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DMTS is an effective treatment in both an inhalation and injection model for cyanide poisoning using unanesthetized mice

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Abstract

Cyanide (CN) is a metabolic poison, halting ATP synthesis by inhibiting Complex IV of the electron transport chain. If exposed at high enough concentrations, humans and most animals can die within minutes. Cyanide is inexpensive to produce and is easily accessible, as it has numerous legitimate industrial applications. Because time is a crucial factor in survival of cyanide poisoning, a rapidly bioavailable, nontoxic, easy to administer CN medical countermeasure could improve morbidity/mortality in a mass CN exposure scenario. Several current candidate CN countermeasures are being developed that act as sulfur donors or CN scavengers and can be given through the intramuscular route. One such countermeasure, dimethyl trisulfide (DMTS), has shown promise as a treatment for cyanide poisoning. Traditionally, evaluation of cyanide medical countermeasures has been conducted in models in which animals are injected or infused with cyanide salts. Even though this type of model is convenient and allows for tight control of exposure parameters, the most likely route of exposure to cyanide is via inhalation. Additionally, acute cyanide exposure models typically require the animals to be anesthetized, which can complicate, confound, or exacerbate the clinical effects of cyanide toxicity. For these reasons, we developed a model of acute cyanide inhalation intoxication, using the Highly Toxic Agent System from CH Technologies for nose-only exposure in unanesthetized mice. Using this model, we show that a newly developed formulation of DMTS is efficacious within two lethal cyanide exposure mouse models (potassium cyanide [KCN] injection and hydrogen cyanide [HCN] inhalation) and is highly effective by intramuscular injection. Within the HCN model, we demonstrate efficacy of DMTS in both a continuous and discontinuous inhalation exposure model. Clinical signs and survival are described in the presence and absence of DMTS using both lethal exposure models.

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Disclosure Statement

The views expressed in this article are those of the author(s) and do not reflect the official policy of the Department of the Army, Department of Defense, or the U.S. Government.

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Keywords

cyanide; hydrogen cyanide (HCN); inhalation exposure; Fourier transform infrared spectrometer (FTIR); Highly Toxic Agent System (HTAS); dimethyl trisulfide (DMTS)

Introduction

Cyanide is a highly toxic, rapidly acting metabolic poison which binds to and inhibits cytochrome a_3 , disrupting the electron transport chain of aerobic respiration [1, 2, 3]. Cyanide has many historical and contemporary industrial applications, including fumigation, precious metal processing, electroplating, and polymer synthesis [4]. Structural fires are a significant source of acute toxic cyanide exposure, as hydrogen cyanide gas is generated by the pyrolysis of polymers commonly found in upholstery and flooring [5]. Additionally, cyanide has been used to intentionally cause harm to self or others. Cyanides were explored as chemical warfare agents during both the First and Second World Wars [6–8]. As a chemical terrorism agent, cyanide has been used for extortion [9], to contaminate acetaminophen and analgesic tampered capsules [10, 11], in state terrorism [12, 13, 14], by nationalist and separatist terrorist groups [15, 16, 17], by left- and right-wing terrorist groups [18, 19, 20], by apocalyptic cults [21] and potentially in Jihadi terrorism [22, 23, 24, 25].

The currently available treatments in the U.S. for cyanide poisoning are the combination of sodium nitrite and sodium thiosulfate (Nithiodote®) and hydroxocobalamin (Cyanokit®). Sodium nitrite is a methemoglobin inducer, which has a higher affinity for cyanide than the ferric iron of cytochrome oxidase a_3 . However, emerging evidence suggests that sodium nitrite may be acting through additional mechanisms [3, 26, 27]. Sodium thiosulfate works as an antidote in cyanide poisoning by acting as a donor of sulfane sulfur, in which endogenous sulfurtransferase enzymes, most importantly rhodanese, combine with cyanide to form thiocyanate. Furthermore, hydroxocobalamin is a cobalt-based direct cyanide-chelating antidote. All three agents are administered by intravenous injection, which has limited application in mass casualty situations. An ideal cyanide antidote for a mass casualty situation would be capable of rapid administration in small volumes via an autoinjector, by intraosseous injection, or by inhalation [28].

Evaluation of cyanide medical countermeasures has traditionally been conducted in models in which animals are injected or infused with cyanide salts [29, 30]. Though this type of model is not necessarily reflective of real-world exposures, it is still an instructive model, allowing for tight control of exposure parameters (i.e., precise volume of CN administration). Additionally, acute cyanide exposure models typically require the animals to be anesthetized. Anesthesia can reduce heart and respiratory rate [31, 32], which is a very different condition from someone who has been exposed to cyanide. Indeed, isoflurane has been shown to protect against acute cyanide exposure, as well as interfere with the protection afforded by sodium nitrite [27]. For this reason, we developed a model of acute cyanide inhalation intoxication, using a Highly Toxic Agent System from CH Technologies, in unanesthetized mice. Use of the nose-only exposure inhalation system allows the ability to execute brief exposures while limiting the conduits of CN entry into the animal. This

model was used to support the development of dimethyl trisulfide (DMTS) as a candidate countermeasure for cyanide poisoning by providing a model of acute CN exposure that better represents a real-world mass casualty scenario. DMTS has previously been demonstrated as an efficacious cyanide countermeasure that can be delivered via intramuscular (IM) injection in an injection-based model of CN toxicity [33]. As a means of comparison, we also include the results of the new formulation in the KCN injection model. Here we show that a new, more concentrated formulation of DMTS is efficacious in a KCN injection model, as well as in two lethal HCN inhalation paradigms, and is highly effective by intramuscular injection.

Materials and Methods

Materials

Male CD-1 mice, aged 35–40 days at exposure, were purchased from Charles River Laboratories (Kingston, NY). Mice were quarantined for 5 days upon arrival on-site. All mice were allowed access to food (LabDiet Laboratory Rodent Diet #5001, St. Louis, MO) and water *ad libitum*, except during periods of experimental manipulation. Animals were group-housed, and maintained in a rodent vivarium where the temperature (68–79°F), humidity (30–70%), and a 12 h light/dark cycle were closely monitored daily. All animals were fasted for a period of 12–16 hours prior to exposure studies to remain consistent with previous experimental parameters performed in-house and with extramural collaborators.

Potassium cyanide (KCN; Sigma-Aldrich, St. Louis, MO) was dissolved in 0.9% sterile saline (Hospira, Lake Forest, IL) immediately before use for the subcutaneous (SC) injection model. The desired concentration of KCN was quantified by a potentiometric assay utilizing a cyanide ion selective electrode (Fisher Scientific, Hampton, NH) and a KCN standard solution (Alfa Aesar, Haverhill, MA). Standard curve data (for 3 concentrations with one replicate each) were entered into Microsoft Excel 2010, and a least squares linear regression was obtained. A slope between –70 and –50 mV/Log(KCN mg/L), and r-squared values between 0.95 – 1.00 were deemed optimal for an accurate concentration reading. No assumptions were tested.

Dimethyl trisulfide (DMTS), Span 80, and Tween 80 were obtained from Sigma-Aldrich (St. Louis, MO). The DMTS antidotal treatment formulation was prepared by adding 2 g DMTS to a surfactant mixture of 0.75 g Span 80 and 2.25 g Tween 80. The mixture was then vortexed, yielding a 40% DMTS solution by weight (440.5 mg/mL). The solution was stored in glass vials and delivered via IM injection in the caudal thigh using glass syringes.

General Animal Handling

In an effort to increase familiarity with experimental manipulations and to minimize stress, mice that were to undergo HCN inhalation were initially acclimated to the nose-only restraint tubes (CH Technologies, Westwood, NJ) used for the inhalation exposure. During acclimation, mice were inserted into restraint tubes (Figure 1A) and monitored for time periods of increasing durations. Durations began at 5 minutes, and increased by 5 minutes

each acclimation period, until a maximum of 15 minutes was reached. Animals were acclimated twice a day for 2 days prior to exposure.

Following cyanide exposure in both models (KCN, HCN), animals were singly housed for 60–90 minutes while being monitored for signs of cyanide intoxication post-exposure. The presence or absence of toxic signs was recorded, including, but not limited to, respiratory distress (i.e., gasping, labored breathing), lethargy, loss of righting ability, tremors, convulsions, and death. Death was defined as apnea without further respiratory effort and loss of palpable cardiac pulsation. Recovery was defined as the ability to ambulate in the absence of observable toxic signs. Surviving animals were returned to their home cage once toxic signs ceased. Animals that survived 24 hours post-experimental procedures were euthanized.

All animal research was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals. Experiments adhered to principles stated in the Guide for the Care and Use of Laboratory Animals, National Research Council, published by the National Academy Press, 2011, and the Animal Welfare Act of 1966, as amended. The study protocol was approved by the Institutional Animal Care and Use Committee, United States Army Medical Research Institute of Chemical Defense (USAMRICD), Aberdeen Proving Ground, MD.

Determination of DMTS LD₅₀

A total of 12 age-matched unanesthetized male mice were challenged with DMTS via IM injection in the left caudal thigh and monitored for toxic signs. The median lethal dose, LD₅₀, of DMTS toxicity in mice was determined via the up-and-down procedure (UDP) sequential testing method [34]. Starting with a dose of 400 mg/kg DMTS, the dose of each successive animal was adjusted either up or down, depending on the outcome of the previous animal. If the previous animal survived, the successive dose in the next subject was increased at 0.1 log unit interval (Table S1). If the animal died, the successive dose was decreased at 0.1 log unit interval. The stopping criterion consisted of 5 reversals in responses. A total of 9 animals were used to achieve the stopping criterion for the DMTS LD₅₀ dose determination. To further characterize the observed clinical signs at specific doses following the LD₅₀ dose determination, 3 additional mice were challenged with DMTS at various doses and examined for the presence or absence of clinical signs within a 24-hour period following exposure.

Paradigm I. Exposure of Mice to Subcutaneous Injection of Potassium Cyanide

Unanesthetized male CD-1 mice were injected subcutaneously with target dosages (5.0–38.1 mg/kg) of KCN solution. Immediately after (within 1 minute sequentially following KCN challenge), mice were administered antidote (DMTS) or vehicle (Span 80/Tween 80 surfactant mixture) via IM injection in the left caudal thigh using a 27–28 gauge, 1” length needle. DMTS antidote doses ranged from 50 mg/kg – 300 mg/kg (0.398 mmol/kg – 2.386 mmol/kg). To generate full dose-response curves, a total of 160 animals were used (30–36 animals per DMTS dose tested, and 30 animals treated with vehicle control).

For the survival and clinical signs study, 13 unanesthetized male mice were injected subcutaneously with 8.0 mg/kg KCN solution. Immediately after (within 1 minute of KCN challenge), mice were administered 100 mg/kg DMTS or vehicle control and monitored for clinical signs. Clinical signs as a function of treatment condition were recorded for 24 hours post-exposure or until recovery (absence of toxic signs). Percent incidences of clinical observations in all animals were recorded as a ratio of total survivors at each time point.

Paradigm II. Exposure of Mice to Inhaled Hydrogen Cyanide

Evaluation of inhalation injury and efficacy of DMTS was accomplished in age-matched unanesthetized mice administered HCN via inhalation. Two models of HCN inhalation were examined in this study: continuous and discontinuous. *Continuous model:* The mice were exposed for 10 minutes continuously to a fixed dose of HCN before removal for antidote administration. *Discontinuous model:* The mice in the discontinuous model were exposed in two stages consisting of an initial 10-minute HCN exposure and a second 30-minute exposure to HCN inhalation, for a total of 40 minutes exposure time; between the stages a 2-minute break occurred wherein the animals were administered countermeasure treatment (or vehicle). Clinical signs were compared as a function of treatment condition. Percent incidences of clinical signs at each time point were determined based on the surviving animals only (i.e., not including observations from animals that died).

Set-Up of HCN Inhalation Apparatus

A representative depiction of our inhalation system is illustrated in Figure 2. An in-line Fourier transform infrared spectrometer (FTIR) (CR4000, Gasmeter Technologies, Helsinki, Finland) generated an infrared absorption spectrum of the gas mixture, which was used to quantify the concentration of HCN flowing through the chamber. An air compressor (Jun-Air, Benton Harbor, MI) was turned on and set to 30 psi to ensure sufficient air flow through the apparatus. LabVIEW (National Instruments, Austin, TX) software was used to set volume of air, not to exceed 95% of the total capacity, to fill a 50 L Tedlar bag (DuPont Tedlar, Buffalo, NY) with a septum port. The total volume of air was regulated via the software and mass flow controllers (MFCs) (SLA5850, Brooks Instruments, Hatfield, PA). After the bag was filled with a set volume of air (e.g., 37.4 L), a sample of HCN gas of desired volume (e.g., 600 mL) was pulled by a gas-tight syringe (Hamilton, Reno, NV) from an HCN cylinder (2% in N₂) (Custom Gas, Durham, NC) and then manually injected via the septum port on the Tedlar bag containing the pre-dispensed volume of dilution air. The HCN cylinder was plumbed to one end of a short, pass-through gas sample tube, the other end of which was connected to the exposure system exhaust and an M18 charcoal canister. After a 5-minute equilibration period, mice in restraint tubes (Figure 1A) were inserted into the exposure chamber (Figure 1B). The exposure system was built around the Highly Toxic Agent System (CH Technologies, Westwood, NJ) which allows for nose-only exposure of up to 12 small animals simultaneously (Figure 1). Gas mixture test atmospheres from the Tedlar bag were pulled through the exposure system under vacuum (to reduce the risk of HCN leaking from unsecured connections in the lines) into the in-line FTIR to quantify HCN concentration in the test atmosphere every 5 seconds.

Exposures were operated by the custom-built LabVIEW program. This software controlled the timing of the exposure stages, volumetric flow rates through mass flow controllers and switching of solenoids. Three mass flow controllers were operated by LabVIEW through a four-channel control panel (0154, Brooks Instruments, Hatfield, PA). These units controlled the flow of compressed air into the Tedlar bag, the flow of test atmospheres through the exposure system, and the purging of HCN from the system after an exposure was completed. Once an exposure was complete, flow from the Tedlar bag was sealed and ambient air was pulled through the exposure system for 60 seconds to remove any residual HCN. Once the animals were removed from the exposure system, the Tedlar bag was purged and rinsed with air before the next test atmosphere was generated. Mouse restraint tubes were airtight, to prevent dilution of test atmospheres with ambient air in the hood. Test atmospheres were pulled through the exposure system at 0.5 L/min. For these inhalation studies, toxicity was expressed as the LC₅₀, the product of the concentration (C; ppm) of the cyanide and the length of exposure (time; min) at which 50% of the animals die.

All gas-handling portions of the exposure system were housed within a certified chemical fume hood. Personnel operating the apparatus wore HCN monitors at all times (Dräger Pac 7000 HCN, Dräger Safety, Lübeck, Germany).

Data Analysis

DMTS LD₅₀ determination.—The LD₅₀ dose with the estimated 95% confidence interval for DMTS was calculated after 5 reversals using software algorithms described in “Implementation of Dixon & Massey UDP,” *Introduction to Statistical Analysis*, 1983, pp 434–438.

KCN Survival Analysis.—KCN data presented as Kaplan-Meier survival curve were plotted and analyzed by a log rank (Mantel-Cox) test with Prism 5 Software (version 5.04; GraphPad, La Jolla, CA). Probability (*p*) values less than 0.05 were considered significant.

Stage-wise KCN and HCN LD₅₀/LC₅₀ determination.—As the discontinuous model of HCN exposure (used in Paradigm II) cannot be accurately expressed as an LC₅₀ value, results from both the continuous and discontinuous HCN models were expressed as LC₅₀ values for the purposes of consistency. The LD₅₀ and LC₅₀ estimations for subcutaneously injected KCN and inhaled HCN, respectively, were determined using the stage-wise adaptive dose method [35], the R-package drc (<https://CRAN.R-project.org/package=drc>), and the SAS PROBSEP program [36]. LD₅₀ and LC₅₀ values with 95% confidence interval (CI) were determined using probit regressions. After each stage, probit dose response models using maximum likelihood methods were fitted to the combined data from all stages as a function of the base 10 logarithms of KCN concentration (mg/kg). The stopping criterion is defined as when half of the width of the LC₅₀ 95% confidence interval to the LC₅₀ drops below 0.4. This is represented as:

$$\begin{aligned} & (95\% \text{ upper confidence interval of the LC50} \\ & - 95\% \text{ lower confidence interval of the LC50}) \div (2 \times \text{LC50}) \leq 0.40 \end{aligned}$$

Results

Paradigm I. Determination of DMTS Dose and Antidotal Efficacy of DMTS in Potassium Cyanide Injection Model of Cyanide Poisoning.

To guide the selection of appropriate DMTS doses in efficacy studies, and to provide an estimate of the safety margin of DMTS, we established the LD₅₀ of DMTS in CD-1 mice to be 598.5 mg/kg (95% CI 477.6 – 750.0 mg/kg) (Table S1). Survival was determined at 24 hours post-injection. Clinical signs were observed continuously for 120 minutes post-injection and at 24 hours. The presence or absence of clinical signs at doses above 400 mg/kg is shown in Table S2. Based upon the estimated median lethal dose (LD₅₀), as well as the presence of toxic signs, we determined the maximal dosage of DMTS to be 300 mg/kg for subsequent efficacy studies. To determine DMTS efficacy against CN challenge using a KCN injection mouse model, mice were exposed to KCN subcutaneously followed immediately by various doses of DMTS (50, 100, 200 and 300 mg/kg). The adaptive dose-response method was used to establish the LD₅₀ of KCN at each dose of DMTS. The LD₅₀ of KCN toxicity in mice in the absence of CN antidote has been previously determined to range from 8.5–12 mg/kg [37, 38], and we established the KCN LD₅₀ in our laboratory to be 8.0 mg/kg KCN (95% CI 6.8–9.4) (Figure 3). The antidotal protection ratio (APR; defined as the ratio of the LD₅₀ of KCN in the presence of DMTS countermeasure to the LD₅₀ of KCN alone) was also calculated for each DMTS dose. The APR of the 50 mg/kg dose of DMTS was determined to be 1.76 (LD₅₀ of 14.1 mg/kg, 95% CI 12.5 – 15.9 mg/kg). A dose of 100 mg/kg DMTS resulted in an APR of 2.21 (LD₅₀ of 17.7 mg/kg, 95% CI 14.7 – 19.9 mg/kg). The 200 mg/kg dose of DMTS resulted in an APR of 3.73 and was considered the most efficacious dose (LD₅₀ of 29.8 mg/kg, 95% CI 25.5 – 34.9 mg/kg). A DMTS dose of 300 mg/kg was also tested to determine if efficacy further improved with increasing concentrations, resulting in an APR of 3.4 (LD₅₀ of 27.2 mg/kg, CI 95% 24.2 – 30.6 mg/kg). As this dose did not provide further efficacy improvement, we determined that 200 mg/kg DMTS provided the greatest protection against subcutaneously injected KCN toxicity. For each DMTS treatment dose evaluated in our study, a dose-response curve was generated (Figure 3).

To examine the capacity of DMTS to ameliorate adverse clinical signs commonly associated with cyanide toxicity (including respiratory distress, convulsions, tremor, etc.), observations were recorded following KCN challenge at the LD₅₀ dosage (8.0 mg/kg) in the presence or absence of DMTS countermeasure treatment. In this evaluation, we used 100 mg/kg DMTS, administered IM. Observations for toxic signs were noted for 24 hours post-challenge, though none of the surviving animals displayed toxic signs, and all survivors appeared normal by 90 minutes post-challenge (Figure 4). Initial signs observed in the vehicle control group included primarily lethargy and/or loss of righting reflex, with respiratory distress observed in 3/13 mice, and convulsions observed in 5/13 mice (Figure 4A). By 5 minutes post-challenge, 8/13 mice had lost the ability to right themselves. In some instances, animals were observed ambulating intermittently with long periods of lethargy between periods of activity. In such cases, the animals were scored with the presence of both. After 60 minutes, the majority of the group had recovered (no observable toxic signs) and were ambulating without issue. During the course of observations, 5/13 animals died, 13/13 animals became

lethargic, and 11/13 lost the righting ability (Table 1). Animals that were challenged at the same LD₅₀ dose of KCN and then treated with 100 mg/kg DMTS demonstrated a markedly increased rate of survival, with only 1 out of 13 animals dying at 5 minutes post-challenge (Table 1). Initial signs observed in the treated group were predominantly lethargy (11/13 mice), with 4 of 13 mice experiencing respiratory distress (Figure 4B). The adverse clinical signs observed in the treated group were completely resolved by 30 minutes post-challenge, with the majority of mice (9/13) demonstrating alertness and ambulating without issue within 10 minutes of treatment. No animals were observed gasping at any point following DMTS treatment, and only 1 of 13 mice experienced convulsions and loss of righting reflex, which is the animal that died (Table 1).

Paradigm II. Characterization of Acute Cyanide Inhalation Intoxication in Unanesthetized Mice.

Two inhalation exposure models were employed (continuous or discontinuous) to simulate different exposure scenarios. In the continuous exposure model, mice were exposed to 10 minutes of HCN without prophylactic antidotal treatment. This exposure was designed to model a scenario in which a person receives a high concentration of HCN and is able to be removed from the area after 10 minutes, upon arrival of emergency medical personnel. The LC₅₀ over a 10-minute continuous exposure was determined to be 421 ppm (95% CI 394–451 ppm) (Figure 5A). The discontinuous exposure paradigm is designed to model a scenario in which a patient or first-responder is exposed to acute cyanide intoxication in an enclosed public area, such as an airport, with 10 minutes required for emergency medical personnel to arrive and administer antidote, and another 30 min required to evacuate the victims from the cyanide intoxication area. The mice in the discontinuous model were exposed to HCN in two stages, first for 10 minutes and then for an additional 30 minutes, resulting in a total exposure time of 40 minutes; between these HCN exposures stages, the animals were administered either countermeasure treatment or vehicle control (Figure 5B). The LC₅₀ over a 40-minute time period was calculated as 324 ppm (95% CI 292–360 ppm) (Figure 5B).

To evaluate the recovery of animals following HCN exposure, surviving mice were examined for the presence or absence of clinical signs of toxicity for up to 24 hours after HCN exposure. The presence of adverse clinical signs in the survivors was recorded in the continuous HCN exposure model, following 10-minute continuous exposure of HCN in the absence of antidotal treatment (Figure 6A). Of 20 mice exposed, 11 survived (55%), with all mortality events occurring either during the HCN exposure or within 2 minutes of removal from the HCN. Of 11 survivors, 82% were immediately observed displaying respiratory distress (gasping, labored or agonal breathing) and loss of righting reflex. Tremors were observed in 73% of the survivors (8/11 mice). Additionally, convulsions were observed in 36% of surviving mice (4/11) within the first 2 minutes following exposure. At 15 minutes post-challenge, 91% (10/11) of surviving mice had once more become ambulatory, though 45% (5/11) were still observed as lethargic, and one subject (1/11) was still observed with tremors/convulsions at that time. At 45 minutes post-challenge, all survivors were observed as normal, with no clinical signs of distress.

Mice exposed to the discontinuous HCN paradigm were observed to have a longer recovery time than those exposed for only 10 minutes (Figure 6B). At the LC₅₀ dose of cyanide (324ppm), 12 of 24 animals survived. All instances of death occurred during the discontinuous HCN exposure (both 1st and 2nd stages) or within 1 minute post-exposure. Among surviving animals, initial clinical signs observed included a loss of righting reflex (10/12), respiratory distress (9/12), tremor (2/12), and lethargy (2/12). Convulsions were observed in 5/12 and 4/12 of surviving mice at 5 and 10 minutes post-exposure, respectively. Beyond that time, no further instances of convulsions were noted. High incidences of respiratory distress persisted through 15 minutes post-exposure. At 30 minutes post-exposure, 3/12 of the surviving mice continued to exhibit respiratory distress. All surviving animals were observed ambulating at 30 minutes post-challenge, 9/12 were observed with lethargy at that time, and 8/12 remained lethargic at 45 minutes post-challenge. All mice were observed to have fully recovered at 60 minutes post-challenge.

Efficacy of DMTS in Inhalation Model of Cyanide Poisoning

The antidotal efficacy of DMTS was assessed against HCN inhalation using both the continuous and discontinuous exposure paradigms. Because the efficacy against KCN challenge was not significantly increased between the 200 mg/kg and 300 mg/kg doses of DMTS (Figure 3), only DMTS doses of 100 mg/kg and 200 mg/kg were further pursued.

Clinical signs were assessed for both exposure paradigms following DMTS treatment and compared to the clinical signs observed in vehicle control groups (Figure 6A-D). Briefly, animals that were treated with 200 mg/kg DMTS following 10-minute continuous HCN regained righting ability at a faster rate (Figure 6A, C). Mice that were exposed to the LC₅₀ dose of HCN for the 40-minute discontinuous paradigm resolved toxic signs at approximately 60 minutes post-challenge. However, the addition of 200 mg/kg DMTS antidote treatment shifted the average recovery time to 30 minutes post-challenge (Figure 6). Additionally, mice that received 200 mg/kg DMTS treatment displayed a reduction in the incidence of convulsions (0/11 survivors), tremors (2/11), and loss of righting reflex (0/11). The majority of mice receiving treatment became ambulatory within 2 minutes post-challenge (10/11). Mice that were treated with 100 mg/kg DMTS displayed incidences of toxic signs and recovery time similar to those in mice that received 200 mg/kg (data not shown).

To examine the ability of DMTS treatment to improve survival, groups of 12 mice were exposed to the LC₅₀ dose for a 10-minute continuous HCN inhalation (421 ppm) and were administered either vehicle control or 200 mg/kg DMTS via IM injection. Of the 12 animals in each group, 7 (58%) survived following HCN challenge in the absence of antidote, and 11 (92%) survived in the presence of 200 mg/kg DMTS (Table 2). Similarly, groups of 12 mice were exposed to the LC₅₀ dose for the 40-minute discontinuous HCN inhalation (324 ppm) and were administered vehicle control, 100 mg/kg DMTS, or 200 mg/kg DMTS via IM injection. Of the 12 mice, 6 (50%) survived in the vehicle control group, 11 (92%) survived in the 100 mg/kg DMTS dosage group, and 9 (75%) survived of those receiving 200 mg/kg (Table 2).

Discussion

DMTS is a sulfur-based molecule found in onions and garlic and is generally recognized as safe by the FDA. A previous formulation of DMTS was demonstrated to be an effective candidate countermeasure against a KCN model of cyanide toxicity, but was limited in its use for intramuscular injection by the relatively high administration volume necessary for efficacy [33]. Here we examine a newer, more concentrated DMTS formulation, which may be more suited to intramuscular injection, and demonstrate its efficacy against a KCN model of toxicity, as well as two models of HCN inhalation toxicity. Future work on DMTS as a novel cyanide antidote will include more extensive characterization of the safety of the countermeasure and will directly compare the efficacy of this more concentrated formulation to other known cyanide countermeasures. Based on these inhalation survival and toxic signs data, this current formulation of DMTS shows promise as a countermeasure for cyanide poisoning.

Testing new therapeutics for cyanide intoxication necessitates the development of multiple well-characterized models of cyanide toxicity. These studies establish two well-controlled HCN inhalation models which address real-world scenarios in which removal from the cyanide intoxication source can happen almost immediately or be delayed for an extended duration before evacuation. These divergent HCN inhalation models may be useful in the development of appropriate therapeutics and therapeutic dosages. We examined the use of these models to determine the antidotal efficacy of DMTS at multiple doses. The survival rates in both models were improved, while the presence of toxic signs was ameliorated, with the addition of DMTS treatment.

Though we observed improved efficacy when we increased the DMTS dose to from 100 mg/kg to 200 mg/kg in our subcutaneous KCN injection model (Figure 3), a higher percentage of animals survived an HCN challenge when administered 100 mg/kg DMTS than those that received 200 mg/kg DMTS (Table 2). This discrepancy could be due to the inherent differences represented in the two paradigms of cyanide toxicity of this study (subcutaneous administration compared to inhalation challenge). For instance, subcutaneous KCN administration of a single bolus injection results in rapid lethality, necessitating high amounts of countermeasure. Because the discontinuous HCN model described (Figure 5B) is designed to reflect a slower administration, more emulative of a real-world scenario of cyanide poisoning, a lower effective DMTS dose may more accurately represent the required dose. Animals exposed to our model of HCN via inhalation tended to experience higher incidences of toxic signs than those exposed to KCN and to exhibit faster clinical recovery (Figures 4 & 6). This may be due to the rapid penetration of HCN into both aqueous and lipid tissue components, allowing for more efficient absorption [39, 40]. The HCN inhalation exposure system model presented here has the benefit of generating the target exposure concentration of HCN gas nearly instantaneously and in a well-controlled manner. The flexibility of this system allows for administration of countermeasure before, immediately following, or during a brief interruption in inhalation exposure, which can provide the benefit of testing multiple parameters of a countermeasure (i.e., its efficacy immediately post-exposure, or its ability to provide continuing protection while cyanide exposure starts, is briefly paused, and restarts, as with our discontinuous exposure

paradigm). A similar discontinuous method has been described previously [41], using HCN gas generated by injecting KCN into a beaker containing sulfuric acid within the exposure chamber. However, this method requires time to achieve desired HCN chamber concentration. Our models provide the additional benefit of utilizing unanesthetized animals. Although this study was limited in scope by the use of fasted animals, which may not necessarily be representative of a real-world toxic gas exposure situation, experiments in progress have focused on addressing this issue.

Our described exposure model is a flexible system and can be appropriately modified for larger animal models (including rats and rabbits) and other gases, as well as to accommodate the delivery of countermeasures before, midway through, or after exposure. Though the current studies presented here do not reflect the respiratory parameters of the animals for the duration of the inhalation exposures, monitoring of respiratory parameters using real-time plethysmography equipment and modified restraint tubes will be employed in future work.

Conclusion

This study demonstrates that DMTS is an effective cyanide antidote in a rodent model for cyanide poisoning. The use of this rodent model in an acute toxicity study provides a relatively inexpensive screen that can be used to greater extent in this and other animal models to examine antidotal efficacy. We present two separate reproducible models of HCN intoxication in adolescent male CD-1 mice. The models demonstrate the rapid and transient onset of effects of HCN in surviving mice, and present the potential to study administration of antidotal treatment before, immediately following, or midway through controlled HCN exposure concentrations. Based on the rapid toxicity of HCN observed, antidotal treatment would need to be initiated as soon as possible for optimal results. We demonstrated that this DMTS formulation treatment is easily administered intramuscularly and protects effectively against HCN inhalation challenge. The effectiveness of DMTS in these scenarios suggests that DMTS is applicable in a mass casualty cyanide exposure setting.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
(a) Mouse restraint tube; (b) rodent inhalation exposure chamber equipped with a total of 12 exposure ports for individual mouse exposure attachments.

Figure 2.

Linear diagrammatic sketch of the inhalation exposure apparatus. Step 1. Air-tight syringe is filled with HCN and injected into Tedlar bag containing air. Step 2. HCN/air mixture pulled through MFCs into FTIR to confirm HCN concentration prior to entering exposure chamber. Step 3. Exposed HCN is pulled through charcoal filter.

Figure 3.

Dose-response curves of subcutaneous KCN in the presence of vehicle control (25% Span80, 75% Tween 80) or DMTS at 50 mg/kg (0.398 mmol/kg), 100 mg/kg (0.795 mmol/kg), 200 mg/kg (1.590 mmol/kg), and 300 mg/kg (2.39 mmol/kg) ($n = 30-36$ male mice per DMTS dose). Treatments administered via IM injection. Survival recorded at 24 hours post-exposure.

Figure 4.

Survival and clinical signs in male CD-1 mice following subcutaneous injection of 1 x LD₅₀ dose of KCN without and with DMTS. (a) Immediately following 8 mg/kg KCN challenge, mice were administered either 100 mg/kg DMTS (gray squares) or vehicle control (black circles) via IM injection (13 animals per condition). The difference between vehicle-treated mice and DMTS-treated mice was significant by a log rank test. (b,c) Clinical signs in both were recorded for 24 hours post-challenge (both survivors and nonsurvivors represented). (b) Animals administered vehicle control (25% Span 80, 75% Tween 80). All surviving animals demonstrated no observable toxic signs at 90 minutes post-challenge. (c) Animals administered 100 mg/kg DMTS. All surviving animals demonstrated no observable toxic signs at 30 minutes post-challenge ($n = 7-13$ mice observed in each condition).

Figure 5.

Dose–response curve following (a) 10-minute continuous HCN nose-only inhalation exposure and (b) 40-minute discontinuous HCN nose-only inhalation exposure ($n = 3–7$ mice per data point). Corresponding representative exposure timelines shown below. LC₅₀: lethal concentration (50% population); ppm: parts per million; CI: confidence interval.

Continuous (10-min) HCN Inhalation Paradigm

Discontinuous (40-min) HCN Inhalation Paradigm

Figure 6.

Clinical signs observed following either 10-minute continuous HCN inhalation exposure at the LC50 for the continuous paradigm (421 ppm), or 40-minute discontinuous HCN inhalation exposure at the LC50 for the discontinuous paradigm (324 ppm). Following 10-minute continuous HCN inhalation exposure, animals were administered either (a) vehicle control or (c) 1.59 mmol/kg (200 mg/kg) DMTS via IM injection. During the 40-minute discontinuous HCN exposure, animals were administered (b) vehicle control or (d) 1.59 mmol/kg (200 mg/kg) DMTS via IM injection. All surviving animals demonstrated no observable toxic signs at 90 minutes post-challenge ($n = 11$ – 12 survivors observed per condition).

Table 1.

Clinical observations following KCN challenge with DMTS treatment (100 mg/kg). Data represented as presence of clinical sign observed during the 24-hour period post-exposure per mouse/total number of mice per group. More than one sign may have been observed per mouse. Recovery was defined as the absence of observable toxic signs. (*n*=13 male mice per treatment group.)

Observed Clinical Signs	Treatment	
	Vehicle Control	100 mg/kg DMTS
Survival ^a	7/13	12/13
Lethargy	12/13	13/13
Labored Breathing	6/13	5/13
Gasping	4/13	0/13
Loss of Righting Reflex	10/13	1/13
Convulsions	9/13	1/13
Tremor	4/13	4/13
Average Recovery Time (min)	48.2	12.5

^aSurvival at 24 h post-exposure

Table 2.**Survival Following LC₅₀ HCN Challenge with DMTS Treatment**

Percent survival following either HCN inhalation exposure at the LC₅₀ (421 ppm) for the 10-minute continuous paradigm or HCN inhalation exposure at the LC₅₀ (324 ppm) for the 40-minute discontinuous paradigm. Discontinuous exposure animals were treated and examined in separate studies and samples were pooled.

Exposure paradigm	Replicate #	Treatment		
		Vehicle Control	100 mg/kg DMTS	200 mg/kg DMTS
<i>Continuous (10-minute)</i>	1	7/12 (58.3%)*	-----	11/12 (91.7%)*
<i>Discontinuous (40-minute)</i>	1	8/13 (61.5%)*	7/8 (87.5%)*	-----
	2	6/12 (50.0%)*	11/12 (91.7%)*	9/12 (75.0%)
	Combined Numbers	14/25 (56.0%)	18/20 (90.0%)	9/12 (75.0%)