Antidotal Effects of the Phenothiazine Chromophore Methylene Blue Following Cyanide Intoxication

Philippe Haouzi,^{*,1} Marissa McCann,^{*} Nicole Tubbs,^{*}
Annick Judenherc-Haouzi,[†] Joseph Cheung,[‡] and Frederic Bouillaud[§]

^{*}Division of Pulmonary and Critical Care Medicine, Department of Medicine and [†]Heart and Vascular Institute, Pennsylvania State University College of Medicine, Hershey, Pennsylvania; [‡]Center of Translational Medicine and Department of Medicine, Lewis Katz School of Medicine of Temple University, Philadelphia, Pennsylvania; and [§]Institut Cochin, INSERM U1016-CNRS UMR8104, Université Paris Descartes, Paris, France

¹To whom correspondence should be addressed at Division of Pulmonary and Critical Care Medicine, Department of Medicine, Pennsylvania State University, College of Medicine, 500 University Drive, H041, Hershey, PA 17033. E-mail: phaouzi@pennstatehealth.psu.edu

ABSTRACT

Our study was aimed at (1) determining the efficacy of the dye methylene blue (MB), following a rapidly lethal cyanide (CN) intoxication in *un-sedated* rats; (2) clarifying some of the mechanisms responsible for the antidotal properties produced by this potent cyclic redox dye. Sixty-nine awake rats acutely intoxicated by CN (IP, KCN 7 mg/kg) received saline, MB (20 mg/kg) or hydroxocobalamin (HyCo, 150 mg/kg) when in deep coma. Survival in this model was very low, reaching 9% at 60 min without any treatment. Methylene blue significantly increased survival (59%, $p < .001$) at 60 min, versus 37% with HyCo ($p < .01$). In addition, 8 urethane-anesthetized rats were exposed to a sublethal CN intoxication (KCN, 0.75 mg/kg/min IV for 4 min); they received MB (20 mg/kg, IV) or saline, 5 min after the end of CN exposure. All MB-treated rats displayed a significant reduction in hyperlactacidemia, a restoration of pyruvate/lactate ratio—a marker of NAD/NADH ratio—and an increase in CO₂ production, a marker of the activity of the TCA cycle. These changes were also associated with a 2-fold increase in the pool of CN in red cells. Based on series of *in vitro* experiments, looking at the effects of MB on NADH, as well as the redox effects of MB on hemoglobin and cytochrome c, we hypothesize that the antidotal properties of MB can in large part be accounted for by its ability to readily restore NAD/NADH ratio and to cyclically re-oxidize then reduce the iron in hemoglobin and the electron chain complexes. All of these effects can account for the rapid antidotal properties of this dye following CN poisoning.

Key words: Cyanide intoxication; Methylene blue; Hydroxocobalamin.

Although cyanide (CN) has multifarious targets, one of its main mechanisms of toxicity is thought to result from its affinity for the mitochondrial cytochrome c oxidase (CCO or complex IV) (Ball and Cooper, 1952; Cooper and Brown, 2008; Jensen et al., 1984; Nicholls et al., 2013). The fixation of CN on CCO prevents its re-oxidation by O₂ (Petersen, 1977). As a consequence (1) the mitochondrial complexes upstream to CCO are all maintained in a reduced state and become unable to transfer protons across the inner mitochondrial membrane leading to an inhibition of mitochondrial ATP synthesis (Kim et al., 2012); (2) the NADH/NAD ratio increases, as NADH is not oxidized anymore by the already reduced complex I. This increase in NADH/NAD ratio

impedes the TCA cycle by negative feedback (LaNoue et al., 1972; Liu et al., 2018), which in turn suppresses the synthesis of molecules of ATP via the mitochondrial substrate-level phosphorylation (TCA cycle). In the cytoplasm, the increase in NADH/NAD ratio catalyzes the transformation of pyruvate into lactate (Burgner and Ray, 1984), preventing pyruvate to be incorporated into the TCA cycle and resulting in severe hyperlactacidemia. The resulting clinical manifestations, which are very similar to those produced by other mitochondrial poisons affecting CCO, include a rapid loss of consciousness, seizures, an irregular breathing pattern—alternating central apnea and gasping—and a rapid and profound depression in cardiac contractility leading

to death. In the surviving victims, the long-term outcome is dictated by the neurological sequelae, which are very similar to those produced by postischemic brain injuries. Finally, CN has a direct pulmonary toxicity when inhaled as hydrogen CN.

The treatment of an acute CN intoxication relies on different families of antidotes aimed at decreasing the concentration of CN in tissues, by either trapping free CN in the blood and in tissues or by increasing CN cellular elimination into thiocyanate. An extensive review of the literature on both symptoms of CN toxicity and its treatment can be found in the recent book from Hall *et al.* (2015).

We have recently revisited the effects of methylene blue (MB) (Haouzi *et al.*, 2018a), an “old” redox dye used for the treatment of methemoglobinemia (Wright *et al.*, 1999) and found that when administered during CN exposure, it produces potent antidotal effects at the dose of 20 mg/kg in anesthetized rats. These observations were consistent with previous clinical reports published more than 80 years ago, wherein CN intoxications were rescued by MB (Eddy, 1930; Geiger, 1932, 1933b). We found that MB also restored isolated cardiomyocyte contractility, intracellular Ca^{2+} homeostasis as well as the activity of K^+ channels altered by toxic levels of CN (Cheung *et al.*, 2018b). Intriguingly, the mitochondrial membrane potential of cells intoxicated by CN was also rescued by MB *in vitro*, along with a decrease in the production of reactive O_2 species (Cheung *et al.*, 2018b; Haouzi *et al.*, 2018a), directly antagonizing the consequences of CN-induced inhibition of CCO activity. We proposed (Haouzi *et al.*, 2018a) that these antidotal effects of MB rely on its redox properties. Our rationale was the following: MB, in the blood and in cells, is immediately reduced into leucomethylene blue (LMB) by NAD(P)H (Buchholz *et al.*, 2008) or by any other reducing molecules (Kelner and Alexander, 1985) that have a redox potential lower than that of MB; leucomethylene blue is then re-oxidized into MB by molecules with a higher redox potential, such as oxidized metallo-proteins or O_2 , giving up to 2 electrons and 1 proton. A new cycle of reduction can then be initiated (Buchholz *et al.*, 2008). A significant and very rapid increase in $\dot{V}\text{O}_2$ (oxygen consumption) can be demonstrated both *in vitro* and *in vivo* (Cheung *et al.*, 2018b; Haouzi *et al.*, 2018a) during and after MB exposure. This “hypermetabolism” is not the result of an increase activity of the mitochondrial electron chain *per se*, but rather reflects the rate at which LMB is directly re-oxidized by O_2 into MB. Because the couple MB/LMB easily diffuses into the cytoplasm and mitochondria of cells in the brain and the heart, where it can concentrate (Peter *et al.*, 2000), we have postulated that MB could, by directly oxidizing NADH, restore the NAD/NADH ratio and thus the production of ATP by a TCA cycle (Komlodi and Tretter, 2017) inhibited by this increase in NADH (LaNoue *et al.*, 1972; Liu *et al.*, 2018) during CN poisoning. In the cytoplasm, a restoration of NAD/NADH ratio also prevents the transformation of pyruvate into lactate (Burgner and Ray, 1984). Secondly, we found that the pool of CN in the blood was higher after adding MB (Haouzi *et al.*, 2018a), suggesting that the couple MB/LMB could alter the redox properties of hemoglobin toward oxidation and trap CN. A similar phenomenon in red cells was found to occur when MB was used during H_2S intoxication (Haouzi *et al.*, 2018b). This proposition is quite counterintuitive, because LMB allows the reduction of oxidized forms of hemoglobin: MB is after all the treatment of methemoglobinemia (Wendel, 1939; Wright *et al.*, 1999). To overcome this apparent contradiction, we proposed that MB/LMB could lead to the cyclic creation of an oxidizing milieu inside the red cells, as was long suggested (Wendel, 1934), due to decrease of the pool in intra-erythrocytaire NADPH (oxidized by

MB), or via the production of H_2O_2 during the re-oxidation of the LMB by O_2 (Tretter *et al.*, 2014). The cyclic consumption of NADPH and production of H_2O_2 (Tretter *et al.*, 2014) could certainly increase the chance of producing molecules of ferric or even ferryl iron (Patel *et al.*, 1996). Because these reactions are opposed by the reducing effect of LMB, molecules of oxidized iron may only be transiently present in the red cells, explaining why stable or significant methemoglobinemia is not observed after MB administration, and that MB allows ferrous iron to be rescued via LMB re-oxidation (Haouzi *et al.*, 2018a; Stossel and Jennings, 1966). As a result, free CN could be trapped at a much higher rate in the presence of MB by these transient forms of oxidized iron. Perhaps more importantly, a similar phenomenon of re-oxidation of metallo-proteins may take place at the level of the mitochondrial electron chain complexes, counteracting the upstream effect resulting from the inability of the complex IV to be oxidized by O_2 in presence of CN. Such a mechanism is cyclically opposed by the capacity of LMB to reduce oxidized cytochrome c, allowing in turn the flow of electrons to resume between the complexes I and III. This hypothesis could account for the restoration of the mitochondrial membrane potential in the presence of a non-operational complex IV (Cheung *et al.*, 2018b; Haouzi *et al.*, 2018a).

We are here reporting efficacy study experiments performed in awake rats that received MB at the dose of 20 mg/kg—a dose corresponding to about 4 mg/kg in humans. Methylene blue was administered when the animals were in a deep coma following an exposure to rapidly lethal levels of CN. Our primary outcome was immediate survival. We also compared the efficacy of MB, to that of a unique dose of hydroxocobalamin (cyanokit, 150 mg/kg) a current CN antidote. Secondly, in a separate group of anesthetized rats, we determined the effects of MB, 5 min after the cessation of a nonlethal CN exposure, on (1) the concentration of CN in red cells, (2) CO_2 production ($\dot{V}\text{CO}_2$) kinetics—a marker of the TCA pathway—and (3) lactate/pyruvate ratio, a surrogate of cytoplasmic NAD/NADH ratio. Finally in a series of *in vitro* studies, we attempted to characterize the capacity of MB and NADH to affect hemoglobin and cytochrome c redox properties directly and via the production of H_2O_2 .

MATERIALS AND METHODS

All experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals, 8th Edition [National Research Council (US) Institute for Laboratory Animal Research]. The study was approved by the Pennsylvania State University College of Medicine Institutional Animal Care and Use Committee. Details on the animal preparation can be found in our previous publications (Haouzi *et al.*, 2018a, 2018b).

MB Following CN-Induced Coma in Awake Rats

Male awake rats (376 ± 41 g) received an IP injection of CN at a dose of 7 mg/kg (Haouzi *et al.*, 2017). Clinical examinations were performed before injection then every minute for 30 min following CN administration. Breathing pattern and the presence of a cardiac pulse were continuously monitored. Coma was defined by the disappearance of the righting reflex. Thirty seconds after an animal fell into a coma, animals were randomly assigned to receive an intravenous administration of 1 of the 3 following solutions: saline, MB (20 mg/kg, MB Akron solution 5 mg/ml), or hydroxocobalamin (HyCo, 150 mg/kg, Cyanokit, Meridian medical technology, 25 mg/ml) infused over a 10-min period. The total disappearance of cardiac pulsations by chest palpation for more than 2 min was considered as pulseless electrical activity (PEA).

Each surviving animal was observed carefully for signs of distress or discomfort for 48 h following KCN injection. Animals showing signs of prostration, inability to walk, eat, or drink, paralysis or visual deficit, were euthanized.

One week following CN exposure, the surviving rats were anesthetized and perfused trans-cardially with phosphate-buffered saline (PBS) followed by 10% neutral buffered formalin, then fixed, stained brain were examined by an ACVP certified pathologist blinded to treatment as previously reported (Haouzi et al., 2017).

MB Administration after Sublethal CN Infusion in Anesthetized Rats

Eight rats were anesthetized (urethane), tracheostomized, and mechanically ventilated as previously described (Haouzi et al., 2018a). A catheter (PE-50 tubing) was inserted into the right carotid artery, and 2 venous catheters one into the right jugular vein for MB or saline infusion and one into the left jugular vein for KCN infusion.

Arterial blood pressure, mixed expired O_2 and CO_2 fractions and respiratory flow were determined for the computation of minute ventilation, O_2 consumption ($\dot{V}O_2$) and CO_2 production ($\dot{V}CO_2$), as previously described (Sonobe and Haouzi, 2016). Cyanide was infused at the rate of 0.75 mg/kg/min (Haouzi et al., 2018a) for 4 min and was stopped. All animals survived the exposure and were euthanized after 1 h. Arterial blood was sampled before and then at 5, 15, and 30 min after the end of CN infusion (recovery period). Methylene blue (20 mg/kg) or saline was administered 5 min into the period of recovery. Blood gases and lactate levels were determined as previously described (Haouzi et al., 2018a; Haouzi et al., 2017). In addition, plasma and red cells were separated for determination of concentrations of CN in red cells by the method of Lundquist and Sorbo (1989). A pyruvate Assay Kit (BioAssay Systems, Hayward, California) was used to determine the level of pyruvate in the plasma. As previously described, we found no direct effect of the presence of MB in the determination of pyruvate, lactate, or CN (Haouzi et al., 2018a).

Effects of MB on NADH, Hemoglobin and Cytochrome c

We have previously investigated the effects of mixing MB and NADH on oxidized hemoglobin and cytochrome c and found that while MB and NADH alone had no effect, mixed together they rapidly reduce these 2 metallo-proteins (Haouzi et al., 2018b). In the present study, we have repeated these experiments and determined the potential effects of H_2O_2 produced during the re-oxidation of LMB, by adding previously reduced cytochrome c in a solution containing MB and NADH, but when all NADH had been oxidized and replaced by H_2O_2 , with no more capacity for MB to form LMB (see Results section). All experiments were done in PBS at a pH ranging from 7.4 to 7.5 (Oakton meter 700) and ambient temperature.

Reduced oxyhemoglobin was identified by the presence of 2 peaks of absorbance at 545 and 575 nm (Horecker, 1943; von Kompen, 1983; Zijlstra and Buursma, 1997) with a positive ratio of these 2 peaks A_{575}/A_{545} ; based on this ratio, we have previously reported that it is possible to identify the concentrations of methemoglobin in solution above 4% (Haouzi et al., 2018a). For cytochrome c (Appaix et al., 2000; Koch and Schneider, 2007; Stotz et al., 1938), the change from the oxidized to the reduced form can be characterized by a shift of the Soret band from 410 to 415 nm with the apparition of 2 peaks at 520 and 550 nm instead of one at 530 nm (Appaix et al., 2000; Koch and Schneider, 2007; Stotz et al., 1938). The changes in concentrations in NADH were evaluated by the peak of absorption at 340 nm (Albani,

2007); the concentrations of oxidized (blue) form of MB were determined by the maximal peak at 660 nm (with a lower peak at 615 nm) (Bergmann and O'Konski, 1963) (see Results section for representative spectra of absorbance).

Production of H_2O_2 in the presence of MB and NADH. We sought to determine whether, at neutral pH and ambient temperature, MB (100 μ M) and NADH (200 μ M) would lead to the production of H_2O_2 . We used a simple spectrophotometric method (hydrogen peroxide assay kit # ab102500, ABCAM, Cambridge, UK) based on the measurement of the absorbance of a solution in presence of horseradish peroxidase (HRP) which catalyzes the reaction of a probe with H_2O_2 present in this solution by producing a red color peak of absorbance at 570 nm. As shown in Figures 6 and 7 in the Results section, MB alone had no direct effects on the reaction produced by HRP and the probe. In contrast, a solution containing NADH gave a color signal that was constant over time (false positive). To avoid any artifact, the determination of H_2O_2 produced by MB/NADH was performed after MB has oxidized all NADH. To establish this timing, we first determined the kinetics of interactions between MB and NADH. In keeping with previous spectra published in the literature, the following criteria were used to identify the reduced and oxidized form of MB (Bergmann and O'Konski, 1963) and NADH (Albani, 2007): The change in concentrations in NADH was evaluated by the peak of absorption at 340 nm, whereas the oxidized form has no absorption at this wavelength (Albani, 2007). Data were obtained in triplicate. In the presence of MB, NADH decreased with an exponential pattern reaching 54% of its original concentration at 60 min and is not measurable anymore at 120 min. According to these kinetics, the presence of H_2O_2 was determined in MB, NADH, or MB+NADH solutions, sampled at 10, 60, 90, and then at 120 min, by spiking an aliquot of each solution with the assay kit. The same analysis was performed while catalase (2500 UI/ml) was added to the solution before being mixed with the assay kit.

Data Analysis

In the awake rat study, survival rate was established and was compared using Kaplan-Meier estimators. In addition, the number of rats dying in each group was compared at 10, 60 min then 24 h using chi-square analysis. In the sedated rat model, the variables of interest were compared before KCN infusion then at 5, 15, and 30 min after the end of CN infusion, using repeated ANOVA, followed by a post hoc multiple comparison procedure.

RESULTS

KCN-Induced Coma

A total of 72 rats received 7 mg/kg KCN IP. Three rats did not meet the criteria for coma (loss of righting reflex) and were not included in the study. All the 69 remaining animals started to hyperventilate within 30 s after CN administration, became agitated before presenting an abrupt coma that was immediately associated with general seizures. The latency to coma averaged 123 ± 90 s and is displayed in Figure 1 in keeping with the type of treatments provided to the animals, a relationship which was retrospectively determined. There was no significant difference in the time to coma between the 3 conditions, ie, saline, MB, or HyCo. Thirty seconds after the diagnosis of coma was established, 23 animals received saline, 24 received MB, and 22 received HyCo. Mortality without treatment (saline group) was very high (Figure 2). 74% and 92% of the animals presented

Figure 1. The left panels display the frequency distribution of the time to coma, that was retrospectively analyzed, following the IP administration of a lethal dose of KCN in each of the 3 groups of animals, ie, saline, MB, or HyCo. There was no difference among the 3 groups, although there was a slight trend in the group that will receive HyCo to have fewer animals presenting a late coma (after 2 min). The right panels show the survival for each time: all the nontreated animals died, except for some of those that presented a late coma. In contrast to the saline group, and to a lesser extend to the group that recovered HyCo, a large number of MB-treated animals survived. Of note, animals that presented a coma within 1 min after CN administration had a very severe outcome in all groups. MB, methylene blue.

an irreversible PEA within 10 and 60 min, respectively, following the onset of coma. Of note, the only 2 animals that survived in the saline group were those presenting with a coma that occurred more than 4 min after CN intoxication (Figure 1).

Administration of MB significantly reduced the mortality down to 29% ($p < .0001$) and 41% ($p < .001$) at 10 and 60 min, respectively (Figure 2). The difference in mortality was significant whether comparing the percentage of surviving animals (chi-square test, $p = .0056$ at 10 min and $p = .00031$ at 60 min) or the survival curves (log-rank Mantel Cox test, $p = .0026$ at 10 min and $p < .0001$ at 60 min).

HyCo also had a clear benefit: mortality was lower than the saline group at 60 min (63.4%, $p = .025$) but, in contrast to MB, not at 10 min (46% survival, $p = .298$). The survival curve (log-rank Mantel Cox test) was significantly different from saline at 60 min ($p < .04$). Although, survival rates were higher with MB than with HyCo, chi-square analysis showed that the difference in survival rate between the 2 groups did not reach significance at 10 ($p = .081$) or at 60 min ($p = .136$). Time to death was significantly longer with MB than HyCo ($p < .005$, Wilcoxon signed-Rank test). Of note, when only the animals that could receive the antidotes were considered, discarding those that died

Figure 2. Survival rate following administration of 1 dose of KCN (7 mg/kg, IP) in 69 rats: 23 rats received saline, 24 rats received MB, and 22 rats received hydroxocobalamin 30 s after presenting a coma. Methylene blue significantly increased the survival rate at 10 and 60 min. Note also that the difference between saline and HyCo was not significantly different at 10 min. All animals that were still alive at 40 min remained alive and well for the following 7 days. MB, methylene blue.

Figure 3. Blood concentrations (mean $\pm$ SD) of lactate and pyruvate/lactate (P/L) ratio before and then 5, 15, and 30 min after sublethal CN intoxication in anesthetized rats (KCN infusion 0.75 mg/kg/min for 4 min). Half of the animals received saline (black bars), whereas the other half received MB (open blue bars) 5 min into recovery. Lactate significantly decreased in the MB group with a complete recovery of the P/L ratio (see text for additional comments).

within 10 min after the onset of infusion, the mortality reached 72% in the control group but only 18% for MB ($p < .019$) and 39% for HyCo (NS).

In each group, all animals that were alive at 60 min (Figure 2) remained alive for the following 7 days with a clinically favorable evolution. They all displayed a progression in body weight, which averaged $0.8\% \pm 0.5\%$ and $1.3\% \pm 0.8\%$ of their pre-intoxication body weight per day, in the MB group and the HyCo group, respectively. No histological lesions were found in the brain of any of the surviving animals, including the cortices, subcortical motor nuclei, cerebellum, or hippocampus.

Physiological Studies of the Effects of MB after Sublethal CN Intoxication in Sedated Rats

A significant increase in blood lactate was produced by CN, reaching approximately 9 mM in both groups, 5 min into the recovery period, ie, before MB or saline was administered. This hyperlactacidemia was associated with a large decrease in the pyruvate/lactate ratio (Figure 3). Four animals received saline and 4 animals received MB 5 min after the end of CN exposure. All MB-treated animals presented a significant drop in the concentration of lactate along with a restoration of the pyruvate/lactate ratio (Figure 3).

Both $\dot{V}O_2$ and $\dot{V}CO_2$ decreased in the 8 intoxicated animals during the administration of CN, reaching their nadir 4–5 min into the recovery, as shown in Figure 4. While in the saline group $\dot{V}O_2$ and $\dot{V}CO_2$ returned to or below baseline during the recovery period, in the MB-treated animals $\dot{V}O_2$ and $\dot{V}CO_2$ increased above the pre-intoxication levels.

Cyanide concentrations in red cells averaged $115 \pm 57 \mu\text{M}$ in the saline group, 5 min into recovery, decreasing over time (Figure 5). In contrast, CN concentrations doubled after MB

Figure 4. $\dot{V}O_2$ and $\dot{V}CO_2$ changes in the group of rats that received a sublethal dose of CN for 4 min (KCN infusion 0.75 mg/kg/min). Half of the animals received MB (open blue circles) or saline (black circles) 5 min into recovery. Note that MB increased $\dot{V}O_2$ and $\dot{V}CO_2$ by 2-fold (see text for further details and discussion).

injection and remained higher than in the saline group for the entire recovery period, with no measurable methemoglobinemia. Of note, 2 additional rats received saline for 4 min instead of CN, and MB was administered following the same protocol. Methylene blue administration did not have any effect on the determination of CN concentrations, as shown in Figure 5.

In Vitro Effects of MB on NADH, Redox Effects on Hemoglobin and Cytochrome c

Mixing NADH with MB (ambient temperature, neutral pH) decreased the concentration of NADH to zero with a kinetics displayed in Figure 6. Methylene blue alone did not produce any peak at 570 nm when mixed with the H_2O_2 assay kit. However, mixing MB and NADH with the H_2O_2 assay kit produced a large peak of absorbance at 570 nm, even when aliquots were sampled and analyzed at 120 min, ie, when no more NADH was present in solution. Of note, pH of the solution remained unchanged at 120 min. This prevented any direct artifact produced by NADH as displayed in Figure 6. The level of H_2O_2 (Figure 6) that we found in the MB-NADH solution corresponded to a concentration that averaged $140 \mu\text{M}$ (triplicate). Adding catalase ($10 \mu\text{l}$, 2500 UI/ml) to the MB-NADH solution at time zero, abolished the peak of absorbance at 570 nm (Figure 7).

Mixing a solution of methemoglobin (Figure 8) or oxidized cytochrome c (Figure 9) with MB or NADH alone had no effects on cytochrome c or hemoglobin absorbance. However, when mixed with MB and NADH, methemoglobin and oxidized cytochrome c were immediately reduced (Figs. 8–10). Conversely, mixing the reduced form of hemoglobin or cytochrome c with a solution of MB-NADH, in which all NADH had been oxidized—when MB has lost its ability to form LMB—produced an immediate re-oxidation of hemoglobin (Figure 10) and cytochrome c (Figure 9). As the only elements present in solution were NAD, MB, and H_2O_2 , we propose that H_2O_2 that had been produced during the re-oxidation of LMB was able to oxidize the reduced form of cytochrome c or hemoglobin. However, in the presence of MB and NADH—and thus LMB—the net reaction was always a reduction.

Figure 5. A, Examples of blood samples obtained after treating red blood cells according to the method of Lundquist and Sorbo (Haouzi et al., 2018a; Lundquist and Sorbo, 1989) which produces a typical purple color with an intensity proportional to the concentration of CN (absorbance at 580 nm). Calibration curves were made using deionized water spiked with known concentrations of potassium CN. Blood was sampled from each animal intoxicated by CN, as described in the Materials and Methods section. The rats received MB or saline 5 min after the end of sublethal intoxication. In 2 animals, saline was administered, instead of CN, followed by MB (see text for further details). Red cells, after separation from the plasma, were treated as described in the Materials and Methods section. Note that the blue coloration due to the presence of MB disappears following the first steps of the reaction with sodium hypochlorite. In rats treated by MB, the concentration of CN clearly increased after administration of MB. B, Mean $\pm$ SD concentrations of CN in the rat erythrocytes ([CN]_{rc}). [CN]_{rc} increased up to approximately 110 μ M, 5 min into the period of recovery, slowly subsiding over time in the saline group (back symbols). The concentrations of CN doubled after MB administration (open blue symbols). Note that in the animals that received MB only (grey symbols), analysis of their blood showed no interference with the determination of CN.

Figure 6. A and B, Kinetics of disappearance of NADH (200 μ M) in the presence of MB (100 μ M) in PBS (phosphate-buffered saline), at pH ranging between 7.4 and 7.5 (values given in the figure) and ambient temperature. The concentrations of NADH were determined based on the peak of absorbance in the ultraviolet range (340 nm). These data were used to determine the time at which all NADH would have disappeared from a solution, which initially contained MB, NADH, and H₂O. As NADH, but not MB, created an artifact for the determination of H₂O₂ (C and D), the reaction of the solution containing MB and NADH with the assay kit, was performed at 120 min, when all NADH was oxidized. In all tests, a peak of absorbance was observed at 570 nm corresponding to concentrations of H₂O₂ in the high μ M range (D).

Figure 7. Absorbance spectrum of a solution of H_2O_2 after reaction with HRP and the assay kit (see method section) showing the peak of absorbance at 570 nm before (A) and after (C) adding a solution of catalase. B, shows the same reaction in the presence of a solution of MB alone (dotted line), which revealed no peak at 570 nm. In contrast, a solution of MB+NADH analyzed after 120 min when all NADH had been oxidized displays a clear peak of absorbance after reacting with HRP and the assay kit. D, Adding catalase at time zero in a solution containing MB+NADH prevented this peak to appear. HRP, horseradish peroxidase;

DISCUSSION

This study shows that 20 mg/kg MB administered following a rapidly lethal CN intoxication in awake rats was able to rescue 60% of the animals, whereas 150 mg/kg hydroxocobalamin saved 40% of intoxicated animals. This study (1) complements our previous report wherein we found an efficacy of MB during and after CN exposure in anesthetized rats, (2) supports the historical observations published in the 1930s suggesting that MB is an effective CN antidote (Geiger, 1933a, 1933b; Hanzlik, 1933; Moldenhauer, 1933), and (3) brings some new information on the potential mechanisms involved, as discussed in the following paragraphs.

IP KCN Administration at the Dose of 7 mg/kg Is a Very Rapidly Lethal Model of CN Intoxication in the Rat

We used a rat model that we have previously validated (Haouzi et al., 2017) and which has already been used in the literature (Boswell et al., 1988; Crankshaw et al., 2007). This model recapitulates some of the systemic symptoms of lethal CN intoxication (hyperpnea, apnea, gasping coma, and rapid cardiac arrest) that can be observed in noninhaled forms of CN intoxication. In our hands, 7 mg/kg KCN IP produced a profound coma in 69 out of 72 animals. This coma was always associated with seizures and a depression in breathing pattern leading to gasping and apnea. As shown in Figure 1, all nontreated (saline) animals that presented a coma within 3 min (90% of the intoxicated animals) died by PEA. This modality of exposure produces a very rapid increase in CN concentration in the blood leading to life-

threatening symptoms often in less than 1 min, offering in turn an extremely short window for treatment. As a consequence, a significant number of animals died before the full dose of the antidotes could be administered (Figure 2). Our studies only used infusion (IV or IP) of KCN in solution; although these exposures do not replicate the toxicity of inhalation studies, they represent reasonably good models for noninhaled CN intoxications, allowing us to calibrate and predict at any given exposure the clinical manifestations and thus compare the efficacy of drugs. Finally, we have previously demonstrated that potassium chloride (instead of CN), at the concentrations used in the present studies, had no measurable effect on circulation or respiration by itself (Haouzi et al., 2017).

Treatment of Life-Threatening CN Intoxication by MB: Mechanisms of Action

Methylene blue is a dye that was synthesized at the end of the 19th century (Clifton and Leikin, 2003; Ginimuge and Jyothi, 2010). We have previously demonstrated that MB could be used against lethal sulfide intoxication (Cheung et al., 2018a; Judenherc-Haouzi et al., 2016; Sonobe et al., 2015), it can rescue from the toxicity of sodium azide (Riha et al., 2011), another mitochondrial poison also affecting the complex IV. We found that MB exerts its antidotal effects against mitochondrial poisons through unique modalities of action, resulting from its very distinctive cyclic redox properties. These properties are currently used to treat methemoglobinemia, because MB allows the transformation of ferric into ferrous iron and to restore the ability of hemoglobin to carry O_2 (Clifton and Leikin, 2003; Ginimuge

Figure 8. Absorbance of a solution containing 100% methemoglobin mixed with PBS (orange lines, A) or with a solution containing MB alone (100 μ M, B), NADH alone (200 μ M, C), or MB+NADH (D) over time. The spectrum of absorbance of NADH (black-dotted line) and MB is also shown for comparison in (A). Neither MB nor MB alone was able to affect the concentration of methemoglobin (B and C)—only reaction at 30 min is shown for clarity. In contrast, when methemoglobin was spiked with MB+NADH, a very rapid reduction of methemoglobin was produced, with the appearance of 2 peaks of absorbance corresponding to the presence of oxyhemoglobin.

and Jyothi, 2010; Mayer et al., 1993; Wright et al., 1999), as illustrated in Figure 8. These redox properties appear to have a major interest during CN intoxication as well: as mentioned in the introduction, as soon as it enters the blood, MB, with a redox potential around zero, is readily reduced into its leucoform, LMB, by many of the reducing agents this molecule will encounter, such as NADH (Buchholz et al., 2008) (Figs. 4 and 5). Leucomethylene blue, on the other hand, is re-oxidized into MB by molecules with a higher redox potential (such as O_2 or most metallo-compounds), giving electrons in the process, then a new cycle of reduction can be initiated (Buchholz et al., 2008). Diffusing rapidly into the cytoplasm and in the mitochondria of any cells, including neurons (Peter et al., 2000), the couple MB/LMB can first restore the TCA cycle and the glycolytic activity by oxidizing NADH and decreasing the NADH/NAD ratio (Komlodi and Tretter, 2017) as NADH is not being oxidized by the complex I like in CN or H_2S intoxication. In such conditions, the electron chain remains “immobilized” in a reduced state, so the reestablishment of the TCA cycle can, in turn, lead to the production of ATP via succinyl-CoA synthase, ie, via mitochondrial substrate-level phosphorylation (Komlodi and Tretter, 2017). This can occur independently of the integrity of the mitochondrial ATPase activity. The restoration of pyruvate/lactate ratio after MB, as well as the increase in $\dot{V}CO_2$, supports such a contention.

It has been previously proposed that LMB could interact directly with the mitochondrial electron chain during a blockage

of complex I by rotenone (Rojas et al., 2012), providing electrons to the complexes downstream to complex I and allowing a partial resuscitation of the mitochondrial electron chain. This could, in turn, maintain the mitochondrial gradient of protons, when complexes are inhibited (Atamna et al., 2008; Daudt et al., 2012; Poteet et al., 2012; Wen et al., 2011; Zhang et al., 2006). However, such a mechanism is irrelevant during CN intoxication as the electron chain is already reduced. We have recently proposed (Haouzi et al., 2018b) that molecules of hydrogen peroxide produced during the re-oxidation of LMB by O_2 (Schirmer et al., 2011; Wainwright and Amaral, 2005), before being “transformed” into H_2O by the large amount of catalase present in cells, could re-oxidize the metallo-proteins (Jancura et al., 2014; Jünemann et al., 2000) of the electron chain complexes “stuck” in a reduced state. Such re-oxidation could restore the capacity of the electron chain to be reduced again (by LMB or NADH) and allow electrons to flow at least up to the complex III. The presence of a large amount of H_2O_2 after reaction of MB and NADH supports this proposition. In addition, we have previously showed that H_2O_2 in the μ M range can re-oxidize reduced cytochrome c (Haouzi et al., 2018b) allowing the complex upstream to complex IV to be reduced again by NADH or LMB.

A similar reasoning could be applied to hemoglobin and the cyclic formation of oxidized forms of iron (ferric or ferryl) followed by their rapid reduction into ferrous iron, which is the only visible effect of MB (Figure 8). A mechanism of cyclic

Figure 9. Examples of the absorbance of a solution of methemoglobin in various conditions. In (A), hemoglobin (methemoglobin) is in its oxidized form (orange line). Mixing methemoglobin with a solution of MB only (purple line) did not affect the level of oxidation of hemoglobin. However, when the methemoglobin was spiked in a solution containing MB and NADH (B), a very rapid reduction was produced with the creation of 2 peaks of absorbance at 545 and 575 nm, specific of oxyhemoglobin (data shown at 5 min similar to the effects described in Figure 8). When methemoglobin was mixed after 120 min with “fresh” MB (C) no change in the redox status of hemoglobin was observed, however, when mixed in a solution containing MB+NADH in which all NADH had been oxidized (MB+NADH solution after 120 min, D), a very rapid re-oxidation of hemoglobin occurred (data are shown at 1 min only). In (C and D) the smaller graph depicts the spectrum of absorbance of the hemoglobin after subtraction of that of MB.

oxidation and reduction of hemoglobin by the couple MB/LMB has already been considered during H_2S intoxication treated by MB (Haouzi et al., 2018b). In our previous study (Haouzi et al., 2018b), we observed that a peak of absorbance of hemoglobin at 620 nm developed in the blood of the animals that received MB and H_2S at the same time, but not in those only receiving H_2S . This effect was reproduced in vitro wherein a peak of absorbance at 620 nm was only present when both MB and H_2S (100 μM) were mixed with hemoglobin (an effect that was never observed when only H_2S was mixed with hemoglobin or methemoglobin). Of note, it is possible to create a peak at 620 nm by mixing hemoglobin with H_2S but to occur, much higher levels of H_2S than those found in vivo must be used (Michel, 1938). How could such a peak, traditionally considered as a marker of the presence “sulfhemoglobin” (Bagarinao and Vetter, 1992; Michel, 1938) require MB, since MB is the treatment of methemoglobinemia (Burrows, 1984; Stossel and Jennings, 1966)? We have proposed (Haouzi et al., 2018b) that although MB by itself is not able to oxidize Fe^{2+} to Fe^{3+} , a cyclic conversion of hemoglobin into methemoglobin, due to the decrease in NADPH (and possibly reduced glutathione in the red blood cells), consumed by MB could be produced. This reaction will be opposed by the reduction of ferric iron (Fe^{3+}) by LMB and its cyclic transformation into a ferrous form (Wendel, 1934). Such enhanced cyclic reaction could increase the chance for molecules of free CN (or free sulfide) to be trapped without any significant and stable increase in mean concentration of methemoglobin. In addition, the production of H_2O_2 during the re-oxidation of LMB by O_2 could lead to the

formation of ferryl iron (Fe^{4+}) (Kassa et al., 2015). In other words, the presence of a peak at 620 nm in our previous studies could have reflected the presence of ferric or ferryl iron resulting from the production of hydrogen peroxide during the re-oxidation of LMB by O_2 (Schirmer et al., 2011; Tretter et al., 2014; Wainwright and Amaral, 2005), ie, before being “transformed” into H_2O by the catalase present in red blood cells. After all, adding H_2S to a solution of hemoglobin and looking for a peak of absorbance at 620 nm is a method used to identify the presence of ferryl iron ($HbFe^{4+}$) (Chintagari et al., 2016).

This phenomenon could also lead to the trapping of CN (or H_2S) on oxidized forms of hemoglobin, in turn impeding CN (or sulfide) diffusion to the tissues. Such a mechanism could certainly account for the lessening of CN toxicity without creating a permanent methemoglobinemia.

All these combined effects could certainly translate into potent antidotal properties following CN intoxication.

MB, $\dot{V}O_2$ and $\dot{V}CO_2$

As briefly mentioned in the introduction, the change in $\dot{V}O_2$ observed after MB administration is not a new observation (Harrop and Barron, 1928). The fact that $\dot{V}O_2$ increases with MB in cell culture, wherein all the complexes are blocked (Haouzi et al., 2018a), strongly supports the view that an increase in $\dot{V}O_2$, in the presence of MB, should not be considered as marker of a change in mitochondrial function, but rather reflects the rate of O_2 consumed during the direct re-oxidation of LMB. The corresponding increase in $\dot{V}CO_2$ however, shows that the

Figure 10. Examples of the absorbance of a cytochrome c solution in various conditions (neutral pH, ambient temperature). Results were qualitatively similar to those shown in Figure 9 for hemoglobin. In (A), cytochrome c is in its oxidized form, showing only 1 peak of absorbance at 530 nm (solid line). Mixing cytochrome c with a solution of MB only (thick line) or NADH only (not shown) did not affect the level of oxidation of cytochrome c. However when the cytochrome c was spiked in a solution containing MB+NADH (B), a very rapid reduction of CC was produced with the creation of 2 new peaks at 520 and 550 instead of 1 peak at 530 nm (data shown at 5 min only). If mixed after 120 min in a solution containing MB (C), no change in the redox status of cytochrome c was observed, however, when mixed in a solution containing MB+NADH in which all NADH had been oxidized (after 120 min), a very rapid re-oxidation of cytochrome c occurred (D).

processes associated with MB reduction (consuming NADH) and LMB re-oxidation (consuming O_2) sped up the TCA. The intriguing control system resulting from this observation is that the oxidation of NADH by a molecule of MB creates a new coupling between CO_2 production (TCA cycle) and extra-mitochondrial $\dot{V}O_2$ reproducing the fundamental matching between mitochondrial $\dot{V}O_2$, NADH oxidation and the TCA cycle.

Clinical Implications

Cyanide is a significant source of intoxication in victims of smoke inhalation (Alarie, 2002; Baud et al., 1991, 2011; Borron et al., 2007a; Hall et al., 1989; Jones et al., 1987; O'Brien et al., 2011). Cyanide also remains an occupational hazard (Prochalska et al., 2014) in various industries (Gidlow, 2017). Of note, in many countries, children are exposed to naturally occurring compounds containing CN, present in their food, such as in apricot kernels (Chaouali et al., 2013; Cigolini et al., 2011; Gerivani et al., 2016; Kupper et al., 2008; Sauer et al., 2015; Vlad et al., 2015) or cassava (Ariffin et al., 1992). Ingestion of CN contained in the cassava plant has been linked not only to dreadful outbreaks of spastic paralysis with myoclonus in tropical regions of Africa in adults (Tagwireyi et al., 2016), but also to death (Ariffin et al., 1992) and developmental deficits (Kashala-Abotnes et al., 2018) during acute exposure in children.

The current strategy of treatment of CN intoxication relies on symptomatic measures and specific antidotes (Baud, 2007; Bebarta, 2013; Borron and Baud, 2012), which act through 2 different mechanisms: (1) "scavenging" the pool of free CN in the blood, using cobalt containing molecules (Bebarta et al., 2012;

Borron et al., 2007b; Lee et al., 2016) or nitrite compounds-induced methemoglobinemia (Baskin et al., 1992; Bebarta et al., 2017; Cambal et al., 2011, 2013), taking advantage of the affinity of CN for oxidized metals or (2) increasing CN elimination as thiocyanate, using sodium thiosulfate (Bebarta et al., 2017; Hall et al., 1987, 2007; Hall and Rumack, 1987) or other sulfur donors, such as sulfanegen (Patterson et al., 2016, 2013).

As discussed in the above paragraphs, MB appears to act through a very different mechanism of action. It is rapidly effective and has a relatively long half-life in the blood (Burrows, 1984; Jünemann et al., 2000). Methylene blue also diffuses extremely rapidly into the brain and the heart (Peter et al., 2000), the most sensitive organs to the toxicity of CN, where it accumulates at higher concentrations than in the blood. 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 (Clifton and Leikin, 2003; Ginimuge and Jyothi, 2010). According to the recommendations for conversion of doses between animals and humans (U.S. Department of Health and Human Services FDA, 2005), a dose of 4 mg/kg in humans, would be equivalent to a dose of about 20 mg/kg in a rat. We previously found that 20 mg/kg MB was extremely safe in rats and produced physiological effects which were all reversible within 30–60 min (Haouzi et al., 2018a). In adult sheep, 7–10 mg/kg has been shown by Burrows (1984) to be innocuous. Finally, MB has been used via intra-osseous route (Hosseinpour and Khodaiari, 2012), even in children, and appeared to be as effective following the IV route with very similar kinetics.

In conclusion, this study performed in rats following a very rapidly lethal CN intoxication without the confounding effects of

anesthesia, shows a clear antidotal effect of MB, when administered at the dose of 20 mg/kg over 10 min. We propose that MB acts in vivo by restoring the NAD/NADH ratio and the activity of TCA cycle ($\dot{V}CO_2$) as well as by trapping CN on hemoglobin through a mechanism that may rely on the cyclic oxidative and reducing effects of the couple MB/LMB on iron. A similar effect may 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 that was previously reported. For all these reasons, MB should be seriously considered as an antidote capable of rescuing victims of life-threatening CN intoxications.

DECLARATION OF CONFLICTING INTERESTS

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

FUNDING

National Institutes of Health, Office of the Director (NIH OD), and the National Institute of Neurological Disorders and Stroke (NINDS; 5R21NS098991-02 and 5U01NS097162-03) to P.H., M.M., N.T., A.J.-H., and J.C., in part.

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