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Antidotal effects of methylene blue against cyanide neurological toxicity: *in vivo* and *in vitro* studies

Philippe Haouzi¹, Marissa McCann¹, JuFang Wang², Xue-Qian Zhang², Jianliang Song², Ilker Sariyer³, Diane Langford³, Maryline Santerre⁴, Nicole Tubbs¹, Annick Haouzi-Judenhers⁵, Joseph Y. Cheung^{2,6}

¹Division of Pulmonary and Critical Care Medicine, Department of Medicine, Pennsylvania State University, College of Medicine, Hershey, Pennsylvania.

²Center for Translational Medicine, Temple University School of Medicine, Philadelphia, Pennsylvania.

³Department of Neurosciences, Temple University School of Medicine, Philadelphia, Pennsylvania.

⁴Fels Institute for Cancer Research and Molecular Biology, Temple University School of Medicine, Philadelphia, Pennsylvania.

⁵Heart and Vascular Institute, Department of Medicine, Pennsylvania State University, College of Medicine, Hershey, Pennsylvania.

⁶Department of Medicine, Temple University School of Medicine, Philadelphia, Pennsylvania

Abstract

The aim of the present study was to determine whether methylene blue (MB) could directly oppose the neurological toxicity of a lethal cyanide (CN) intoxication. KCN, infused at the rate of 0.375 mg/kg/min intravenously, produced 100% lethality within 15 min in unanaesthetized rats ($n = 12$). MB at 10 ($n = 5$) or 20 mg/kg ($n = 5$), administered 3 min into CN infusion, allowed all animals to survive with no sequelae. No apnea and gasping were observed at 20 mg/kg MB ($P < 0.001$). The onset of coma was also significantly delayed and recovery from coma was shortened in a dose-dependent manner (median of 359 and 737 seconds, respectively, at 20 and 10 mg/kg). At 4 mg/kg MB ($n = 5$), all animals presented faster onset of coma and apnea and a longer period of recovery than at the highest doses (median 1344 seconds, $P < 0.001$). MB reversed NaCN-induced resting membrane potential depolarization and action potential depression in primary cultures of human fetal neurons intoxicated with CN. MB restored calcium homeostasis in the CN-intoxicated human SH-SY5Y neuroblastoma cell line. We conclude that MB mitigates the neuronal toxicity of CN in a dose-dependent manner, preventing the lethal depression of respiratory medullary neurons and fatal outcome.

Address for correspondence: Philippe Haouzi, MD, PhD, Division of Pulmonary and Critical Care Medicine, Department of Medicine, Pennsylvania State University, College of Medicine, 500 University Drive, H041, Hershey, PA 17033. phaouzi@pennstatehealth.psu.edu.

Competing interests

The authors declare no competing interests.

Keywords

cyanide; antidote; neurons; methylene blue

Introduction

The current strategy for treating acute cyanide (CN) intoxication relies on symptomatic measures, comprising ventilatory and circulatory support^{1,2} and specific antidotes.^{3–5} The latter can act through two different mechanisms¹: by “scavenging” the pool of free CN in the blood, using, for instance, cobalt-containing molecules^{2,6,7} or nitrite-induced methemoglobinemia^{8–11}-taking the advantage of the affinity of CN for “metals”-or² by increasing CN elimination as thiocyanate, using sodium thiosulfate^{9,12–14} or other sulfur donors, such as sulfanegen.^{15,16} These antidotes can be combined.

Methylene blue (MB) is a dye that was synthesized at the end of the 19th century.^{17,18} We have recently revisited the effects of MB, which was briefly proposed in the 1930s^{19–22} as a treatment of CN intoxication, and found that this molecule exerts potent antidotal effects against CN toxicity through a unique modality of action that results from its distinctive cyclic redox properties.^{23–25} These properties are currently used to treat the excessive formation of ferric iron in hemoglobin (methemoglobin), restoring the ability of hemoglobin to carry O₂ in patients with methemoglobinemia.^{17,18,26,27} After being administrated, MB is immediately reduced into its leucoform, leucomethylene blue (LMB),²⁸ by reducing agents present in blood and cells, such as NAD(P)H.²⁹ LMB, on the other hand, is reoxidized into MB by molecules with a higher redox potential (such as O₂ or most metallocompounds), giving up to two electrons in the process (Fig. 1), and a new cycle of reduction can be initiated.²⁹ Diffusing rapidly into the cytoplasm and in the mitochondria of any cells, including neurons,³⁰ the MB/LMB couple can restore the TCA cycle and the glycolytic activity by oxidizing NADH and decreasing the NADH/NAD ratio,³¹ rescuing the TCA cycle when inhibited by the accumulation of NADH, like for instance during an impediment of the electron chain activity by CN or H₂S intoxication.^{25,28} The reestablishment of the TCA cycle can, in turn, lead to the production of ATP via succinyl-CoA synthase-the mitochondrial substrate-level phosphorylation³¹-independently of the integrity of the mitochondrial ATPase activity. Also, MB/LMB redox properties appear to be able to restore the mitochondrial membrane potential, depressed by CN, through mechanisms that are not fully understood.^{25,28} Indeed, recent studies have shown a decrease, by MB, of postanoxic brain injury,^{32–36} as well as in various conditions associated with altered mitochondrial functions.^{37–41} It has been proposed that LMB could interact directly with the mitochondrial electron chain,⁴² providing electrons to the complexes I and III and allowing a partial resuscitation of the mitochondrial electron chain. Although such a mechanism would be difficult to reconcile with the effects of CN intoxication, which result, if anything, in a *reduction* of the electron chain complexes, MB does restore the mitochondrial membrane potential and decrease the production of reactive O₂ species in cells intoxicated by CN.^{24,25}

These effects translate into a drastic decrease in mortality following MB administration after lethal CN intoxication in the rat.^{24,25} This increased survival is mediated through the rescue

of cardiac contractility,^{23,43} an effect also observed with other mitochondrial poisons.^{28,44,45} Whether MB is also able to counteract or rescue the neurological manifestations of CN toxicity, which precede CN-induced cardiac arrest, remains an outstanding question. The acute neurological manifestations of CN intoxication clearly mimic those observed in hypoxia/anoxia, consisting of rapid coma and seizures with an early depression in medullary neurons producing apnea and gasping.⁴⁶ Neurological long-term sequelae are rare, ranging from minor cognitive dysfunctions to profound motor (including extrapyramidal syndrome), visual or memory deficits, akin to the effects of postanoxic injury.^{47,48} We have recently observed that rats with neurological deficits after acute CN intoxication presented lesions that affected areas of the brain similar to those found after ischemia: diffuse lesions of the hippocampus, cerebellum, cortex, and subcortical nuclei.⁴⁹ Lesions were only present in rats with a clinical deficit.

In this study, we sought (1) to determine if MB could alter or prevent CN-induced coma and apnea/gasping in awake rats, (2) to establish the doses of MB required to produce such an effect, and (3) to ascertain whether such a protective mechanism, if present *in vivo*, could be ascribed to a direct rescue of neurons by MB, that is, independently of any improvement in circulatory status. This was achieved by studying the effects of MB in two neuronal models: a primary culture of human fetal neurons or an established human SH-SY5Y neuroblastoma cell line exposed to CN toxicity, using concentrations of CN compatible with those observed in human intoxication (100 micromolar).

Materials and methods

In vivo experiments

Animal preparation.—A total of 32 male Sprague-Dawley rats (Charles River Laboratories) weighing 0.392 ± 109 kg were studied (see power analysis below). All experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals, 8th Edition (the National Research Council (United States) Institute for Laboratory Animal Research). The Penn State Hershey Medical Center Institutional Animal Care and Use Committee approved the study. The rats were housed at the Animal Resource Services at the Penn State College of Medicine, which conforms to the requirements of the U.S. Department of Agriculture and the Department of Health, Education and Welfare. Rats were provided with food and water *ad libitum*, on a standard 12-h (7 AM–7 PM) light/dark schedule, under the direct supervision of veterinarians.

Solutions.—Potassium cyanide (KCN; Sigma, ref. no. 60178) solutions were prepared at the concentration of 0.5 mg/mL (dilution in sterile saline) in keeping with our previous report.⁴⁶ We have previously observed that KCN infused in rats at the dose of 0.375 mg/kg/min would produce a sequence of symptoms that leads to depression in medullary neurons and death by cardiac arrest (CA) within less than 15 minutes.⁴⁶ In addition, an infusion of potassium chloride (KCl) at the same concentrations of KCN⁴⁶ had no measurable effects. MB (10 mg/mL, Akron) was diluted in sterile saline (5–1 mg/mL), to keep the volume of saline constant (2 mL) regardless of the dose of MB that was used.

Protocol and data analysis.—On the day of the study, rats were briefly sedated (3.5% isoflurane) for about 15 min to place a heparinized, double lumen, venous catheter in the dorsal vein of the tail. Two to three hours were given for full recovery before doing the study.

KCN was infused intravenously using a high-precision infusion pump (Fusion 100; Chemyx Inc; Stafford, TX) at the rate of 0.375 mg/kg/min for 13 minutes. The duration of exposure was selected based on the fact that shorter exposures, using the same rate of CN infusion, were unable to lead to a lethal outcome. More specifically, when 0.375 mg/kg for 7 min ($n = 3$, total dose 2.6 mg/kg) was used, the coma was always reversible. Also, exposure up to 10 min at the same rate (total dose 3.7 mg/kg) was not always lethal (one out of three animals). The total dose of 4.8 mg/kg (infused over 13 min) was always lethal and was, therefore, chosen for our present study.

Intravenous CN infusion allows reproducible and stereotyped sequences of symptoms consisting of (1) a short phase of locomotor agitation and hyperventilation followed by (2) a period of general weakness leading to (3) a coma, which we define by the loss of righting reflex, often associated with (4) seizing; the coma is rapidly followed by (5) a period of apneas (cessation of visible breathing activity for more than 3 seconds) and gasping (large breathing activity associated with grunting sounds) leading to (6) a CA defined by the disappearance of the perception of any cardiac pulsation^{1,46} and complete whitening of the pupils.

MB was infused 3 min after the onset of CN infusion, that is, when the first neurological symptoms developed (agitation, weakness, and ataxia). Three different doses were used in keeping with doses we have previously used in rats, that is, 4, 10, or 20 mg/kg^{24,25,28,44,45,50,51} for 5 minutes. The rationale for the doses of MB is developed in the Discussion section, and these doses, which correspond to a range of doses, currently recommended for the treatment of methemoglobinemia in humans,^{17,18} in keeping with allometric considerations.⁵² In addition, in a separate group of animals, MB was infused at the dose of 20 mg/kg, but over 10 minutes. Animals were monitored every 15 seconds for 20 min, then every minute for the following hour in the surviving rats. All surviving animals were examined and weighed every day for 2 weeks.

Sample size estimation was established as follows: Mortality was our primary outcome; since the model is always lethal, 80% survival in the MB-treated group would require five animals per group to identify a significant effect (alpha 0.05, power of 80%), while three animals per group would be enough if the treated group allowed 90% survival. The study was initially designed with five animals per group. Our secondary outcomes were the onset of each of the following symptoms: 1-agitation, 2-general weakness or ataxia, 3-coma, 4-seizures, 5-apnea and gasping, and 6-asystole. The duration of coma of the animals that survived the intoxication was determined. Data are presented as median and range. Comparison between groups was done using nonparametric statistics (Mann-Whitney *U* test for two-group comparisons).

Comparisons between the effects of the doses were performed using the Kruskal-Wallis test for multiple comparison and the Mann-Whitney *U* test for two-group comparisons. Statistical analyses were done with GraphPad Prism® 6 (Graphpad Software, La Jolla, CA). $P < 0.05$ was regarded as significant for any of these comparisons.

In vitro experiments

Action potential measurement in primary human fetal neuron cultures.—

Human fetal neuron cultures were obtained from the Basic Science Core I of the Comprehensive NeuroAIDS Center at Temple University Lewis Katz School of Medicine. Neurons were isolated and cultured as described previously.⁵³ Briefly, primary neurons were prepared directly from fetal human brains by differentiation. Fetal human brain cells were plated in culture dishes coated with laminin and poly-L-ornithine. Cells were differentiated into neurons by neuronal differentiation media consisting of neurobasal media supplemented with B27, glutamax, recombinant human brain-derived neurotrophic factor, glial-derived neurotrophic factor, and penicillin/streptomycin for up to 4 weeks. Neurons were characterized by microtubule-associated protein 2 and synaptophysin immunostaining. Neuronal differentiation and function were monitored electrophysiologically by multielectrode arrays measuring local field potentials weekly in culture. The medium was changed every 3 days.

Action potential (AP) was measured in neurons after 2 weeks in culture with whole-cell patch clamp. Fire-polished pipettes (tip diameter 2 μm) with resistances of 5–6 M Ω when filled with pipette solutions were used. Pipette solution consisted of (in mM) 125 KCl, 4 MgCl₂, 0.06 CaCl₂, 10 HEPES, 5 K⁺-EGTA, 3 Na₂ATP, and 5 Na₂-creatine phosphate (pH 7.2). The external solution consisted of (in mM) 132 NaCl, 5.4 KCl, 1.8 CaCl₂, 1.8 MgCl₂, 0.6 NaH₂PO₄, 7.5 HEPES, 7.5 Na⁺-HEPES, and 5 glucose, pH 7.4. Phosphate-buffered saline (PBS) or NaCN (100 μM) was added at time zero, and AP was measured at 10 minutes. In another group of neurons, MB was added 3 min after NaCN and AP was measured at 10 minutes. APs were recorded using the current clamp configuration at 1.5 $\times$ threshold stimulus, 0.5-ms duration, and at 30 °C.^{54–57}

[Ca²⁺]_i measurement in differentiated SH-SY5Y neuroblastoma cells.—

SH-SY5Y cells were plated at the low confluence in a 1:1 mixture of Ham's F12 and Dulbecco Modified Eagle Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 2 mM glutamine, in a humidified atmosphere of 5% CO₂ in the air at 37 °C. After 24 h of cell plating, differentiation into neuron-like phenotype was triggered by exposure to 10 μM retinoic acid (RA) in DMEM containing 1% FBS for 10 days. DMEM-containing RA was changed every 3 days to replenish RA in culture media. Under these conditions, SH-SY5Y cells expressed the neuronal marker neuron-specific enolase by 4 days and persisted to 10 days in culture⁵⁸ at which time cells were used for [Ca²⁺]_i measurements. Fura-2-loaded (0.67 μM fura-2 AM, 15 min) differentiated SH-SY5Y cells on glass coverslips were incubated in media containing (in mM) 87 NaCl, 40 KCl, 1.25 CaCl₂, 1.2 MgCl₂, 1.2 KH₂PO₄, 20 HEPES, and 5.6 glucose, pH 7.4. Differentiated SH-SY5Y cells were exposed to excitation light (360 and 380 nm) only during data acquisition, and epifluorescence (510 nm) was recorded. PBS (CTL) or NaCN (100 μM) was added at

time zero, and $[Ca^{2+}]_i$ was measured at 0, 2, 5, and 10 minutes. In another group of neurons, MB was added 3 min after NaCN and $[Ca^{2+}]_i$ was measured at same time points. Daily calibration of fura-2 signals to convert fura-2 fluorescence intensity ratios (F_{360}/F_{380}) into $[Ca^{2+}]_i$ values was performed as previously described.^{59–63}

Data analysis.—All results are expressed as the means $\pm$ standard error of the mean. For the analysis of $[Ca^{2+}]_i$ as a function of group (control, NaCN, and MB+NaCN and time), a two-way ANOVA was used. For the analysis of AP parameters, a one-way ANOVA was used. A commercially available software package (JMP® version 14 Pro, SAS Institute, Cary, NC) was used. In primary analyses, $P = 0.05$ was taken to be statistically significant. In subgroup analyses, the Bonferroni correction ($P = 0.05/3$ or $P = 0.0167$ was deemed significant since there were three subgroup comparisons) was applied.

Results

Time course of the effects of CN in unanaesthetized rats (n = 12)

As illustrated in Figure 2, the infusion of CN produced a rapid change in behavior consisting of an increased locomotor activity, typically associated with a phase of hyperventilation, starting 194 seconds (median) after the onset of infusion (range 150–212 seconds). More than 1 min later, 238 seconds (range 239–306 seconds) into CN infusion, all animals started to present some difficulty walking, which led to a coma ~6 min into the exposure (386 seconds, range 328–461 seconds). This coma was followed by seizures at 403 seconds (range 310–555 seconds), and then all the animals became apneic, with a typical gasping pattern 516 seconds (range 401–623 seconds) into CN exposure. Cardiac pulsations disappeared with a clear whitening of the pupils at 783 seconds (range 706–946 seconds).

Effects of MB 20, 10, and 4 mg/kg

Responses were similar at 20 mg/kg ($n = 5$) and 10 mg/kg ($n = 5$), showing a dramatic change in the neurological outcomes and survival. As displayed in Figure 3, none of the treated animals presented an apnea or a gasping pattern reflecting the absence of depression of medullary respiratory neurons at either dose. The time to coma was significantly delayed and of briefer duration at the highest dose of MB with a median of 359 and 737 seconds for 20 and 10 mg/kg MB, respectively. Twenty milligrams per kilogram of MB, whether infused over 10 or 5 min, produced the same effects. Individual data are shown with P values in Figure 3. All the animals survived, and none of them presented any specific issue with feeding or behavior after recovery from the coma, except for a somnolence that lasted for less than 2 hours. MB, at 4 mg/kg, still had a significant impact on the development of the neurological toxic effects of CN, and the animals survived the exposure (Fig. 3); however, the time to coma and apnea was significantly shorter than at the dose of 10 or 20 mg/kg (Fig. 4). The duration of coma was four times longer than for the highest dose of MB as shown in Figure 4. Also, in the group that received the lowest doses of MB, one animal displayed difficulty walking after the exposure that did not resolve within 24 h following the end of CN exposure, requiring the euthanasia of the animal. Finally, a transient, but significant, loss of weight was observed for 1 day following CN exposure and treatment, and then all the animals regained weight at a rate ranging from 3 to 6 g per day.

Effects of NaCN on AP in human embryonic neurons and effects of MB

Compared with control human embryonic neurons treated with PBS, exposure to NaCN (100 μ M) for 10min resulted in E_m depolarization from -70.5 ± 0.7 to -59.1 ± 0.5 mV ($P < 0.0001$), reduction in AP amplitude from 108.6 ± 1.7 to 90.8 ± 1.0 mV ($P < 0.0001$), and prolongation of AP duration at 100% repolarization (APD_{100}) from 1.78 ± 0.06 to 2.69 ± 0.15 ms ($P = 0.0007$) (Fig. 5). Compared with neurons exposed to NaCN, MB (20 μ g/mL) added 3 min after NaCN significantly repolarized E_m ($P < 0.0001$), increased AP amplitude ($P = 0.0005$), and shortened APD_{100} ($P = 0.0014$) (Fig. 5) so that there were no longer any significant differences when compared with AP parameters measured in control neurons (E_m , $P = 0.1708$; AP amplitude, $P = 0.0623$; and APD_{100} , $P = 0.0427$).

NaCN increases $[Ca^{2+}]_i$ in differentiated SH-SY5Y neuroblastoma cells; effects of MB

Depolarization of differentiated SH-SY5Y cells by incubation in high K^+ (40 mM) media did not result in increases in $[Ca^{2+}]_i$ over 10 min of observation (Fig. 6). Addition of NaCN (100 μ M) in high K^+ media caused progressive increases in $[Ca^{2+}]_i$ ($P = 0.0095$; group $\times$ time interaction effect) (Fig. 6). When added 3 min after NaCN, MB (20 μ g/mL) prevented the $[Ca^{2+}]_i$ elevation observed in neurons exposed to NaCN alone ($P = 0.0170$; group $\times$ time interaction effect) such that there were no longer any differences in the time courses of $[Ca^{2+}]_i$ between control and NaCN + MB neurons ($P = 0.2644$; group $\times$ time interaction effect) (Fig. 6).

Discussion

This study has established for the first time that MB is able to mitigate the neurological toxicity of CN not only *in vivo* but also *in vitro*. In unanaesthetized rats, 20 or 10 mg/kg of MB was able to antagonize CN neurological toxicity by not only reducing the time and severity (duration) of coma but also by preventing the life-threatening depression in medullary respiratory neurons in an extremely lethal form of intravenous CN intoxication. All the treated animals survived with no sequelae in an otherwise fatal exposure. To determine whether the protection by MB against CN neurotoxicity *in vivo* was mediated by a direct protective effect on neurons, rather than or in addition, to a restoration of systemic circulation,²³ two neuronal models were used: a primary culture of human fetal neurons or an established human SH-SY5Y neuroblastoma cell line. Since SH-SY5Y neuroblastoma cells resemble immature sympathetic neuroblasts in culture, SH-SY5Y cells were differentiated into more mature dopaminergic neuron-like cells⁵⁸ before subjecting them to our experimental protocols: MB administered several minutes after CN exposure did restore normal neuronal activity of neurons intoxicated with CN.

Methodological considerations and the question of the doses

We used KCN, as in our previous studies,^{24,25,46} following many other studies that used KCN to produce lethal or sublethal CN intoxication.^{2,64} We have previously established that KCl infused at the same rate as KCN in the present study, with equimolar concentrations of potassium salt, had no effect on the concentration of potassium in the blood.⁴⁶ Perhaps, more importantly, KCl infusion was unable to reproduce any of the effects of KCN on circulation or breathing.

The timing of the antidote injection was dictated by the time course of the development of the symptoms and the time to death. It is possible that a benefit could be observed even at the lowest dose (4 mg/kg) MB, which showed less efficacy in the rapidly lethal scenario created by our protocol.

The doses of MB that are currently used in humans for the treatment of methemoglobinemia are about 1–2 mg/kg to be repeated, if needed.^{17,18} According to the recommendations for the conversion of doses between animals and humans provided in the The Guidance for Industry: Estimating the Maximum Safe Starting Dose in Initial Clinical Trials for Therapeutics, published by the Department of Health and Human Services and the FDA,⁶⁵ a dose of 4 mg/kg in humans, corresponding to about 150 mg/m², would, therefore, be equivalent to a dose of about 20 mg/kg in a rat, while 10 mg/kg will correspond to the lowest dose currently used in the clinical setting in humans. In terms of toxicity, a deficit in G6PD is regarded as a classical contraindication to the utilization of MB;¹⁸ however, MB appears to be very safe in children presenting a deficit in G6PD,⁶⁶ as well as in adults with nonsymptomatic G6PD deficit.^{67,68} Although MB has no serotonin “toxicity” by itself, MB can also interact with selective serotonin reuptake inhibitors (SSRIs).⁶⁹ The incidence of “serotonin syndrome” in patients treated by SSRI agents receiving MB appears, however, to be relatively low.^{69,70} Based on the present data, it seems reasonable to assume that the doses currently used for the treatment of methemoglobinemia will be effective against life-threatening CN intoxication. Of note, MB, at the lowest doses of 4 mg/kg (corresponding to less than 1 mg/kg in humans), also shows efficacy, although less potent than at 10 or 20 milligrams per kilogram. This makes possible the use of small doses of MB in combination with other families of antidotes at levels wherein no toxicity related to potential oxidative stress is to be expected.

The in vivo model

The effects of MB significantly delayed and shortened, in a dose-dependent manner, the onset of coma and seizures, while preventing the potentially lethal depression of medullary neurons produced by CN. The latter, akin to the effects of hypoxia^{71–74} or other mitochondrial poisons,⁷⁵ has been shown to occur before a depression in cardiac contractility develops. Gasping and apnea were easy to clinically identify and distinguish from a regular breathing pattern. Of note while at 20 mg/kg, breathing was not affected, at 10 mg/kg, a very transient episode of apnea was observed in one animal, which was still delayed when compared with all untreated animals. At 4 mg/kg, all the animals had episodes of apneas that were not sustained preventing the animals to die. None of these animals presented a cardiac arrest, in striking contrast with the untreated group wherein all the animals died before or within a few minutes following the end of CN exposure (Fig. 2). All the treated animals recovered from their coma following 10 and 20 mg/kg with no sequelae. This was also true at the dose of 4 mg/kg, but for one animal that presented difficulty walking and had to be euthanized. This demonstrates a clear dose-effect of MB with maximal efficacy at 20 milligrams per kilogram. Whether higher doses, which may be associated with oxidative, and thus some toxic, effects of MB could be even more effective remains to be determined.

The in vitro model

The concentration (100 μM) of CN chosen for the present study was 1–2 orders of magnitude lower than that used in previous studies.^{76,78} The reason for selecting this CN dose was twofold. First, our CN concentration utilized corresponded to a realistic level of intoxication compatible with survival. For instance, Baud *et al.*⁷⁹ have shown that clinically toxic effects were associated with CN concentrations between 40 and 100 μM in victims of smoke inhalation, while concentrations $>100 \mu\text{M}$ were potentially lethal. In both mouse⁸⁰ and rabbit⁷ models of CN intoxication, peak blood CN concentrations (80–120 μM) achieved with CN doses that produced 50% mortality (LD_{50}) were similar to those observed in humans. Similarly, in a swine study in which 4% KCN was infused intravenously to effect a 50% reduction in baseline mean arterial pressure, the blood CN level achieved was $3.6 \pm 0.7 \mu\text{g/mL}$ or $\sim 140 \mu\text{M}$,⁸¹ similar to the concentration of CN used in the present study. Second, at this CN dose, the depressed cardiac contractility could be rescued by antidotes: by hydroxocobalamin *in vivo*⁸¹ or MB *in vitro*.²³

Our observation that NaCN (100 μM) depolarized human fetal neurons in primary culture is similar to that observed in rat hippocampal slices in which NaCN (1–2 mM) resulted in rapid depolarization.^{76,78} By contrast, in isolated rat hippocampal neurons in which the fast Na^+ current was blocked by tetrodotoxin, NaCN (2 mM) induced hyperpolarization, rather than depolarization of the membrane potential.⁷⁷ These considerations suggest that the responses to CN by our human fetal neurons in primary culture, in the absence of tetrodotoxin, were similar to those observed in freshly isolated neuronal tissues. One major finding is that NaCN exposure not only resulted in membrane depolarization but also reduction in AP amplitude and prolonged repolarization: all these adverse effects may conspire to render the neurons more excitable and impair nervous impulse conduction, accounting for the coma and depression in breathing as well as the early seizures consistently observed with CN. Another major finding is that MB was efficacious in reversing the deleterious effects of NaCN on neuron AP morphology; thereby providing the evidence for a direct cellular protective effect against NaCN neurotoxicity.

We used another neuronal model (established SH-SY5Y neuroblastoma cell line but differentiated into neuronal phenotype) to evaluate if MB exerted protection against CN neurotoxicity. Our baseline $[\text{Ca}^{2+}]_i$ values of 30–60 nM in differentiated SH-SY5Y cells are similar to those reported for neonatal rat hippocampal neurons (50–130 nM).⁸² NaCN (100 μM) did not change $[\text{Ca}^{2+}]_i$ in differentiated SH-SY5Y cells (data not shown), in agreement with the observations in neonatal rat hippocampus neurons⁸² and primary rat cerebellar granule cells.⁸³ We, therefore, attempted to increase Ca^{2+} influx by incubating SH-SY5Y cells in high K^+ media. Surprisingly, depolarizing the membrane with a high K^+ solution did not increase $[\text{Ca}^{2+}]_i$ in differentiated SH-SY5Y cells, in contrast to that observed in primary culture of rat cerebellar granule cells.⁸³ Lack of $[\text{Ca}^{2+}]_i$ increase may be due to more effective Ca^{2+} extrusion and Ca^{2+} uptake into intracellular organelles in differentiated SH-SY5Y cells. Interestingly, exposure to NaCN (100 μM) in high K^+ medium resulted in significant increases in $[\text{Ca}^{2+}]_i$ over 10 min; an effect that was reversed by MB added 3 min after NaCN. These data indicate that MB was efficacious in normalizing ion homeostasis in CN-intoxicated neurons.

Mechanisms of action of MB during CN intoxication

First, one should acknowledge that the mechanisms of CN toxicity are multifarious. Although signs of CN intoxication are usually attributed to the combination of ion CN with the mitochondrial complex IV (cytochrome c oxidase (CCO)), in turn preventing its reoxidation by O₂,⁸⁴ the mechanisms of CN toxicity are still poorly understood. As a consequence of the complex IV-CN interaction, the mitochondrial complexes upstream to CCO are all maintained in a reduced state and are unable to transfer protons across the inner mitochondrial membrane leading to a rapid depression of the membrane potential, preventing, in turn, mitochondrial ATP synthesis.⁸⁵ The corollary reduction in the rate of O₂ utilization and production of reactive O₂ species, by complex I or III, are considered as the hallmarks of CN poisoning. In addition, since NADH is not oxidized anymore, by an already reduced mitochondrial complex I, the NADH/NAD ratio increases and virtually stops the TCA cycle.^{86,87} This, in turn, suppresses ATP synthesis via mitochondrial substrate-level phosphorylation (TCA cycle). The rise in NADH/NAD ratio also catalyzes the transformation of pyruvate into lactate in the cytoplasm.⁸⁸ We have recently suggested that the manifestations of CN intoxication are not directly caused by a *decrease* in ATP *concentration*-at least in the forms where victims can survive-as a rapid decrease in ATP turn over and consumption occurs balancing any reduction in ATP production.²³ This is well exemplified for CN cardiac toxicity, wherein CN toxicity⁸⁹⁻⁹⁴ produces a reduction in cardiac contractility⁹³ via reversible inhibition of LCa channel activity, reducing in turn ATP needed for contractions.²³ A profound alteration of K⁺ channels⁹⁵ is also present. We have recently found that they result from abnormal phosphorylation of L-type Ca²⁺ channel subunit α_{1c} at T96, Na⁺-K⁺-ATPase subunit α_2 -peptide at S461 and S464, and voltage-gated K⁺ channel subfamily KQT member 2 at S429.⁴³ We found that all these changes that could lead to a fatal outcome were rescued by MB.⁴³

MB, a very potent redox compound, can oppose CN toxicity via different mechanisms.^{24,25} We have recently proposed that MB acts *in vivo* by increasing/restoring the NAD/NADH ratio and the activity of the TCA cycle, as well as by trapping CN on hemoglobin.^{23,24} The latter appears to rely on the cyclic oxidative and reducing effects of the couple MB/LMB on iron. A similar mechanism could take place at the level of the electron chain complexes upstream to the complex IV, which may account for the restoration of the mitochondrial function. We have suggested that molecules of hydrogen peroxide produced during the reoxidation of LMB by O₂,^{96,97} before being “transformed” into H₂O by a large amount of catalase present in cells, could oxidize various metalloproteins^{98,99} of the electron chain complexes “stuck” in a reduced state.^{24,25} Such reoxidation could restore the capacity of the electron chain to be reduced again (by LMB or NADH). Of note, MB appears to be effective not only against CN^{23,24} but also in intoxications affecting mitochondrial functions, including H₂S,^{45,51} sodium azide,¹⁰⁰ and rotenone.¹⁰¹

Conclusions and perspectives

In a lethal model of CN intoxication, we found that MB shows antidotal properties against CN intoxication-induced coma, seizure, and the lethal depression in breathing in a dose-dependent manner (from 4 to 20 mg/kg). The doses of MB used in this study are

commensurate with those proposed in humans and currently used for the treatment of methemoglobinemia. Our *in vitro* data provide direct support for our premises; that is, MB directly counteracts the toxicity of CN-exposed neurons, restoring normal AP morphology, resting potential, and intracellular Ca^{2+} homeostasis. Efficacy studies must be confirmed in other species, and certainly in larger mammals, in keeping with the FDA animal rules and the issues related to the specificity of the response of rodents to hypoxic stress. Finally, due to the necessity of a rapid intervention and a high number of victims potentially involved, the efficacy of the intraosseous route for administering MB should be investigated.

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Figure 1.

(A) Structure of MB. (B) Redox reactions illustrating the reaction of MB and NADH, as an electron donor. The reduction of MB into its leucoform results in oxidation of NADH.

LeucoMB is reoxidized, in the presence of O₂, into MB producing a molecule of H₂O (via the formation of H₂O₂).

Figure 2.

(A) The protocol used in the present study. (B) Effects of KCN infusion (0.375 mg/kg/min for 13 min) on the occurrence of the various symptoms of CN toxicity (median and range of onset of symptoms are shown). The signs of toxicity developed in a very reproducible manner in keeping with the level of CN starting with a phase of agitation followed by a period of weakness, coma, and seizures than a depression in breathing leading to cardiac asystole (CA). The intoxication was always lethal within 15 min in all animals.

Figure 3.

Effects of the different doses of MB, infused 3 min into CN exposure, on the symptoms of CN toxicity. Open boxes show the median and range of the effects in untreated animals (displayed in Fig. 1). Individual data are shown for each dose of MB. The effects of MB were similar at 10 and 20 mg/kg, whether infused for 5 or 10 minutes (A-C). None of the animals presented apnea and they all survived. The duration of coma was significantly delayed at either dose, but was, however, significantly longer following 10 mg/kg than after 20 mg/kg. Following 4 mg/kg (D), the effects of MB were also significantly different from untreated intoxication, but (1) one of the rats that survived, presented with difficulty walking, had to be euthanized within 24 h (red dot) and (2) all animals presented a period of apnea and gasping, while the duration of coma was significantly longer than at the highest doses of MB (untreated versus MB $*P < 0.001$, and $\&P < 0.05$). CA, cardiac arrest.

Figure 4.

Comparison of the effects of three doses of MB (20 mg/kg, administered over 10 or 5 minutes; 10 mg/kg; and 4 mg/kg) at the onset of coma (A), apnea (B), and seizures (C) along with the duration of coma (D). (* $P < 0.001$, \$ $P < 0.01$, and & $P < 0.05$).

Figure 5.

NaCN depolarizes resting membrane potential (E_m), reduces action potential (AP) amplitude, and prolongs action potential duration (APD). These changes were rescued by methylene blue (MB). AP parameters were measured in cultured human embryonic neurons by whole-cell patch clamp (see the materials and methods section). PBS (CTL) or NaCN (100 μ M) was added at time zero, and AP was measured at 10 minutes. In another group of neurons, MB (20 μ g/mL) was added 3 min after NaCN and action potential was measured at 10 minutes. *Top:* Representative AP tracings from neurons treated with PBS (CTL) (A), NaCN (B), and NaCN+MB (C). *Bottom:* The means $\pm$ SE of resting E_m , APD at 100% repolarization (APD_{100}), and AP amplitude from five CTL (D), seven NaCN (E), and six NaCN + MB (F) neurons (two human fetal brain preparations). * $P < 0.0007$, CTL versus NaCN; and # $P < 0.0015$, NaCN versus NaCN + MB.

Figure 6.

Methylene blue reverses $[Ca^{2+}]_i$ elevation in neurons exposed to NaCN. $[Ca^{2+}]_i$ was measured in fura-2-loaded neurons in 40 mM K^+ media (Methods). PBS (CTL) or NaCN (100 μ M) was added at time zero, and fura-2 signals were measured at times indicated. In another group of neurons, MB (20 μ g/mL) was added 3 min after NaCN. Time courses of $[Ca^{2+}]_i$ in neurons exposed to PBS (CTL, open circles), NaCN (closed circles), NaCN + MB (open square), ($n = 3$) are shown.