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REVIEW

Past, present and future of cyanide antagonism research: From the early remedies to the current therapies

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Abstract

This paper reviews milestones in antidotal therapies for cyanide (CN) spanning early remedies, current antidotal systems and research towards next generation therapies. CN has been a part of plant defense mechanisms for millions of years. It became industrially important in the nineteenth century with the advent of CN assisted gold mining and the use of CN as a pest control agent. The biochemical basis of CN poisoning was actively studied and key mechanisms were understood as early as 1929. These fundamental studies led to a variety of antidotes, including indirect CN binders that generate methemoglobin, direct CN binders such as hydroxocobalamin, and sulfur donors that convert CN to the less toxic thiocyanate. Research on blood gases at the end of the twentieth century shed new light on the role of nitric oxide (NO) in the body. The discovery of NO's ability to compete with CN for enzymatic binding sites provided a previously missed explanation for the rapid efficacy of NO generating antidotes such as the nitrites. Presently used CN therapies include: methemoglobin/NO generators (*e.g.*, sodium nitrite, amyl nitrite, and dimethyl aminophenol), sulfur donors (*e.g.*, sodium thiosulfate and glutathione), and direct binding agents [(*e.g.*, hydroxocobalamin and dicobalt salt of ethylenediaminetetraacetic acid (dicobalt edetate)]. A strong effort is being made to explore novel antidotal systems and to formulate them for rapid administration at the point of intoxication in mass casualty scenarios. New antidotes, formulations, and delivery systems are enhancing bioavailability and efficacy and hold promise for a new generation of improved CN countermeasures.

Key words: Cyanide; Hydrocyanic acid; Antagonist; Antidote; Cobinamide; Sulfanegen; Sulfane sulfur donor

Core tip: This paper reviews milestones in antidotal

therapies for cyanide (CN) spanning early history, current antidotal systems, and research towards next generation therapies. Presently used CN therapies include: methemoglobin/nitric oxide generators (*e.g.*, sodium nitrite, amyl nitrite, and dimethyl aminophenol), sulfur donors (*e.g.*, sodium thiosulfate and glutathione), and direct binding agents (*e.g.*, hydroxocobalamin and dicobalt edetate). New antidotes, formulations, and delivery systems are presently being developed for rapid administration at the point of intoxication in mass casualty scenarios. These hold promise for a new generation of improved CN countermeasures.

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INTRODUCTION

Cyanide (CN) refers to both the anion CN^- and the undissociated form of hydrogen cyanide (HCN). It is a weak acid with a pKa of 9.2, therefore at the body's pH it exists mainly in the HCN form. HCN can easily cross cellular, and subcellular membranes such as the blood brain barrier, and mitochondrial membranes.

CN is formed and is present in various spheres of Nature, such as the cosmos, volcanic eruptions, and lightning[1]. Certain plants, fungi, bacteria, and animals synthesize CN as a component of cyanogenic glycosides to provide a source of nitrogen and for self-defense[2,3]. Consuming cyanogenic plants (*e.g.,* cassava roots, yams, sorghum, maize) without proper preparation can lead to CN intoxication. The cyanogenic glycoside amygdalin is present in some pitted fruits like bitter almond, and apricot cherries[4]. Cyanogenic glycosides can eliminate HCN through a hydrolytic reaction[5]. Metabolism of other cyanogenic chemicals such as the cyanogen halides, and nitriles also leads to CN intoxication[6].

CN is present in smoke from fires (especially burning acrylonitrile, polyurethane, polyamide, wool, silk, rubber) and cigarettes[7-9]; in the vasodilating agent Nitroprusside (formulated as a CN complex)[10,11]; and in industrial settings where CN is widely used to complex metals - for example, CN is widely used in industry for gold extraction from ores[12].

CN has been used as a poison from antiquity. It was first isolated from cherry laurel[13]. Early CN poisonings were reported more than 200 years ago by Wepfer in 1679, and by Fontana in 1795[13], however, the first attempt to antagonize CN was reported later, in the 19th century[14]. Theories, based on biochemical mechanisms of CN antagonism were reported in the mid-20th century[15-20] and therapeutic agents became available for clinical application. After the recent recognition of CN as a military weapon and terror agent, intense research has led to a deeper understanding of CN biochemistry, and has fostered the development of novel CN countermeasures.

CN was used as a chemical weapon for the first time in World War Ⅰ. In World War Ⅱ the Nazi's used CN in gas chambers[21]. In the 1980's Iraq used CN against the Kurds[22]. CN was also involved in the Tokyo subway attack in 1995[23], in the first World Trade Center attack in 1993[24], and in the Chicago Tylenol disaster in 1982[25].

FROM RESEARCH TO THERAPY

Mechanism of cyanide toxicity/search for diagnostics

The primary biochemical basis of CN poisoning was known and published as early as 1929[26]. CN binds and inactivates several metal-containing enzymes, but the most important effect is attributed to the binding of Cyt c Oxidase, which is the terminal oxidase of the mitochondrial electron transport chain. This results in histotoxic hypoxia, due to the inhibition of cell oxygen utilization. As the result of suppressing the aerobic metabolic pathway, the less efficient anaerobic pathway becomes dominant, and pyruvate is reduced to lactic acid. The resultant acidosis leads to central nervous system (CNS) and myocardial depressions.

Higher CN doses (higher than 5XLD50) generate more complex responses[27]. For example, at higher doses, CN induces pulmonary arteriolar and coronary vasoconstriction that can result in cardiogenic shock/pulmonary edema[28], and stimulates chemoreceptors in the carotid artery and aorta resulting in hyper apnea. The antidotal effects of the vasodilators (*e.g.,* nitrites) and alpha-adrenergic blockers (*e.g.*, phenoxybenzamine) confirmed the vascular effects of CN, that are present along with the earlier discovered biochemical enzyme binding effects[29].

Lower doses of CN cause responses such as dizziness, headache, nausea, and vomiting as a result of inhibiting and interfering with the cellular enzymes. Chronic low dose CN exposure can result in Parkinson-like syndromes, confusion, and intellectual deterioration[30]. It is suggested that the wide-spread pathologic condition of tropic ataxic neuropathy is associated with the intense consumption of cassava with high CN-Glycoside content[31].

HCN absorbs rapidly through the mucus membranes and the skin. Inhaling HCN results in rapid intoxication, and if concentrations are high enough, death. When salts are ingested, the absorption from the GI tract is slower. About 60% of the absorbed CN binds to protein. It has high affinity to Cobalt and Fe^{3+}, but it also reacts with sulfur containing molecules in the body (see next paragraphs).

The diagnosis of CN intoxication is difficult in the absence of a direct assay for CN; however, cardiovascular and CNS depressions in smoke inhalation victims always suggest CN involvement. Generally,

symptoms, such as metabolic acidosis, coma, shock, seizures, bradycardia, are not specific, but if the patient is randomly collapsed, and not responding to oxygen treatment, CN exposure should be suspected. Laboratory tests can show saturated hemoglobin (Hgb) (oxygen is not utilized), lactic acidosis (blocked oxidative phosphorylation), and hyperglycemia (toxic effects on pancreatic beta-cells)[32]. CN caused death is confirmed chemically using a colorimetric test at the scene of the crime[33], followed by analysis by GC-MS[34]. Due to the relatively short half-life of CN in the blood[35], detecting major metabolites such as thiocyanate (SCN) and 2-aminothiazoline-4-carboxylic acid (ATCA) are recommended[36,37]. ATCA, as a stable CN metabolite has been established as an important forensic CN biomarker[38]. A recent review of the analysis of CN and its metabolites was published by Logue *et al*[39] in 2010.

Cyanide metabolism

Small amounts of CN can be metabolized in the body by various endogenous metabolic pathways, however, the rate of detoxification is slow (about 0.017 mg CN/body weight/min). The body can metabolize CN at even the lethal dose, if that is given slowly over the course of several hours[40]. In the most important endogenous detoxification reaction CN is converted to the less toxic SCN by reaction with thiosulfate and other endogenous sulfur donors in the presence of a sulfurtransferase enzyme, Rhodanese (Rh). Since Rh is a mitochondrial enzyme, and the inorganic thiosulfate has limited cell penetration capability, the serum albumin-sulfane complex plays a major role in ferrying sulfur donors to Rh[41]. Endogenous sulfur donors are formed from cysteine and methionine in the presence of sulfurtransferases such as Rh, Mercaptopyruvate Sulfur Transferase and Cystathionase. The second most important metabolic pathway is the formation of the chemically stable CN metabolite, ATCA, when CN reacts with endogenous cysteine[37]. Another important metabolic pathway is the formation of cyanocobalamin from hydroxocobalamin. These metabolic end-products are excreted by the urine, while unreacted CN is excreted in breath, urine and sweat. The most recent understanding of CN metabolism, the role of nitric oxide (NO) will be discussed in the next section.

Milestones in CN antagonism

At the end of the 19th and beginning of the 20th century scientists began a systematic search for ways of neutralizing the toxic effects of the deadly chemical. These investigations of CN antagonism prior to the 1930s resulted in the development of the classic CN therapies such as of sodium thiosulfate and sodium nitrite that are still in use worldwide. Table 1 lists some important therapies and the countries where they are presently employed in treating CN intoxication.

Methemoglobin formers: Historically the first CN poisoning remedy was the methemoglobin former amyl nitrite[42]. In studies on the biochemical mechanism of CN antagonism, it was found that while CN has low affinity to Hgb, it has high affinity to the oxidized form of Hgb, methemoglobin (MetHgb), resulting in the formation of the relatively stable cyano-MetHgb complex[15,16]. Amyl nitrite, the first MetHgb former used for CN antagonism, was followed subsequently by sodium nitrite. As early as 1933, the combination of the MetHgb formers amyl nitrite, sodium nitrite and sodium thiosulfate was reported to show significant enhancement in antidotal protection[15,16]. To overcome the disadvantage of the slow MetHgb formation by nitrites, a fast MetHgb former, 4-dimethylaminophenol (DMAP), was developed and investigated[43-45]. When DMAP was administered intravenously, CN was entrapped within the red blood cells with relatively high efficiency[46]. This fast acting MetHgb former was developed and used in Germany in the military and civilian population. It produced 2-3 x LD50 protection in a dog model when the MetHgb level was kept around 30%[47]. Similar CN antidotal protections were found with other MetHgb formers such as p-aminopropiophenone (PAPP), p-aminoheptanoylphenone, and hydroxylamine[48].

Sulfane sulfurs and sulfur donors as CN antidotes: As early as 1894 Lang[49] reported that sodium thiosulfate antagonizes CN intoxication by converting it to a biologically less active metabolite in rabbits. Pascheles[50] and Kahn[51] pointed out that in the liver enzymes enhanced the transformation of CN to SCN. During that time other endogenous organo sulfur molecules, such as cysteine, cystine and glutathione were also investigated as CN antidotes[52-54].

In parallel with the MetHgb binding theory reported by Chen *et al*[15] in 1933, Lang[55] reported the biotransformation reaction between CN and thiosulfate, in which thiosulfate served as a sulfur donor substrate for the mitochondrial sulfurtransferase, Rh. Investigating the substrate specificity of Rh, Sorbo reported that sulfur donors, such as aliphatic and aromatic thiosulfonates are superior sulfur donors than thiosulfate[56]. Rh (E.C. 2.8.1.1; thiosulfate:cyanide sulfurtransferase) was the first sulfurtransferase that was studied in detail[57,58]. The utilization of thiosulfate as CN antidote was reported to be limited by its short biological half-life, small volume of distribution, and limited ability to penetrate the mitochondrial membranes to reach the Rh enzyme[41]. Frankenberg *et al*[59] reported in 1975 that the presence of CN enhanced the cell penetration capability of thiosulfate, making thiosulfate more efficient as a therapeutic agent, rather than as a prophylactic agent. Sulfane sulfur molecules (containing multiple divalent sulfur atoms bound to each other) can serve as a sulfur donor to the Rh reaction. In 1981, Westley[60] reported that a series of sulfane sulfur compounds, such as thiosulfonates, polythionates persulfides, polysulfides, and elementary sulfur serve as substrates for the Rh reaction, however, the pharmacokinetic parameters and toxicity of some of these compounds limited their usage

Table 1 Present cyanide therapies worldwide

Antidotal therapy	Country availability
Sodium nitrite, amyl nitrite and sodium thiosulfate	Europe, Asia (Lilly kit; Talor kit; Pasadena kit) United States (Nithiodote™)
Hydroxocobalamin	European Union/United States (Cyanokit®)
4-Dimethylaminophenol	Germany/Austria/Netherlands
Dicobalt edetate	Netherlands/France/United Kingdom/Australia/Israel (Kelocyanor)

as CN antidotes.

The Westley lab reported that the reaction between CN and mercaptopyruvate (another type of sulfur donor), was catalyzed by 3-Mercaptopyruvate sulfurtransferase (E.C. 2.8.1.2) in the cytosol and mitochondria[61]. When mercaptopyruvate reacts with CN, both SCN and cyanohydrin are formed. Mercaptopyruvate enhances the antidotal effect of thiosulfate, or the thiosulfate + nitrite combination[19]. Westley *et al*[57] reported that serum albumin may also act as an endogenous sulfane sulfur donor, and can react with CN to form SCN.

Sodium thiosulfate and nitrite are the active components of the present CN antidotal combination therapy in the United States (Nithiodote™).

Cobalt compounds: Because cobalt has high affinity to CN, cobalt containing compounds can be used as CN antidotes. Hydroxocobalamin can react with CN by forming cyanocobalamin, which is excreted in the urine[62]. In a dog model, dicobalt edetate is superior to the classic nitrite + thiosulfate combination[63]. When cobalt chloride was administered in combination with thiosulfate or nitrite, it was reported that there was a striking enhancement between cobalt and thiosulfate, but not between the cobalt and the nitrite[64]. Due to its toxicity, cobalt chloride is not used as an antidote for humans[65].

Carbonyl compounds: CN is a nucleophile that reacts with carbonyl containing molecules, such as aldehydes and ketones, to form cyanohydrines. Sodium pyruvate was reported first to antagonize CN in a mice model[66]. The advantage of sodium pyruvate over the classic thiosulfate + nitrite antidotal combination is that it is actively transported intracellularly and can distribute to sites of CN localizations[67]. Sodium pyruvate has been reported to significantly enhance the protective effects of nitrite, but with thiosulfate this enhancement is negligible. However, by adding pyruvate to the combination of nitrite and thiosulfate, a striking protection was found[19]. The strong CN antidotal effects of alpha-ketoglutaric acid were reported as a result of cyanohydrine formation with CN[68].

Clorpromazine: Chlorpromazine was reported as a CN antidote as early as 1958[69]. The mechanism of its action is not known. It does not form MetHgb, bind CN, nor serve as sulfur donor, but it does enhance the antidotal effects of thiosulfate, and the thiosulfate + nitrite combination[70].

Oxygen/hyperbaric oxygen: The Way lab reported first the CN antidotal effect of Oxygen/Hyperbaric oxygen[71-73]. Oxygen alone does not protect against CN, but it does enhance the effects of thiosulfate and the thiosulfate + nitrite combination[74].

Way - classification of CN antidotes: Way[19] summarized the mechanisms of CN antagonism and classified the CN antagonists known at that time. For example, he described the four major steps involved in the antagonism of CN by nitrites and thiosulfate: (1) CN binds to Cytochrome C Oxidase to form CN-Cytochrome C Oxidase; (2) nitrite reacts with Hgb to form MetHgb; (3) CN binds to MetHgb to form Cyano-MetHgb; and (4) thiosulfate reacts with CN to form SCN. This mechanism explained how the classic CN therapy (thiosulfate + nitrite) works, how CN partitions between Cytochrome c Oxidase and MetHgb until it is eventually converted to SCN and eliminated from the body by the urine.

Way described four basic classes of CN antagonists: (1) class Ⅰ: Cyanide Binders: Class IA: MetHgb formers (such as nitrites and DMAP); Class IB: Cobalt compounds (such as dicobalt edetate and Hydroxocobalamin); Class IC: Carbonyl compounds (such as pyruvate); (2) class Ⅱ: Sulfur Donors: Class ⅡA: Thiosulfate; Class ⅡB: Thiosulfonates; Class ⅡC: Other Sulfur sulfanes; (3) class Ⅲ: Cyanide Binders and Sulfur Donors: such as mercaptopyruvate; and (4) class Ⅳ: Unknown Mechanism: Class ⅣA: Oxygen; Class ⅣB: Chlorpromazine.

New antidotal approach and new sulfur donor combination studies in the 20th century: The lower *in vivo* antidotal efficacy of thiosulfate relative to its *in vitro* efficacy highlighted how the limited cell penetration capability of thiosulfate adversely impacted its ability to reach the mitochondrial Rh[75]. Early investigations indicated the importance of externally administered Rh directly to the circulation to enhance the CN antidotal effect of thiosulfate[43,76-78]. However, when proteins (enzymes) are injected directly to the bloodstream, they are rapidly destroyed by proteolytic enzymes and the body's immune system. Therefore, the efficiency of this approach is limited[79]. To minimize the adverse immunologic reactions, a protective

environment is needed for the externally administered enzymes. The two major challenges of this approach of placing sulfur donor and Rh in a close proximity are: finding an appropriate sulfur donor with high sulfur donor reactivity, and developing an appropriate scheme for protecting Rh against macrophage recognition in the circulation[80]. Early investigations following this approach focused on Rh encapsulation within Carrier Erythrocytes[79-81]. To enhance the antidotal efficacy of the CN antidotal system of sulfur donor + externally administered Rh, organic thiosulfonates with superior sulfur donor reactivity were employed. When butane thiosulfonate was encapsulated with Rh in Carrier Erythrocytes, and administered in combination of sodium nitrite, a 14 x LD50 prophylactic protection was found[81]. To overcome the disadvantage of carrier erythrocytes (labor demanding encapsulation, prior blood typing), biodegradable, synthetic polymeric nano-delivery systems were employed[82].

The importance of this approach became suppressed when sulfur donors with higher lipophilicity, and higher cell penetration capability were employed. In 1999 Baskin *et al*[83] reported results on the *in vitro* and *in vivo* efficacy studies with various synthetic sulfur donors with different chemical structures and greater lipophilicity than thiosulfate. In 2006 Ashani *et al*[84] reported that garlic, and its main component, allicin, were beneficial in acute CN intoxications. Allicin breaks down spontaneously to form a variety of organosulfur molecules, such as diallyl-sulfide, diallyl-disulfide and diallyl trisulfide. In the presence of oil, allicin is transformed to ajoene and vinyl-dithiins[85,86]. Investigations of these specific garlic components proved that they are not superior to thiosulfate *in vitro* nor *in vivo,* even when they were applied with nano-intercalated Rh[82]. More recent investigations have examined naturally occurring sulfur donors from garlic and onion that have lower Rh dependence. These sulfur donors demonstrate superior sulfur donor reactivity to thiosulfate. As a result of these investigations, an advanced formulation and superior therapeutic antidotal protection from intramuscularly administered methylpropyl trisulfide was reported in a mice model[87]. Very recent investigations are focused on other garlic compounds as sulfur donors that can improve the ancient, but still clinically used thiosulfate + nitrite combination (Nithiodote™). Publications of the results of these investigations are presently in progress.

Mystery of nitrite/nitric oxide: Turning point in mechanism of CN antagonism: The traditional nitrite theory of MetHgb formation provided a simple explanation of the role of nitrites (sodium nitrites and amyl nitrites) in CN antagonism: Nitrites produce MetHgb; since MetHgb has higher affinity to CN than Cyt c oxidase, MetHgb removes CN from the binuclear heme center (Fea3-CuB) of Cyt c Oxidase, and the mitochondrial electron transport chain is able to return to its job of transferring electrons to oxygen and generating ATP. In this picture nitrites act as indirect CN scavengers[19,88]. When the nitrite + thiosulfate combination is applied, the CN that nitrite displaces reacts with thiosulfate to form SCN that is excreted in the urine[89,90]. However, additional research revealed that the blood MetHgb content needed to be around 15% to effectively antagonize CN[91]. However, when the recommended amyl nitrite dose is applied, only about 5%-7% of Hgb is oxidized to MetHgb, and at these low doses amyl nitrite still acts as an efficacious CN antidote.

The fact that rapid onset of antidotal efficacy by nitrites could not be explained by MetHgb formation suggested one or more additional therapeutic mechanisms. A turning point in understanding this mystery came when the mitochondrial NO synthase was discovered and the function of NO as a regulator in the electron transport chain was characterized[92].

NO regulates the conversion of oxygen to water by Cyt c Oxidase as follows: Ferri-heme a3 takes up electrons and is reduced to ferro-heme a3, NO enters its active site pocket before oxygen does, a nitrosyl-ferro-heme a3 derivative is formed that reacts with dioxygen. NO then dissociates from ferro-heme a3 (rate limiting step), and in the presence of an additional electron donor, nitrite is formed *via* an intermediate peroxynitrite. The cycle's last step is the conversion of peroxynitrite to nitrite and water. In this way, the cycle results in the reduction of oxygen by NO to form water and nitrite. When CN enters the mitochondria, it binds to Cyt c Oxidase. NO can alter the CN binding to Cyt c Oxydase and displace CN from the Cyt c Oxidase's binding site thus restoring its availability for Oxygen binding[93,94]. Pearce *et al*[95] 2003, suggested the reversal of CN inhibition of Cyt c Oxidase by NO occurs in the presence of excess reduced (ferro cyt c Oxidase) and oxygen. They followed the CN substitution by NO in the ferri-heme a3, through a 5-coordinate structure by electron paramagnetic resonance spectroscopy. They stated that NO does not simply act as a reversibly bound competitive inhibitor, but it is also an auxiliary substrate that is consumed and converted to readily releasable nitrite. The displaced CN may then be converted to SCN, or be scavenged by the circulating MetHgb. When external nitrite is added, it boosts the availability of the auxiliary substrate for the Cyt c Oxidase. Along with the NO and MetHgb mechanisms, the vasodilation effects of nitrites also contribute to the their antidotal effects against CN. These results by the Pearce *et al*[95] have provided strong evidence toward solving the puzzle of the mechanisms by which nitrite/NO antagonize CN.

The regulatory effect of NO on Cyt c Oxidase became a well-studied area[96,97]. The signaling molecule NO is generated endogenously from L-Arginine by nitric oxide synthase (NOS)[98]. In CN intoxication, due to the histotoxic hypoxia and lactic acidosis, the NOS activity is decreased, and an exogenous NO source is necessary to protect the cellular respiration. Exogenous nitrites can be transformed to NO even in the condition of

hypoxia[99,100].

Studies on isolated cells (where no Hgb/MetHgb) confirmed that NO inhibits CN by displacing it from Cyt c Oxidase[94]. When the NO donor S-nitroso-N-acetyl-DL-penicillamine was applied to the CN inhibited Cyt c Oxydase, CN was replaced by NO. However, when a NO scavenger 2-phenyl-4,4,5,5-teramethylimidazoline-1-oxy-3 oxide (PTIO) was present, the CN antagonism was blocked, providing strong experimental support for the role of NO as a CN displacement agent[96]. In 2010, Leavesley *et al*[101] used a selective NO scavenger, PTIO to explore CN inhibition by nitrites. In the presence of CN, both the PTIO consumption and the Cyt c Oxidase were inhibited. However, nitrite pretreatment reversed the CN inhibition of NO production and Cyt c Oxidase activity. Conversely, pretreatment with the NO scavenger PTIO negated the ability of the nitrites to antagonize CN. Cumulatively, these studies conclude that a key mechanism of CN antagonism by nitrites involves the generation of NO that competitively displaces CN from Cyt c Oxydase. The NO mechanism runs parallel to the MetHgb mechanism. The formation of each of these species, NO and MetHgb, plays an independent role in the *in vivo* antagonism of CN by nitrites.

Modern theory for old molecules (Nitrites): In 2013 Cambal *et al*[102] compared CN antidotal effects, and blood NO concentration when equimolar amounts of sodium nitrite and amyl nitrite were given to mice intraperitoneally or by inhalation after sub lethal CN doses. They reported that the toxic effects of iso-amyl alcohol formation from amyl nitrite, and the more efficient antidotal effects by sodium nitrite favored the use of sodium nitrite over iso-amyl nitrite. Agreeing with prior studies the authors noted that MetHgb formation was insufficiently rapid to explain the full antidotal effects of amyl nitrite[103]. They also noted that the sodium nitrite maintained efficacy even when MetHgb formation was suppressed. These studies suggested that the NO mechanism was primary and MetHgb formation secondary to the antidotal action of these nitrites. The authors also discussed the complexity of experimental interpretation when interferences due to anesthesia are present.

When the CN antidotal effects of the clinically used anti-angina drug isosorbide dinitrate (ISDN) were reported, NO formation was declared as the major mechanism of its CN antidotal efficacy. It was reported that ISDN has potential advantages over sodium nitrite because it is relatively non-toxic, and is less likely to form MetHgb[104].

Present therapies and recent research investigations in the United States

For treating CN poisoning, multiple antidotes exist and vary in regional availability: MetHgb generators (*e.g.,* sodium nitrite, amyl nitrite, and dimethyl aminophenol), sulfur donors (*e.g.,* sodium thiosulfate and glutathione), and direct binding agents (*e.g.,* hydroxocobalamin and dicobalt edetate). All currently marketed antidotes appear to be effective. In the United States, sodium nitrite and sodium thiosulfate and hydroxocobalamin are used as cyanide antidotes, while in France and several other European countries, only hydroxocobalamin is favored[105]. Antidotal mechanisms include chelation, formation of stable, less toxic complexes, MetHgb induction, and sulfane sulfur reaction with endogenous Rh enzyme. Research with the goal of finding new, safer and more effective cyanide antidotes continues[106].

Table 2 Novel cyanide therapies in development for rapid IM delivery in mass casualty situations

Novel therapy	Mechanism of action	Older analog
Cobinamide	Direct CN Scavenger	Hydroxocobalamin
Sulfanegen	CN Transformer (Leverages the mercaptopyruvate sulfur transferase enzyme)	Mercaptopyruvate
Next generation sulfane sulfur donors	CN Transformer (Leverages the rhodanese enzyme)	Thiosulfate

CN: Cyanide.

The two currently FDA approved CN countermeasures, Nithiodote™ (sodium nitrite and sodium thiosulfate) and Cyanokit® (hydroxocobalamin), each have limitations [*e.g.,* sodium nitrite: intravenous (IV) administration, hypotension, methemoglobinemia; thiosulfate: IV administration, slow onset of action; hydroxocobalamin: IV administration, large volume]. Furthermore, the common requirement for IV administration renders broad use of these CN countermeasures unrealistic in a mass CN exposure event. The distinct need remains to develop a CN countermeasure suitable for mass casualties. Recent investigation efforts focus on developing efficient, easy to administer (*e.g.,* intramuscular) CN countermeasures, which may also be used in combination with other new generation countermeasures. Table 2 shows the classification of recent CN antidotes under development in the United States related to the two present CN therapies of Cyanokit® and Nithiodote™.

Hydroxocobalamin

Hydroxocobalamin is a vitamin B derivative known as vitamin B_{12a}. The compound is a hygroscopic, odorless, dark red crystalline powder which is freely soluble in water and ethanol, and practically insoluble in acetone and diethyl ether. It is the hydroxylated active form of vitamin B_{12} differing in that hydroxocobalamin has a hydroxo moiety linked to a cobalt ion while cyanocobalamin, known as vitamin B_{12} has a cyano moiety. The latter is used to treat pernicious anemia while hydroxocobalamin, based on its strong cyanide binding ability is a potent antidote against cyanide. Hydroxocobalamin has a large molecular weight and a trivalent

cobalt ion that is coordinated by a tetrapyrrol ring. Its mode of action is attributed to its ability to form cyanocobalamin through binding cyanide ion by substituting the aforementioned hydroxo ligand[107,108].

Its pharmacokinetic properties can be characterized by significant plasma protein binding and the formation of various cobalamin-(Ⅲ) complexes after intravenous administration. Free and total cobalamin-(Ⅲ) complexes have half-lives of 26-31 h and overall urinary excretion accounts for 60%-70% of the administered dose[108].

The first reports of its antidotal efficacy were documented in 1952 when an experimental cyanide poisoning of mice was conducted. Ever since, hydroxocobalamin has proved its efficacy in combating cyanide intoxication both in animal models and humans. Mice, rats, guinea pigs, beagle dogs and Yorkshire pigs are some of the species that were included in hydroxocobalamin studies. Alongside the large variety of species included in the investigations various administration methods, such as intravenous, intraperitoneal and intracerebral micro dialysis were also tested. The studies also included pre- and post-poisoning set-ups and cyanide intoxications of various natures including inhalation and parenteral[109-114].

The efficacy of hydroxocobalamin was also seen in human poisonings. In these cases the antidotal effect of the compound, although originally developed as a monotherapy was seen either alone or in combination with other agents, such as 100% oxygen, sodium nitrite and sodium thiosulfate. The therapy was applied in cyanide poisonings originating from various sources including ingestion (*e.g.,* cyanide salts and hydrogen cyanide) and inhalation (*e.g.,* smoke). Survival rate depended on many factors but most notably hydroxocobalamin therapy was especially helpful when administered before anoxic brain damage occurred due to the cardiac arrest of the patients[114-116].

Hydroxocobalamin is available as an United States Food and Drug Administration (FDA) and European Medicines Agency (EMA) approved formulation. The development of the medication was initiated in the 1980s based on request from the Fire Brigade of Paris and antipoisoning center of Paris. The product was approved in France in 1996 and approved in the United States fast track in 2006, followed by EMA approval in 2007. The antidotal kit comprises two glass vials. Each vial contains 2.5 g of hydroxocobalamin in the form of lyophilized powder, a transfer spike for reconstitution, and an intravenous tubing system. Prior to administration the product is reconstituted with 100 mL of preferably 0.9% sodium chloride solution for injection, or lactated Ringer or 5% glucose solution for injection. The mixture is shaken and then administered intravenously over a period of 7.5 min. Hydroxocobalamin has shown itself to be an effective CN antidote *via* intravenous administration. In the pivotal studies which due to ethical reasons were not controlled studies but included patients with suspected or known cyanide-poisoning a survival rate of 58% was seen[108,117].

Cobinamide

Cobinamide is the penultimate precursor in the biosynthesis of cobalamin, lacking the dimethylbenzimidazole nucleotide tail coordinated to the cobalt atom in the lower axial position.

Thus, whereas cobalamin has only an upper ligand binding site, cobinamide has two - both an upper and a lower - ligand binding sites. Moreover, the dimethylbenzimidazole group has a negative trans effect on the upper binding site, thereby reducing cobalamin's affinity for ligands. As a result of these cobinamide has a higher affinity with an overall K_a of 10^{22} mol/L for cyanide ion (binding affinity for first cyanide ion is 10^{14} mol/L and for second ion is 10^{8} mol/L) than cobalamin; for the sake of comparison K_a is 10^{12} mol/L for cobalamin. On the basis of K_a values cobinamide should be a more effective cyanide detoxifying agent than cobalamin[118]. Furthermore, from the aspect of antidote formulation an advantageous feature of cobinamide is that it is at least five times more water soluble (as aquohydroxocobinamide) than hydroxocobalamin[119].

As *in vitro* affinities are not always in line with the *in vivo* conditions (*e.g.,* intracellular proteins may bind cobinamide and cobalamin to varying degrees), many *in vivo* investigations on cell lines and animal models were carried out to examine the cyanide detoxifying properties of cobinamide and to compare it with cobalamin and other antidotes.

The ability of equimolar doses of cobinamide and hydroxocobalamin to reverse the effects of cyanide exposure in New Zealand white rabbits (n = 16) was investigated by Brenner *et al*[119]. CN toxicity was induced by intravenous infusion and the animals were monitored continuously noninvasively by diffuse optical spectroscopy.

Rabbits were divided into three groups: controls (n = 5) received intravenous saline, 6 rabbits were treated with intravenous hydroxocobalamin, and 5 rabbits with intravenous cobinamide following CN exposure. At the end of the sodium cyanide (NaCN) infusion (10 mg in 60 cc normal saline for 60 min), 5 cc of normal saline or 0.0816 millimoles of cobinamide or hydroxocobalamin dissolved in 5 cc of normal saline were infused over 30 s. Cobinamide caused significantly faster and more complete recovery of oxy- and deoxyhemoglobin concentrations in CN-exposed animals over hydroxocobalamin with a recovery time constant of 13.8 ± 7.1 min.

Broderick *et al*[118] found cobinamide to be several-fold more effective than cobalamin in reversing CN inhibition of oxidative phosphorylation in mammalian cells; in rescuing mammalian cells and Drosophila melanogaster from cyanide toxicity; and in reducing CN inhibition of Drosophila Malpighian tubule secretion. Cobinamide was as effective when administered up to 5 min post-CN exposure as when used pre-exposure for prophylaxis[118].

Currently marketed antidotal formulations for CN poisoning must be given by IV administration, limiting their use in treating mass casualties. It is advantageous that cobinamide could be delivered by other routes, *e.g.,* oral ingestion, inhalation, too[118,120].

Cobinamide was studied in both an inhaled and intraperitoneal model of CN poisoning in mice by Chan *et al*[120]. They found cobinamide more effective than hydroxocobalamin, sodium thiosulfate, sodium nitrite, and the combination of sodium thiosulfate-sodium nitrite. Compared to hydroxocobalamin, cobinamide was 3 and 11 times more potent in the intraperitoneal and inhalation models, respectively.

The use of intramuscular cobinamide sulfite to reverse CN toxicity-induced physiologic changes in a sub lethal CN exposure animal model was investigated by Brenner *et al*[121]. New Zealand white rabbits (n = 11) were given 10 mg sodium cyanide intravenously over 60 min. To follow the effect of antidote, tissue oxy- and deoxyhemoglobin concentrations were monitored using quantitative diffuse optical spectroscopy and continuous-wave near-infrared spectroscopy. After completion of the CN infusion, the rabbits were treated intramuscularly with cobinamide sulfite (n = 6) or vehicle (controls, n = 5). Intramuscular administration led to rapid absorption of cobinamide and the molecule was extremely effective at reversing the physiologic effects of CN. Recovery time to 63% of their baseline values in the central nervous system occurred within a mean of 1032 min in the control group and 9 min in the cobinamide group, with a difference of 1023 min. In muscle tissue, recovery times were 76 and 24 min, with a difference of 52 min[121].

When hydroxocobalamin and cobinamide were compared on a nonventillated swine model it was reported that both hydroxocobalamin and cobinamide rescued severely CN-poisoned swine from apnea, however, the dose of cobinamide was one fifth that of hydroxocobalamin to get the same protection[122].

In another study in which cobinamide sulfite was administered *via* intramuscular injection, cobinamide was rapidly absorbed. Mice recovered from a lethal dose of CN even when cobinimide was injected after they had been apneic for over 2 min. Cobinamide sulfite at doses up to 2000 mg/kg exhibited no clinical toxicity[120].

Broderick *et al*[123] observed that cobinamide is highly effective in neutralizing CN ions released by nitroprusside in cultured mammalian cells, Drosophila melanogaster, and mice. Sodium nitroprusside is used to treat hypertensive emergencies and acute heart failure. It acts by releasing NO, a highly potent vasodilator, but for each NO molecule released, five cyanide ions are released, too; thus limiting the safe use of this therapy. To avoid this side effect a CN scavenger could be beneficial when administering nitroprusside. It was reported that cobinamide could neutralize nitroprusside-released CN without having any effect on nitroprusside-released NO, thus it could be a valuable adjunct to nitroprusside therapy[123].

It is worth mentioning that in addition to cyanide, cobinamide is capable of reacting with NO. However, the binding constant of cobinamide is substantially lower for NO than it is for cyanide ($\approx 10^{22}$ mol/L for cyanide; $\approx 10^{10}$ mol/L for NO)[124]. On this basis when given in excess of available CN, cobinamide may potentially induce vasoconstriction and increase blood pressure, as has also been observed with hydroxocobalamin[125].

It can be concluded that cobinamide and its sulfite-salt are effective cyanide detoxifying agents that have the potential to serve as CN antidotes for smoke inhalation victims and persons exposed to CN used as a weapon of mass destruction.

Sulfanegen

Sulfanegen is the water-soluble prodrug of 3-mercaptopyruvate that was developed in the early 1990's to overcome the problem of the relatively low serum stability of the sulfur donor 3-mercaptopyruvate[126]. Sulfanegen is a dimer that dissociates non-enzymatically in physiological systems to two equivalents of the monomer 3-mercaptopyruvate. The endogenous enzyme 3-mercaptopyruvate sulfur transferase (3-MPST) catalyzes the transfer of reactive sulfane sulfur from 3-mercaptopyruvate to CN resulting in the formation of thiocyanate and pyruvate[127,128]. Compared to Rh, the 3-MPST enzyme is available in both the mitochondria and cytoplasm, whereas rhodanese is present only in the mitochondria of hepatic and renal tissues. It was shown that a high amount of 3-MPST exists in the tissue of the brain, specifically in the cerebellum. This strong presence of detoxifying enzyme in the brain holds therapeutic promise, because much of the damage from CN intoxication is localized in the heart and brain.

The other potential advantage with sulfanegen is the fact that it exerts its effects in less than 3 min. With hydroxocobalamin, a fifteen-minute intravenous infusion is required to deliver a standard dose. The discovery of the highly water-soluble sulfanegen triethanolamine for development as an intramuscular injectable antidote was reported by Patterson *et al*[129]. The potential of intramuscular and intravenous sulfanegen sodium treatment to reverse CN effects was evaluated in a rabbit model (n = 35). Changes associated with CN exposure and reversal were monitored by diffuse optical spectroscopy and continuous wave near infrared spectroscopy. Sulfanegen sodium was shown to reverse the effects of CN exposure on oxy- and deoxyHgb rapidly, significantly faster than in case of control animals. Red blood cell CN levels also returned to normal levels faster with both intramuscular and intravenous sulfanegen sodium treatment than with control treatments[126].

Severe CN toxicity - occurrence of severe lactic acidosis accompanied by significant elevation in blood CN levels - was induced in juvenile pig models to demonstrate the CN antagonism capability of sulfanegen[127]. Anesthetized pigs (n = 8) received a high-dose intravenous infusion of sodium nitroprusside SNP (100 mg/h) for 2 h to induce CN toxicity. Then, four

pigs received 3 doses of sulfanegen sodium (2.5 g *i.v.*) and four pigs received placebos. Administration of the sulfanegen antidote resulted in progressively significant reduction in blood lactate and CN levels with 100% survival ($P < 0.05$), whereas the placebo-treated pigs deteriorated and did not survive ($P < 0.05$). In another group of pigs ($n = 6$) severe CN toxicity was induced by NaCN and at peak toxicity (value determined during preliminary measurements), the animals were given sodium sulfanegen (2.5 g *i.v.*) followed by a repeat dose 60 min later in surviving animals. Without sulfanegen the NaCN injection used in this study resulted in CN toxicity, accompanied by severe lactic acidosis, and mortality in all the pigs. Sodium sulfanegen reversed NaCN-toxicity and prevented mortality in all the pigs treated with this antidote[127].

The combination of cobinamide and sulfanegen was explored by Chan *et al*[120] using a non-lethal and two different lethal models - a CN injection and a CN inhalation - of CN poisoning in mice. The effect of the two antidotes was found to be at least additive when used together in all the models used in this study. At doses where all animals died with either drug alone, the combination yielded 80% and 40% survival in the injection and inhalation models, respectively. Similarly, drug doses that yielded only 40% survival with either drug alone, yielded 80% and 100% survival with the combination therapy in the injection and inhalation models, respectively[120].

New sulfur donors

As indicated earlier, very recent investigations in the Petrikovics lab have focused on naturally occurring sulfur donors with lower Rh dependence (scavenger type mechanism), that have superior sulfur donor reactivity to thiosulfate. These sulfur donors were first extracted from garlic compounds and show great promise as successors to the ancient antidote thiosulfate, which is still clinically used in the thiosulfate + nitrite combination (Nithiodote™). Publications of the results of these investigations are presently in progress.

CONCLUSION

Table 1 shows the recent therapies in the US and some European countries, and Table 2 indicates the relations of the new generation CN antidotes to the recent CN therapies in the US. The roots of the present clinically employed therapies reach back to research efforts initiated in the early 20th century. CN intoxication from suicides, homicides, fires, industrial accidents, and potential terrorist attacks, presents a tremendous need for new, rapidly acting and efficient tools to antagonize/treat the toxic and lethal effects of CN in both isolated and mass casualty scenarios.

The lead new generation CN countermeasure cobinamide is a successor of the ancient, but still employed therapy of hydroxocobalamin. Sulfanegen is a next generation successor of mercaptopyruvate. The new generation of sulfane sulfur donors are successors of the classic sulfur donor sodium thiosulfate. These novel sulfane sulfur donors are organo-sulfur molecules that were originally found in garlic and onion. They have other potentially positive health effects[130] and work efficiently against CN intoxication without requiring the catalytic mediation of sulfurtransferases.

New combinations, formulations and nano-delivery systems of these next generation antidotes with enhanced bioavailability, and efficacy, provide strong hope for the gradual replacement of present therapies with improved new drugs and therapies for CN intervention.

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