Novel, Orally Effective Cyanide Antidotes

Herbert T. Nagasawa,*[,†,‡] David J. W. Goon,[†,‡] Daune L. Crankshaw,[†,‡] Robert Vince,[†] and Steven E. Patterson[†]

Center for Drug Design, Academic Health Center, University of Minnesota, Minneapolis, Minnesota 55455, and Medical Research Laboratories, DVA Medical Center, Minneapolis, Minnesota 55417

Received September 13, 2007

Abstract: A series of prodrugs of 3-mercaptopyruvate (3-MP), the substrate for the enzyme 3-mercaptopyruvate/cyanide sulfurtransferase (3-MPST) that converts cyanide to the nontoxic thiocyanate, which are highly effective cyanide antidotes, have been developed. These prodrugs of 3-MP are unique in being not only orally bioavailable, but may be administered up to an hour prior to cyanide as a prophylactic agent and are both rapid- or slow-acting when given parenterally.

Antidotes for the therapeutic management of cyanide poisoning, especially in the U.S., have relied mainly on the enzyme rhodanese (thiosulfate/cyanide sulfurtransferase, EC 2.8.1.1) for detoxification. This enzyme extracts the sulfane sulfur from its substrate, thiosulfate, to form an activated-sulfane complex, which is susceptible to nucleophilic attack by cyanide with the formation of the less-toxic thiocyanate, that is excreted in the urine.[1] Rhodanase is concentrated in the liver and kidneys where it is found in the mitochondrial matrix, a site of low accessibility for ionized, inorganic species, such as thiosulfate.[2] This compartmentation of rhodanase in mammalian tissues leaves major targets of cyanide lethality, namely, the heart[3] and central nervous system[4–6] unprotected. (Rhodanase is also found in red blood cells, but its relative function has not been clarified.[3])

Clinically, the vasodilator sodium nitrite is used in combination with sodium thiosulfate, both administered intravenously in tandem.[1,6,7] Sodium nitrite oxidizes hemoglobin to methemoglobin, which has a high affinity for cyanide, thereby allowing the sequestration of cyanide as cyanomethemoglobin and preventing it from binding to cytochrome oxidase.[1] However, this combination is far from a simple antidote. Methemoglobin formation by nitrite is slow and must be accelerated by intranasal administration of a more rapid acting and volatile amyl nitrite, although this may not be the only mechanism of action by this compound.[8] In addition, nitrite must be carefully monitored to avoid toxicity and is contraindicated when the formation of carboxyhemoglobin by carbon monoxide inhalation (as in victims of fires) further compromises the oxygenation of hemoglobin. Recently, a cyanide-trapping agent used in France, namely, hydroxocobalamin,[9] has been approved by the FDA for use in the U.S. Hydroxocobalamin (MW 1355), which is administered intravenously, sequesters one mole of cyanide (MW 27) per mole of antidote to form cyanocobalamin (vitamin B-12).[10]

The need for a new antidote capable of being administered in a timely manner to the mass-exposed cyanide victims in the event of a chemical disaster or terrorist action is acute, and the present lack of preparedness for such a disaster is alarming.[11]

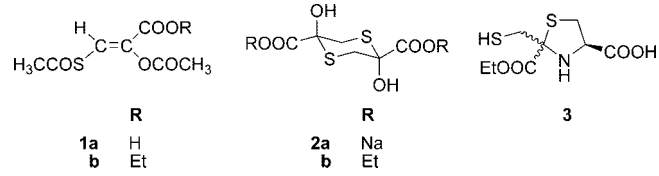

Figure 1. Some prototype prodrugs of 3-mercaptopyruvate (3-MP).

Accordingly, the accelerated development of an alternative cyanide antidote is of high priority. The exploitation of the ubiquitous enzyme present in both the cytoplasm and mitochondria of cells, namely, 3-MPST,[a] whose natural substrate is the cysteine catabolite, 3-MP, has been suggested by Porter and Baskin[12] and by Nagahara et al.[13] However, 3-MP is chemically unstable, and attempts at intravenous administration to counteract the toxicity of cyanide were unsuccessful due to this instability,[14] hence, our prodrug approach.

The three structurally diverse prototype prodrugs of 3-MP that we developed are depicted in Figure 1. Compounds **1** and **2** are sulfhydryl-protected on the mercaptopyruvate, whereas for compound **3**, only the cysteine sulfur is protected. However, the thiomethyl group of **3** is relatively unreactive; for example, we were unable to acetylate this group using standard reagents. Other derivatives related to this series will be reported in a later paper. Compounds **1a** and **1b** require sequential cleavage of the enol acetate and the thiol acetate functional groups (or vice versa) by esterase action in vivo to liberate 3-MP, whereas the prototype compounds **2** and **3** dissociate *nonenzymatically to* liberate 3-MP or its ethyl ester. In addition, the prototype compound **3** was designed to provide not only 3-MP (or its ethyl ester) in vivo, but also to release an equivalent of L-cysteine, the immediate biochemical precursor of and the endogenous reservoir for 3-MP. 2-Substituted thiazolidine-4-carboxylic acids similar to **3** are known to undergo nonenzymatic, hydrolytic opening of the thiazolidine ring in vivo to provide L-cysteine, the rate-limiting amino acid for the biosynthesis of glutathione.[15,16] L-Cysteine can also be oxidized in vivo to L-cystine, which can be further converted metabolically to L-thiocystine, a known sulfane sulfur-generating cyanide antagonist.[17] Compounds **1** and **2** (Figure 1) were readily prepared from commercially available starting materials, while the prototype **3** was synthesized from compound **2b** by condensation of its dissociated, monomeric form (of ethyl-3-MP) in situ with L-cysteine (Supporting Information). The configurations of compounds **1a**, **2a**, and **2b** were verified by X-ray crystallography, while the structure of **1b** (an oil) was assigned by analogy to **1a**. Of interest is the 6-fold alternating axis (center) of symmetry[18] displayed by compound **2** (Supporting Information).

These prototype prodrugs of 3-MP were evaluated for their antidotal efficacy against a toxic, but nonlethal, cyanide dose in mice, using a test paradigm that we developed to allow for 24 h survival of the animals, a requirement imposed by our Institutional Animal Care and Use Committee (IACUC). Thus, instead of attempting the duplication of a historical cyanide LD_{50} dose and measuring the shift in this LD_{50} by preadministration of the putative cyanide antidote, we measured the righting reflex

* To whom correspondence should be addressed. Phone: 949-854-6125. Fax: 612-625-8154. E-mail: nagas001@umn.edu.
† Center for Drug Design.
‡ DVA Medical Center.

[a] Abbreviations: 3-MP, 3-mercaptopyruvate; 3-MPST, 3-mercaptopyruvate sulfurtransferase (EC 2.8.1.2); N/T, sodium nitrite-sodium thiosulfate combination; H, hydroxocobalamine.

10.1021/jm7011497 CCC: $37.00
Published on Web 11/27/2007

Table 1. Effect of Prodrugs on Righting Times: 30 Min Pre-Cyanide, Oral Administration, Screen Righting Time (Mins ± SE)[a]

treatment[b]	avg ± SE	*n*	*%S*	*p* value	P.I.
CN	69.9 ± 3.4	8	100		1.0
CN+1a	24.6 ± 3.9	8	100	<0.0001	2.8
CN+1b	13.0 ± 4.1	7	100	<0.0001	5.4
CN+2a	17.0 ± 3.3	11	100	<0.0001	4.0
CN+2b	15.3 ± 2.5	9	100	<0.0001	4.6
CN+3	33.5 ± 1.3	4	100	<0.0001	2.1

[a] Symbols are *n* = number; *%S* = percent survivors; P.I. = protective index, that is, ratio of avg recovery time for CN + carrier-treated mice over avg recovery time for CN + antidote-treated mice. Dimethyl sulfoxide (DMSO) was used as solvent for the hydrophobic CN prodrugs. Dead animals were excluded in the statistical analyses. Administration of DMSO, prodrugs, N/T, or H alone had no effect on the righting reflex. [b] The 3-MP prodrugs were administered by gavage 30 min pre-CN at 1.45 mmol/kg body weight. Values are means ± SE and statistical analysis was by ANOVA with Scheffe post hoc.

Table 2. Effect of 3-MP Prodrugs on Righting Times: 60 Min Pre-Cyanide, Oral Administration, Screen Righting Time (Mins ± SE)

treatment[a]	avg ± SE	*n*	*%S*	*p* value	P.I.
CN	69.8 ± 3.2	4	100		1.0
CN+1a	33.9 ± 2.2	8	100	<0.0001	2.1
CN+3	35.3 ± 1.6	8	100	<0.0001	2.0

[a] The prodrugs were administered by gavage 60 min pre-CN at 1.45 mmol/kg body weight. Other parameters are the same as Table 1.

recovery times of animals given cyanide alone and cyanide-plus-antidote, using minimal (but sufficient) numbers (*n*) of mice commensurate with a statistical power that allowed for detailed analyses of the data.[19]

Table 1 documents our observation that compounds **1a**, **1b**, **2a**, **2b**, and **3**, when administered orally by gavage 30 min *prior* to the dose of cyanide, were fully protective against cyanide toxicity according to our recovery time criteria, with an average Protective Index (P.I., the ratio of the righting time of the cyanide-only controls divided by the righting times of the cyanide-plus-antidote-treated animals) of 3.78, with the P.I. for compounds **2a** and **2b** averaging 4.3. Because compounds **1b** and **3** were specifically designed as double prodrugs of 3-MP, that is, requiring two activating steps for the release of 3-MP in vivo, they were evaluated for their oral prophylactic efficacy against cyanide by giving these compounds to mice a full *one hour before* the administration of the cyanide. As can be seen (Table 2), the results were still very significant, with P.I.s of 2.0 and 2.1 ($p < 0.0001$). Prophylactic agents against cyanide, heretofore nonexistent, are highly desirable for firemen and rescue workers responding to industrial and residential fires where the presence of cyanide in the smoke is life-threatening.

The comparative antidotal efficacies of several of our prototype 3-MP prodrugs against a standard (human) dose of the currently available cyanide antidotes in the U.S., namely, nitrite/thiosulfate (N/T) combinations and hydroxocobalamin (H), are shown in Table 3. The antidotes were administered intraperitoneally (i.p.; N/T and H cannot be given orally) 5 min before cyanide, and the average righting times of the mice were analyzed statistically. Except for compound **3**, which was expected to be slow-acting by design, compounds **1a**, **1b**, and **2b** were found to be rapid-acting and superior to either the N/T combination or hydroxocobalamin based on their relative protective indices (Table 3).

When administered i.p. 5 min postcyanide, compound **2b** with a P.I. of 3.9 was found to be highly effective in countering the toxic effect of cyanide, with compound **1a** being somewhat less (Table 4). This is contrasted to the minimal protection, offered by H and the N/T combination.

Table 3. Effect of 3-MP Prodrugs on Righting Times: 5 Min Pre-Cyanide; i.p. Administration, Screen Righting Time (Mins ± SE)

treatment[a]	avg ± SE	*n*	*%S*	*p* value	P.I.
CN	65.9 ± 3.4	22	76		1.0
CN+1a	8.7 ± 1.2	10	100	<0.0001	7.6
CN+1b	10.3 ± 1.3	9	100	<0.0001	6.4
CN+2b	8.7 ± 1.0	10	100	<0.0001	7.6
CN+3	42.5 ± 3.4	6	100	<0.001	1.6
CN+N/T	16.4 ± 2.2	7	100	<0.0001	4.0
CN+H	12.6 ± 3.3	7	100	<0.0001	5.2

[a] All prodrugs (0.29 mmol/kg) were administered intraperitoneally (i.p.) at 5 min pre-CN (0.10 mmol/kg or 4.8 mg/kg). N/T = nitrite/thiosulfate (1.45/6.32 mmol/kg). H = hydroxocobalamin (0.217 mmol/kg = 300 mg/kg).

Table 4. Effect of 3-MP Prodrugs on Righting Times: 5 Min Post-Cyanide, i.p. Administration, Screen Righting Time (Mins ± SE)

treatment[a]	avg ± SE	*n*	*%S*	*p* value	P.I.
CN	61.3 ± 5.5	17	94		1.0
CN+1a	36.9 ± 4.1	7	100	0.08 [b]	1.7
CN+1b	27.0 ± 3.6	8	100	<0.002	2.3
CN+2b	15.8 ± 2.3	8	100	<0.0001	3.9
CN+3	42.8 ± 4.0	8	100	0.3 [b]	1.4
CN+N/T	46.0 ± 5.8	8	100	0.5 [b]	1.3
CN+H	44.0 ± 4.0	7	100	0.4 [b]	1.4

[a] All prodrugs were administered intraperitoneally (i.p.) at 5 min post-CN. Other parameters are the same as Table 3. [b] NS = not significant.

In summary, by providing cells directly with *prodrug forms* of 3-MP, the substrate for 3-MPST, thus effectively bypassing the necessity for its enzymatic generation from L-cysteine, an amino acid whose tissue concentrations are known to be tightly controlled at low levels,[20] a unique series of highly effective cyanide antidotes has been discovered. Although it has been reported that, except for 3-MP itself and its oxidized disulfide form, there are no other substrates for 3-MPST, the fact that all of the compounds that initially liberate the ethyl ester of 3-MP showed good and sometimes better potency than those that liberated 3-MP in protecting mice against cyanide, suggest that ethyl 3-mercaptopyruvate must also be a substrate for this enzyme as well. Further proof requires kinetic analysis with purified 3-MPST. These 3-MP prodrugs were shown to be not only protective by the intraperitoneal route in mice, but also when given orally, even 30–60 min *prior* to cyanide. Thus, they are amenable for use as prophylactic agents by first responders in domestic fires, as well as by military personnel in preparation for an impending cyanide threat. Also, because they are synthesized from readily available starting materials, they can be produced in quantity for wide distribution to public health agencies for stockpiling to protect the populace in the event of a major cyanide disaster resulting from an industrial accident or terrorist activity.

Acknowledgment. We thank Jacquie Briggs and Mary Mullett for technical assistance, Jonathan Cohen for recording the NMR spectra, and Victor G. Young, Jr. and Benjamin E. Kucera for the x-ray data. This study was supported by a Seed Grant from the Center for Drug Design, Academic Health Center, University of Minnesota and by the National Institutes of Health CounterACT Program through the National Institute of Neurological Disorders and Stroke (Award #UO1NS058087-01).

Supporting Information Available: Experimental details for the preparation of **1a**, **1b**, **2a**, **2b**, and **3**. The X-ray crystal structures of **1a**, **2a**, and **2b** are also provided. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Baskin, S. I.; Horowitz, A. M.; Nealley, E. W. The antidotal action of sodium nitrite and sodium thiosulfate against cyanide poisoning. *J. Clin. Pharmacol.* **1992**, *32*, 368–375.

(2) Hol, W. G.; Lijk, L. J.; Kalk, K. H. The high resolution three-dimensional structure of bovine liver rhodanese. *Fundam. Appl. Toxicol.* **1983**, *3*, 370–376.

(3) Alexander, K.; Baskin, S. I. The inhibition of cytochrome oxidase by diaminomaleonitrile. *Biochim. Biophys. Acta* **1987**, *912*, 41–47.

(4) Sylvester, M.; Sander, C. Immunohistochemical localization of rhodanese. *Histochem. J.* **1990**, *22*, 197–200.

(5) Westley, J. Rhodanese and the sulfane pool. In *Enzymatic Basis of Detoxification*., Jacoby, W. B., Ed. Academic Press: New York, 1984; pp 245–262.

(6) Isom, G. E.; Johnson, J. D. Sulfur donors in cyanide intoxication. In *Clinical and Experimental Toxicology of Cyanides*., Ballantyne, B. M., Marrs, T. C., Eds.; Wright: Bristol, 1987; pp 413–426.

(7) Way, J. L. Cyanide Intoxication and its mechanism of antagonism. *Annu. Rev. Pharmacol. Toxicol.* **1984**, *24*, 451–481.

(8) Bhattacharya, R. Antidotes to cyanide poisoning: Present status. *Indian J. Pharmacol.* **2000**, 94–101.

(9) DesLauriers, C. A.; Burda, A. M.; Wahl, M. Hydroxocobalamin as a cyanide antidote. *Am. J. Ther.* **2006**, *13*, 161–165.

(10) Megarbane, B.; Delahaye, A.; Goldgran-Toledano, D.; Baud, F. J. Antidotal treatment of cyanide poisoning. *J. Chin. Med. Assoc.* **2003**, *66*, 193–203.

(11) Kein, M. R. Terrorism involving cyanide: The prospect of Improving Preparedness in the prehospital setting. *Prehosp. Disaster Med.* **2006**, *11*, s56–s60.

(12) Porter, D. W.; Baskin, S. I. Specificity studies of 3-mercaptopyruvate sulfurtransferase. *J. Biochem. Toxicol.* **1996**, *10*, 287–292.

(13) Nagahara, N.; Ito, T.; Minami, M. Mercaptopyruvate sulfurtransferase as a defense against cyanide toxication: molecular properties and mode of detoxification. *Histol. Histopathol.* **1999**, *14*, 1277–1286.

(14) Westley, J. In *Methods of Enzymology*; Jakoby, W. B., Ed.;Academic Press, Inc: San Diego, 1981; Vol. 77, pp 285–291.

(15) Nagasawa, H. T.; Goon, D. J.; Muldoon, W. P.; Zera, R. T. 2-Substituted thiazolidine-4(*R*)-carboxylic acids as prodrugs of L-cysteine. Protection of mice against acetaminophen hepatotoxicity. *J. Med. Chem.* **1984**, *27*, 591–596.

(16) Roberts, J. C.; Nagasawa, H. T.; Zera, R. T.; Fricke, R. F.; Goon, D. J. Prodrugs of L-cysteine as protective agents against acetaminophen-induced hepatotoxicity. 2-(Polyhydroxyalkyl)- and 2-(polyacetoxyalkyl)thiazolidine-4(*R*)-carboxylic acids. *J. Med. Chem.* **1987**, *30*, 1891–1896.

(17) Huang, J.; Niknahad, H.; Khan, S.; O'Brien, P. J. Hepatocyte-catalysed detoxification of cyanide by L- and D-cysteine. *Biochem. Pharmacol.* **1998**, *55*, 1983–1990.

(18) Eliel, E. L. *Stereochemistry of Carbon Compounds*. McGraw-Hill: New York, 1962; *30*, p 10ff.

(19) Crankshaw, D. L.; Goon, D. J. W.; Briggs, J. E.; DeLong, D.; Kuskowski, M.; Patterson, S. E.; Nagasawa, H. T. A novel paradigm for assessing efficacies of potential antidotes against neurotoxins in mice. *Toxicol. Lett.* **2007** in press.

(20) Stipanuk, M. H.; Dominy, J. E., Jr.; Lee, J. I.; Coloso, R. M. Mammalian cysteine metabolism: New insights into regulation of cysteine metabolism. *J. Nutr.* **2006**, *136*, 1652S–1659S.

JM7011497