction between tetrathionate and cyanide.

For an agent to be a viable drug candidate, it must have an acceptable therapeutic index. We found that the therapeutic index of tetrathionate for treating cyanide poisoning in juvenile and young adult mice is at least 5, and old adult mice is at least 3.3. Data from rat toxicity studies confirmed that renal dysfunction is the major basis of tetrathionate toxicity, but that up to 250 mg/kg, the renal injury appears to be reversible.

Cyanide and methanethiol both inhibit cytochrome C oxidase in complex IV of the mitochondrial electron transport chain, which would be expected to reduce intracellular ATP. Consistent with this mechanism of action, cyanide has been shown to reduce the intracellular ATP content in the brains of cyanide-poisoned rats, and we now show that methanethiol

reduces intracellular ATP in COS-7 cells [30]. No antidote currently exists for methanethiol poisoning, although we showed recently that the vitamin B_{12} analog cobinamide rescues pigs in a lethal model of methanethiol exposure [31]. In the current work, we found that both sodium tetrathionate and sodium thiosulfate reacted with methanethiolate to produce dimethyldisulfide *in vitro*. These are highly plausible reactions based on the oxidizing power of both tetrathionate and thiosulfate, and methanethiol oxidation produces dimethyldisulfide according to the following reaction, where [O] represents an oxidizing agent: $CH_3SH + [O] \rightarrow CH_3S\text{-}SCH_3$. In cultured cells, tetrathionate was about 3-fold more potent than thiosulfate at recovering intracellular ATP, and, in mice, tetrathionate was 2-fold more potent than thiosulfate in rescuing the animals from a lethal exposure to methanethiol. Thus, tetrathionate appears to be a better methanethiol antidote than thiosulfate.

Limitations

A limitation to these studies is that the mice and rabbits were anesthetized, as required by the IACUCs at our respective institutions due to the possibility of the animals suffering during cyanide or methanethiol exposure. Since control non-antidote-treated animals were also anesthetized, we think it is unlikely the anesthetic interfered with the interpretation of results. A second limitation is that we used only male animals in the efficacy studies. It is possible we would have obtained different results with female animals.

Conclusions

We conclude that sodium tetrathionate could be a good drug for emergent treatment of both cyanide and methanethiol poisoning. A major advantage of tetrathionate is that it could be administered by intramuscular injection *via* an autoinjector in the pre-hospital setting.

Acknowledgments

Funding

The work was supported, in part, by the Office of the Director, National Institutes of Health, Grant #U01NS105057 to GRB.

References

[1]. Cyanide: understanding the risk, enhancing preparedness. Clin Toxicol 2006;44 (Suppl 1):47–63.

[2]. Cummings TF. The treatment of cyanide poisoning. Occup Med 2004;54(2):82–85.

[3]. Kelly DP, Chambers LA, Trudinger PA. Cyanolysis and spectrophotometric estimation of trithionate in mixture with thiosulfate and tetrathionate. Anal Chem 1969;41(7):898–901.

[4]. Baskin SI, Kirby SD. The effect of sodium tetrathionate on cyanide conversion to thiocyanate by enzymatic and non-enzymatic mechanisms. J Appl Toxicol 1990;10(5):379–382. [PubMed: 2254590]

[5]. Hebting J. Versuche überentgiftung der Blausäure durch schwefe-labspaltende substanzen. Biochem Zeitschrift 1910;28:208–212.

[6]. Binet L, Wellers G, Dubrisay J. [Value and mechanism of the antidote action of sodium tetrathionate in hydrocyanic poisoning. Presse Med 1951;59(32):641–643.]. [PubMed: 14853831]

[7]. Nitescu II, Craiu R, Wassermann N. Cyanides and anticyanides. The action of sodium tetrathionate as an antidote in hydrocyanic poisoning. Rum Med Rev 1961;5:203–204. [PubMed: 13729279]

[8]. Chen KK, Rose CL, Clowes GHA. Comparative values of several antidotes in cyanide poisoning. Am J Med Sci 1937;188:767–781.

[9]. Chistoni A, Foresti B. L'antidotismo del tetrationato sodico nelle intossicazioni da acido cianidrico. Arch Internat de Pharmacod 1932;42:140–171.

[10]. Hatch RC, Laflamme DP, Jain AV. Effects of various known and potential cyanide antagonists and a glutathione depletor on acute toxicity of cyanide in mice. Vet Hum Toxicol 1990;32:9–16. [PubMed: 2301155]

[11]. Gilman A, Philips FS. The metabolic reduction and nephrotoxic action of tetrathionate in relation to a possible interaction with sulfhydryl compounds. Am J Physiol 1946;147:115–126. [PubMed: 21000729]

[12]. Hendry-Hofer TB, Witeof AE, Ng PC, et al. Intramuscular sodium tetrathionate as an antidote in a clinically relevant swine model of acute cyanide toxicity. Clin Toxicol 2020;58(1):29–35.

[13]. Tansy MF, Kendall FM, Fantasia J, et al. Acute and subchronic toxicity studies of rats exposed to vapors of methyl mercaptan and other reduced-sulfur compounds. J Toxicol Environ Health 1981; 8(1–2):71–88. [PubMed: 7328716]

[14]. Fairchild EJ, Stokinger HE. Toxicologic studies on organic sulfur compounds. I. Acute toxicity of some aliphatic and aromatic thiols (mercaptans). Am Ind Hyg Assoc J 1958;19(3):171–189. [PubMed: 13559131]

[15]. Wilms J, Lub J, Wever R. Reactions of mercaptans with cytochrome c oxidase and cytochrome c. Biochim Biophys Acta 1980; 589(2):324–335. [PubMed: 6243968]

[16]. Waller RL. Methanethiol inhibition of mitochondrial respiration. Toxicol Appl Pharmacol 1977;42(1):111–117. [PubMed: 201048]

[17]. Kangas J, Jappinen P, Savolainen H. Exposure to hydrogen sulfide, mercaptans and sulfur dioxide in pulp industry. Am Ind Hyg Assoc J 1984;45(12):787–790. [PubMed: 6517022]

[18]. Occupational safety and health guideline for methyl mercaptan Washington, D.C.: US Department of Health and Human Services National Institute for Occupational Safety and Health Division of Standards Development and Technology Transfer; 1988. p. 1–6.

[19]. Toxicological profile for methyl mercaptan Atlanta, GA: Agency for Toxic Substances and Disease Registry US Public Health Service; 1982. p. 1–62.

[20]. Widener A. Four killed at DuPont plant. Chem Eng News 2014; 92:4. Available from: https://cen.acs.org/articles/92/i47/Four-killed-DuPont-plant.html

[21]. Atofina settlement. Miosha News 2002;6:1–18. Available from: https://www.michigan.gov/documents/CIS_WSH_minwssm02_59600_7.pdf

[22]. Brenner M, Kim JG, Mahon SB, et al. Intramuscular cobinamide sulfite in a rabbit model of sublethal cyanide toxicity. Ann Emerg Med 2010;55(4):352–362. [PubMed: 20045579]

[23]. Chan A, Jiang J, Fridman A, et al. Nitrocobinamide, a new cyanide antidote that can be administered by intramuscular injection. J Med Chem 2015;58(4):1750–1759. [PubMed: 25650735]

[24]. Bebarta VS, Brittain M, Chan A, et al. Sodium nitrite and sodium thiosulfate are effective against acute cyamide poisoning when administered by intramuscular injection. Ann Emerg Med 2017; 69(6):718–725. [PubMed: 28041825]

[25]. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. Anal Biochem 1976;72:248–254. [PubMed: 942051]

[26]. Dutta S, Sengupta P. Men and mice: relating their ages. Life Sci 2016;152:244–248. [PubMed: 26596563]

[27]. US Department of Health and Human Services F. Product development under the animal rule Washington, D.C.: Guidance for Industry; 2014. p. 1–48.

[28]. Bloom N, Forbes G, Policoff L. Toxicity of sodium tetrathionate. Proc Soc Exp Biol Med 1949;72(1):207–209. [PubMed: 15391715]

[29]. Sloan H. Production of experimental uremia by sodium tetrathionate. Proc Soc Exp Biol Med 1951;76(2):344–346. [PubMed: 14827921]

[30]. MacMillan VH. Cerebral energy metabolism in cyanide encephalopathy. J Cereb Blood Flow Metab 1989;9(2):156–162. [PubMed: 2921290]

[31]. Hendry-Hofer TB, Ng PC, McGrath AM, et al. Intramuscular cobinamide as an antidote to methyl mercaptan poisoning. Inhal Toxicol 2021;33(1):25–32. [PubMed: 33356664]

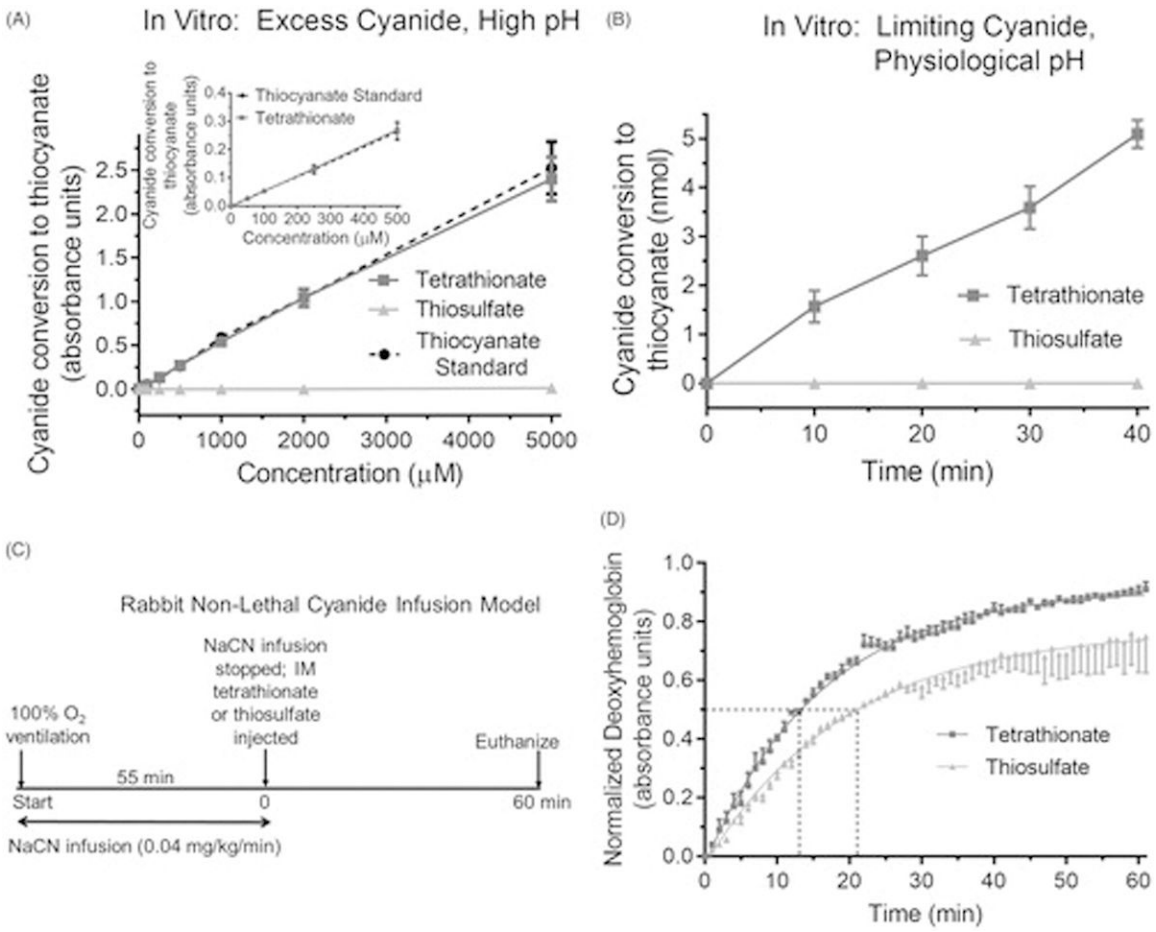


Figure 1.

Sodium tetrathionate reacts directly with cyanide. (A) The indicated concentrations of sodium tetrathionate (dark grey squares, solid line) and sodium thiosulfate (light grey triangles, solid line) were incubated at 37 °C in 50 mM sodium phosphate buffer, pH 11.0 with a 5-fold excess of potassium cyanide. After 15 min, thiocyanate formation was measured by adding ferric nitrate and measuring absorbance at 460 nm. Sodium thiocyanate incubated under identical conditions in the absence of potassium cyanide is shown (black circles, dashed line). The main figure shows data from zero to 5 mM of sodium tetrathionate, sodium thiosulfate, and sodium thiocyanate, and the inset shows data from zero to 500 μM of sodium tetrathionate and sodium thiocyanate. (B) Similar experiments as those shown in Panel A were conducted except the sodium tetrathionate (dark grey squares, solid line) and sodium thiosulfate (light grey triangles, solid line) were at 1 μM, the potassium cyanide was held constant at 100 μM, and the sodium phosphate buffer was pH 7.4. The amount of thiocyanate produced over 40 min is shown. (C) Diagrammatic representation of a rabbit non-lethal cyanide infusion model. Rabbits received a continuous intravenous infusion of sodium cyanide at 0.04 mg/kg/min for 55 min. The infusion was stopped and they were injected immediately with equimolar amounts of either sodium tetrathionate or sodium thiosulfate. Throughout the experiment, the tissue concentration of oxy- and deoxy-hemoglobin was monitored by continuous-wave near-infrared spectroscopy. (D) Rabbits

were injected intravenously with sodium cyanide according to the protocol shown in panel (C). The rate of return of deoxy-hemoglobin towards baseline values is plotted *vs.* time for animals injected with sodium tetrathionate (dark grey squares) or sodium thiosulfate (light grey triangles). Time zero is the same as in panel (C). The dotted lines show the time to 50% recovery of deoxy-hemoglobin, which was 13.5 min for tetrathionate and 21.6 min for thiosulfate. In panels (A,B,D), the data are the mean ± SEM of three independent experiments.

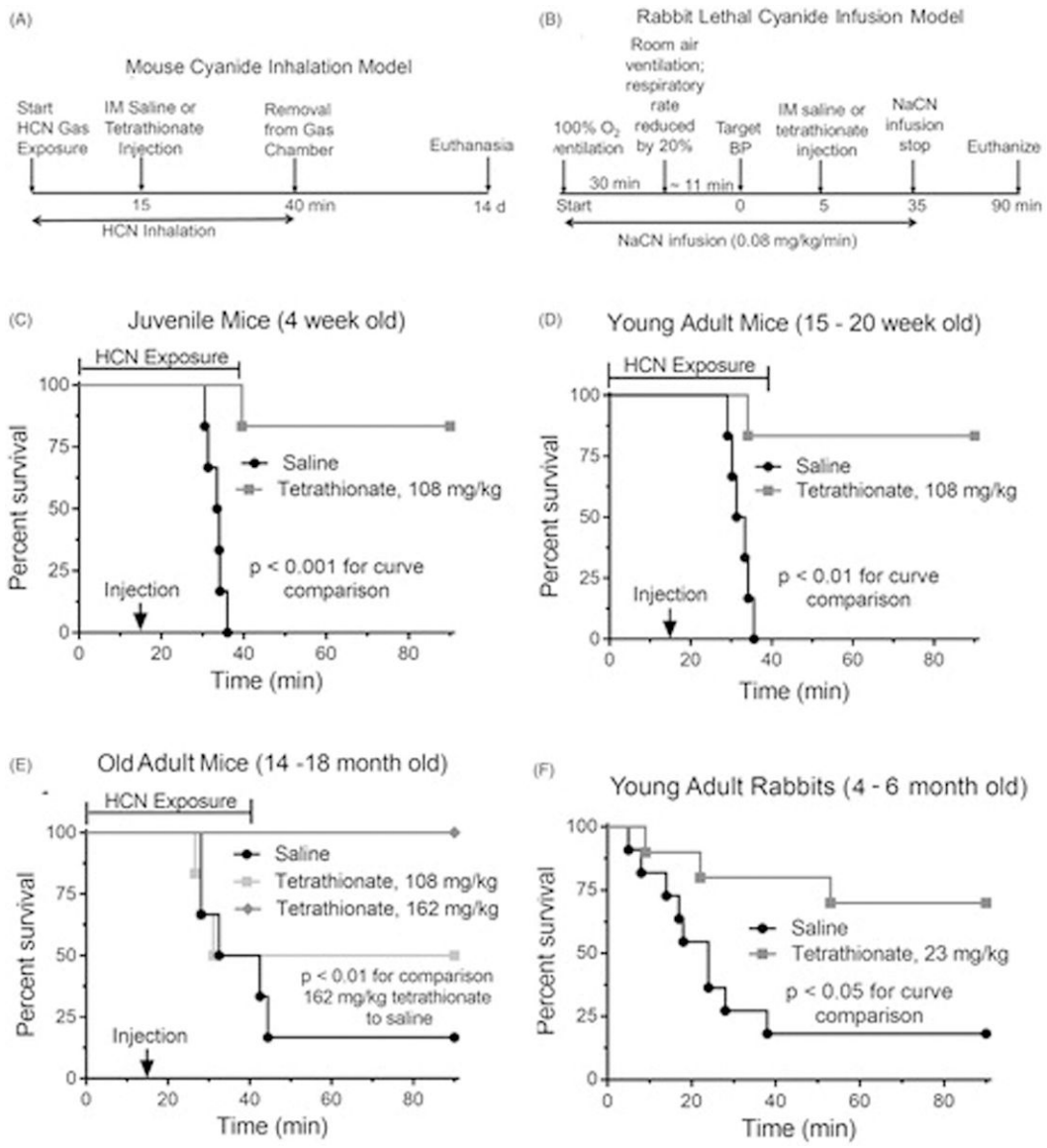


Figure 2.

Sodium tetrathionate rescues juvenile, young adult, and old adult mice, and young adult rabbits from lethal cyanide poisoning. (A,B) Diagrammatic representations of the mouse cyanide inhalation model (panel A) and rabbit lethal cyanide infusion model (panel B). (C–E) Juvenile mice (4 week old, panel C), young adult mice (15–20 week old, panel D), and old adult mice (14–18 month old, panel E) were exposed to hydrogen cyanide gas in an airtight chamber as depicted in panel (A). After 15 min of gas exposure, they were removed from the chamber and injected intramuscularly with saline (black circles), 108 mg/kg sodium tetrathionate (grey squares), or 162 mg/kg sodium tetrathionate (old adult mice, grey diamonds). They were placed back in the chamber for an additional 25 min of cyanide gas exposure. Animals that lived until 90 min were observed for an additional two weeks, and all of them appeared fully normal. *N*=6 mice in each group. (F) Rabbits received a continuous intravenous infusion of sodium cyanide as depicted in panel (B). When the

mean arterial blood pressure was <70% of baseline, the animals received an intramuscular injection of saline (black circles) or 23 mg/kg sodium tetrathionate (grey squares). The cyanide infusion was continued for 30 min more, and the animals were then observed for an additional 60 min. Zero time is the time of saline or sodium tetrathionate injection. N=11 for saline-injected animals and N=10 for tetrathionate-injected animals. In panels (C–F), the difference between saline-injected and tetrathionate-injected animals is compared by a log-rank test.

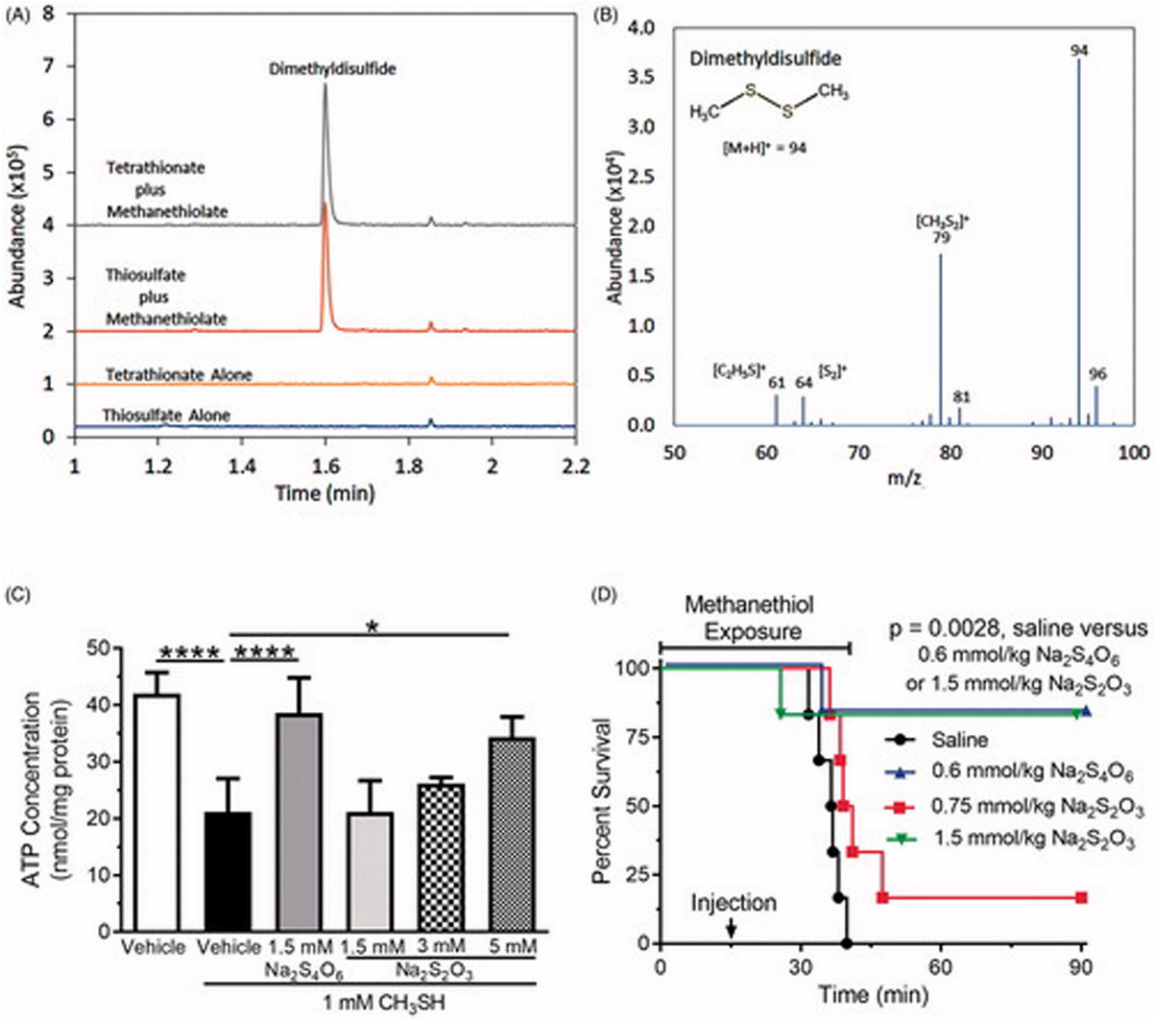


Figure 3.

Sodium tetrathionate and sodium thiosulfate react with sodium methanethiolate, and reverse methanethiol toxicity in cells and mice. (A) Sodium methanethiolate was mixed at a 1:1 molar ratio with sodium tetrathionate (grey line) or sodium thiosulfate (red line), and the product(s) of the reaction were analyzed by dynamic headspace gas-chromatography mass-spectrometry. As negative controls, the mass spectra of sodium tetrathionate (orange line) and sodium thiosulfate (blue line) alone are shown. (B) The peak eluting at 1.6 min in panel (A) was identified as dimethyldisulfide by mass spectrometry and fragmentation analysis; the structure of dimethyldisulfide is shown in the upper left. (C) COS-7 cells were incubated for 3 h with 1 mM methanethiol; due to its volatility, it was added at zero time and again at 1.5 h. At the time of the second methanethiol addition, either 1.5 mM sodium tetrathionate ($Na_2S_4O_6$) or 1.5, 3, or 5 mM sodium thiosulfate ($Na_2S_2O_3$) were added to the cells. At the end of the 3 h incubation, the cells were extracted *in situ*, and ATP was measured by a chemiluminescent method. * and **** indicate $p < 0.05$ and <0.001, respectively, by a one-way ANOVA for the indicated comparisons. (D) Mice were exposed to 1400 ppm methanethiol gas for 15 min, and were removed from the chamber and injected with either saline (black circles), 0.6 mmol/kg sodium tetrathionate ($Na_2S_4O_6$,

blue triangles), or 0.75 or mmol/kg sodium thiosulfate ($Na_2S_2O_3$, red squares, and green inverted triangles, respectively). They were placed back in the chamber for an additional 25 min of methanethiol exposure. The saline-injected animals all died before removal from the chamber at 40 min. Animals that were alive at 90 min remained alive and well over a two-week follow-up period. $N = 6$ mice per group. The difference between saline-injected animals and animals that received 0.6 mmol/kg sodium tetrathionate or 1.5 mmol/kg sodium thiosulfate was significant by a log-rank test ($p = 0.0028$).

Table 1.

Renal parameters in rats injected with sodium tetrathionate.

Gender	Dose (mg/kg)	Basal		24h		48h		72h		96h	
		BUN	Cr	BUN	Cr	BUN	Cr	BUN	Cr	BUN	Cr
		(mg/dl)									
M	200	17	0.3	15	0.2	14	0.3	16	0.3	15	0.2
F	200	15	0.2	35	0.7	23	0.4	15	0.3	14	0.4
M	250	16	0.3	12	0.3	14	0.4	15	0.4	13	0.4
F	250	16	0.3	139	3.2	>180	4.1	163	2.9	79	1

Male and female rats were injected with the indicated dose of sodium tetrathionate, and 24, 48, 72, and 96 h later, blood was drawn and blood urea nitrogen (BUN) and creatinine (Cr) were measured in the serum. The basal value was obtained 24 h before sodium tetrathionate injection. All of the rats appeared clinically well throughout the study, and all other laboratory tests showed no change over the 96 h.