Synthesis and Evaluation of the Thiosemicarbazone, Dithiocarbazonate, and 2′-Pyrazinylhydrazone of Pyrazinecarboxaldehyde as Agents for the Treatment of Iron Overload

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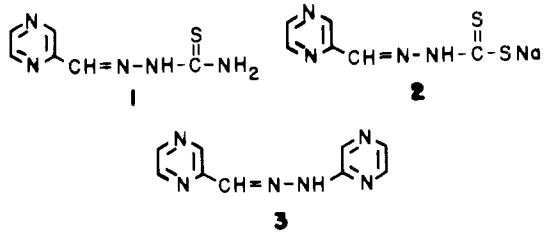
Three prototype tridentate ligands (i.e., pyrazinecarboxaldehyde thiosemicarbazone, sodium pyrazinecarboxaldehyde dithiocarbazonate, and pyrazinecarboxaldehyde 2′-pyrazinylhydrazone) were prepared and evaluated for their relative abilities to remove iron from model systems designed to mimic particular aspects of chronic transfusional iron overload. These compounds were synthesized by condensation of pyrazinecarboxaldehyde with the appropriate substituted hydrazide. Iron-binding properties were determined, and the ability to remove iron from the proteins transferrin and ferritin was ascertained. An in vivo model system employing iron-loaded mice was used to demonstrate that all three compounds were effective at reducing tissue iron levels.

The genetic disease Cooley's anemia is characterized by an inability to form functional red blood cells;[1] therefore, to maintain the hematocrit at a level compatible with the survival of these patients, regular transfusions of erythrocytes are required. These transfusions, coupled with increased intestinal absorption of iron by individuals with this disease and the lack of excretory processes capable of dealing with large amounts of iron, cause an accumulation and an abnormal tissue distribution of this metal.[2] Thus, iron is accumulated in tissues such as the liver, spleen, and ultimately the heart, where its toxicity leads to death, usually before age 20.[3] In an effort to prolong the life span of individuals afflicted with Cooley's anemia, therapy includes the employment of chelating agents to remove excess iron. The current drug of choice, deferoxamine, has not proven to be completely satisfactory, although new approaches are being studied to increase its clinical efficacy.[4]

5-Hydroxypyridine-2-carboxaldehyde thiosemicarbazone has been shown to be capable of producing excretion of relatively large amounts of iron in humans,[5] but since this compound was developed to be a cytotoxic antineoplastic agent its relative toxicity to normal tissues appears to be too great to allow its routine use in the treatment of Cooley's anemia. However, the demonstrated great affinity of this class of agents for iron[6] suggests that an agent of this series with reduced toxicity, but which retains high iron-binding affinity, might be of considerable utility in the treatment of this disease. Out of a series of 13 analogues synthesized,[7] three compounds were selected for study on the basis of reduced toxicity in tests of anticancer activity in animals (Spingarn and Sartorelli, unpublished observations). The synthesis and biological properties of these three agents are reported in this article.

Chemistry. Condensation of thiosemicarbazide with pyrazinecarboxaldehyde in a weakly acidic solution gave 1. Hydrazinopyrazine, synthesized by refluxing chloropyrazine with hydrazine, reacted with the aldehyde to form 2, and reaction of sodium dithiocarbazinate[8] with pyrazinecarboxaldehyde provided 3.



All of the synthesized agents formed strong 2:1 ligand–metal complexes with both iron(II) and iron(III), as shown in Table I. The method of continuous variation was used to determine the stoichiometries of binding and also to calculate approximate stability constants.[9] Most of the complexes were too strong to be quantitated by this technique; however, minimum constants could be derived by assuming less than a 2% deviation from linearity. The data in Table I indicate that the ligands form complexes which are very strong and support the concept that these compounds are tridentate chelating agents.

Table I. Stoichiometry and Minimum Stability Constants of Iron Chelates[a]

compd	iron valence	pH	stoichiometry	λ_{max}, nm	stabil const (log β)
1	Fe(II)	7.4	2:1	630	>17
	Fe(III)	7.1	2:1	630	>25
2	Fe(II)	7.5	2:1	685	>17
	Fe(III)	7.5	2:1	680	>25
3	Fe(II)	7.4	2:1	480	16.4
	Fe(III)	7.4	2:1	480	>27
deferoxamine	Fe(III)		1:1		30.6[16]
tropolone	Fe(III)		1:1		10.5[17]

[a] Stoichiometry and minimum stability constants were determined by continuous variation methodology,[9] except for deferoxamine and tropolone which were derived from the literature.

An effective iron-chelating agent for the treatment of Cooley's anemia should be capable of removing iron from both transferrin in the serum and ferritin in the liver and/or spleen. Since a variety of factors, in addition to the stability constants of the metal complexes, influence the competition between a chelating agent and these iron-binding proteins, direct competition between chelating agents and these proteins was assayed by dialyzing the iron-containing protein against a solution of the ligand to be tested (Table II). Positive controls of the known iron-chelating agents, tropolone and deferoxamine, were added to permit assessment of relative potencies. Of the compounds studied, only tropolone removed large amounts of iron from transferrin under the conditions employed; this property of tropolone has been reported previously.[10] This finding appears to be paradoxical, since tropolone has the lowest stability constant for iron of all the compounds tested and suggests that equilibrium processes are not dominant in this assay. In contrast, tropolone was not exceedingly effective when competing for iron present in ferritin. At time periods less than 72 h, compound 2 was the most active of the agents tested at removing iron from ferritin; deferoxamine was also relatively effective but less potent than 2. Both compounds 1 and 3 were relatively weak as competitors for the iron present in ferritin.

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Table II. Competition between Ligands and Proteins for Iron

compd	removal of radioact from ^{59}Fe-transferrin, % of total cpm removed/h[a]	removal of iron from ferritin, μM[b]
PT[c]	0.3	ND[d]
1	0.3	1.4
2	0.4	48.8
3	0.1	<1.0
deferoxamine	1.0	25.2
tropolone	19.7	4.1
none	<0.1	<1.0

[a] ^{59}Fe-labeled transferrin was dialyzed against a 1.00 mM solution of ligand in 0.1 M Hepes buffer (pH 7.0) at room temperature; initial rates were determined by removing aliquots of the dialysate at various intervals of time and by determining the amount of radioactivity therein. [b] Ferritin was dialyzed against a 1.00 mM solution of ligand, and the iron content of the dialysate was assayed by atomic absorption spectroscopy at 20 h. [c] PT, 2-pyridinecarboxaldehyde thiosemicarbazone. [d] ND, not determined.

Biological Results and Discussion. An in vivo test system consisting of an iron overloaded mouse was adapted from previously developed methodology[11] to evaluate further the potential of the synthesized chelating agents (Table III). All of the three newly synthesized compounds removed significant amounts of iron from iron-loaded mice and were more efficacious than deferoxamine in reducing the degree of saturation of transferrin by iron. None of the drugs reduced the hematocrit of animals when measured at necropsy. Comparison of these results with the in vitro systems employed implies that simple competition between these agents and both ferritin and transferrin was not responsible for in vivo efficacy. It is important to stress that in vivo potency can be significantly affected by the presence of interfering ions in the biological milieu, with hydrogen, calcium, and hydroxide ions being among the most significant.

When animals were loaded with iron, their levels of urinary iron excretion increased almost twofold. Deferoxamine induced an increase in the rate of iron excretion, but this action was not statistically significant. The two dosage levels of pyrazinecarboxaldehyde thiosemicarbazone (1) employed appeared to cause renal shutdown, resulting in excretion of only small volumes of urine; thus, little iron was eliminated by this route in mice treated with this agent. These results differed from those of French et al.[6] who reported the urinary excretion of relatively large amounts of iron by non-iron-loaded animals treated with similar doses of this agent. Animals receiving pyrazinecarboxaldehyde dithiocarbazonate (2) or pyrazinecarboxaldehyde 2′-pyrazinylhydrazone (3), at daily doses of 80 and 100 mg/kg, respectively, excreted relatively large amounts of urinary iron. These animals, as well as those treated with 1, had highly colored urine, the color being characteristic of the iron chelates of these compounds. The iron present in urine did not appear to be heme iron, since tests for hematurea were negative.

The major site of iron storage in the body is the liver. The iron-loading technique used increased liver iron levels of mice to almost threefold that of normal. All of the drugs employed reduced the levels of iron in the liver. Deferoxamine is known to diminish liver iron levels in metal-overloaded animals but not splenic iron;[12] treatment with this ligand was included to ensure that liver iron of animals in this test system was susceptible to this agent.

Iron levels of mouse spleen were also elevated by the iron-loading regimen. Splenic iron was significantly reduced by treatment with compounds 1–3; however, as with liver iron, none of the regimens employed reduced the iron level to that of the non-iron-loaded control animals. This result is probably due to the relatively short (i.e., 6 day) treatment course used, rather than to an inherent deficiency in the properties of these drugs.

The iron-loading regimen used did not significantly increase the levels of this metal present in the heart and lungs of mice. Interestingly, however, pyrazinecarboxaldehyde 2′-pyrazinylhydrazone appeared to be capable of reducing the iron content of these organs.

Experimental Section

Melting points were determined with a Thomas-Hoover capillary melting point apparatus and are uncorrected. IR absorption spectra were obtained using a Perkin-Elmer Model 257 spectrophotometer. NMR spectra were determined with a Varian T-60A spectrometer employing tetramethylsilane as an internal standard. Elemental analyses were performed in duplicate by the Baron Consulting Co., Orange, Conn., and were within ±0.4% of theoretical values.

Pyrazinecarboxaldehyde Thiosemicarbazone (1). Pyrazinecarboxaldehyde was synthesized by the method of Rutner and Spoerri.[13] The aldehyde (2.7 g, 0.015 mol) was added to 2 g (0.02 mol) of thiosemicarbazide dissolved in 35 mL of 70% EtOH, and the resulting product was dried at 75 °C to provide 2.1 g (78%) of yellow powder 1 decomposing at 236–237 °C (lit.[14] 237 °C): IR

Table III. Distribution of Iron in Chelating Agent Treated Iron Overloaded Mice

treatment[a]	daily dosage,[b] mg/kg	% transferrin saturation	iron levels, μg/mouse[c]				
			daily urinary iron	liver	spleen	heart	lung
C_1		82	1.2 ± 0.5[d]	82 ± 19[d]	8.8 ± 1.5[d]	8.1 ± 1.4	14.5 ± 2.1
C_2		ND	2.2 ± 1.6	222 ± 39	21.4 ± 5.6	9.6 ± 2.5	15.5 ± 2.0
deferoxamine	125	75	3.0 ± 1.0	132 ± 35[d]	19.2 ± 3.7	9.4 ± 2.1	16.4 ± 2.2
1	40	58	1.1 ± 0.2	171 ± 40[d]	16.7 ± 3.6[d]	9.2 ± 1.7	15.4 ± 2.0
	80	44	1.1 ± 0.6	156 ± 39[d]	14.6 ± 3.0[d]	9.4 ± 2.6	16.1 ± 1.3
2	40	51	1.4 ± 0.5	193 ± 40	14.0 ± 3.8[d]	8.5 ± 2.3	14.8 ± 1.9
	80	27	5.5 ± 2.8[d]	176 ± 38[d]	18.6 ± 4.2	10.6 ± 3.0	17.7 ± 2.5
3	50	44	3.4 ± 1.8	174 ± 21[d]	16.4 ± 3.2[d]	7.4 ± 1.3[d]	14.6 ± 1.6
	100	38	4.9 ± 2.6[d]	164 ± 22[d]	14.9 ± 3.5[d]	7.6 ± 1.2[d]	12.8 ± 1.8[d]

[a] Treatment groups consisted of 10 mice each. C_1 were the results from non-iron-loaded, vehicle-treated animals and C_2 represents the findings using iron-loaded, vehicle-treated animals. [b] Drugs were administered intraperitoneally for 6 consecutive days with treatment being initiated 3 days after the last iron-loading injection. Deferoxamine and 2 were administered in solution, whereas 1 and 3 were given as suspensions in vehicle. Doses selected were approximate LD_{10} values and one-half of that level. [c] Organ homogenates were analyzed by atomic absorption spectroscopy at necropsy. The results represent mean values ± SD. [d] Significantly different from control group C_2, $p < 0.05$; ND, not determined.

(KBr) 3130 (N-H stretch), 1610 cm^{-1} (C=N stretch). Anal. ($C_6H_7N_5S$) C, H, N.

Sodium Pyrazinecarboxaldehyde Dithiocarbazonate (2). Hydrazine hydrate (5.0 g, 0.10 mol) and NaOH (4.0 g, 0.10 mol) were dissolved in 85 mL of EtOH. To this stirred mixture was added 5.8 mL of CS_2 (0.10 mol) over a 10-min period. After an additional 10 min, the white solid was removed by filtration and washed with EtOH to yield 10.5 g (80%) of a white powder, sodium dithiocarbazinate: mp 63–64 °C; IR (KBr) 3390, 3240 (N-H stretch), 1635, 1610 (N-H bend), 1450 cm^{-1} (C=S stretch).

Sodium dithiocarbazinate (6.5 g, 0.05 mol) and pyrazinecarboxaldehyde (5.4 g, 0.04 mol) were dissolved in a small amount of 70% EtOH and warmed to about 50 °C. The mixture was cooled and filtered to yield an orange precipitate of **2** weighing 5.0 g (55%), which decomposed at 238–240 °C: IR (KBr) 3070 (N-H stretch), 2910 (aldehydic C-H stretch), 1640, 1590–1560 (multiple, aromatic C=N stretch), 1505 (C-H bend), 1460 cm^{-1} (C=S stretch). Anal. ($C_6H_5N_4S_2Na \cdot H_2O$) C, H, N.

Pyrazinecarboxaldehyde 2′-Pyrazinylhydrazone (3). Chloropyrazine (4.0 g, 0.035 mol) and hydrazine hydrate (12.0 g, 0.24 mol) were refluxed until a bright yellow homogeneous mixture was formed (30–45 min). The solution was then maintained at 4 °C for 2 days. A white precipitate was collected by filtration and recrystallized from benzene to yield 2.6 g (70%) of hydrazinopyrazine: mp 107 °C; IR (KBr) 3300 (N-H stretch), 1630 cm^{-1} (C=N stretch); NMR (Me_2SO-d_6) δ 3.8 (d, J = 52 Hz, NH_2), 7.3 (s, NH), 7.7–8.1 (m, $C_4N_2H_3$).

Hydrazinopyrazine (0.2 g, 1.8 mmol) and pyrazinecarboxaldehyde (0.20 g, 1.8 mmol) were dissolved in 10 mL of EtOH. After warming for a few minutes, the mixture was chilled and filtered. The orange-yellow precipitate that formed was collected, washed with water, and dried to provide 265 mg (90%) of **3**: mp 260–261 °C; IR (KBr) 3190 (aldehydic C-H stretch), 1560–1600 cm^{-1} (multiple C=N stretch). Anal. ($C_9H_8N_6$) C, H, N.

Stoichiometry of chelates was determined by the method of continuous variation.[9] Ligand solutions (1.00 mM, 0 to 3.00 mL) were mixed with 0 to 3.00 mL of 1.00 mM $FeSO_4$ or $Fe(NO_3)_3$ in various proportions, such that the total final volume was 3.00 mL. One hour later, the solutions were diluted to 10.00 mL with 50 mM barbital buffer (pH 7.4). All solutions contained 0.100 M KNO_3. Absorbance at the λ_{max} in the visible region was ascertained at various time intervals to ensure equilibrium, and results were plotted as absorbance vs. concentrations of metal/metal + ligand. The complexes were stable within the time period employed.

Measurement of competition between transferrin and chelating agents employed ^{59}Fe-labeled transferrin.[15] Approximately 10^6 cpm of radioactive transferrin was placed in a dialysis sack with 1.0 mL of 0.1 M Hepes buffer (pH 7.00) and dialyzed against 25.0 mL of 1.00 mM ligand solution in buffer at room temperature. Aliquots of 1.00 mL were removed and radioactivity present in the dialysate was measured using a Beckman Gamma 8000 spectrometer with windows set at 800–1400 keV.

Measurement of competition between ferritin and chelating agents was conducted by dialysis of ferritin (containing a total amount of 1.34 mg of Fe) against 25.0 mL of 1.00 mL of ligand solution at room temperature. Between 0 and 200 h, 1.00-mL aliquots of dialysate were removed and stored in tightly stoppered plastic tubes. All samples were analyzed for iron content with a Perkin-Elmer Model 303 atomic absorption spectrophotometer.

Hypertransfused Mouse System. The methodology employed was a modification of that developed by E. Gralla and H. Rosenkrantz, Mason Research Institute.[11] A canine red blood cell suspension was prepared by washing cells three times with 0.9% NaCl and heating in 0.9% NaCl for 30 min at 50 °C. Six-week-old male BDF1 mice were injected ip on days 1, 3, and 5 with 0.6 mL of the erythrocyte suspension (240 μg of Fe/injection). Groups of ten mice constituted one treatment group. Metabolic cages were provided with standard lab chow and tap water ad libitum. On days 8 to 13, ip injections of 0.1 mL of the test drugs in either suspension (**1** and **3**) or solution (**2** and deferoxamine) were administered. Dose levels employed were the approximate LD_{10} values of each agent and one-half of that amount. Vehicle contained 5% EtOH and 0.5% Tween 80 in 0.9% NaCl. The control group C_1 consisted of non-iron-loaded vehicle-treated mice and C_2 of iron-loaded vehicle-treated animals. The urine of experimental animals was collected, centrifuged at 1000g for 15 min, and analyzed by atomic absorption spectroscopy for iron content on a daily basis. On day 15, animals were anesthetized with Et_2O and exsanguinated by cardiac puncture. The blood from each treatment group of 10 mice was pooled and assayed for the degree of transferrin saturation employing a standard hematologic kit (American Monitor Co., Indianapolis, Ind.). The liver and spleen of each animal was removed and, after washing, stored frozen until analyzed. The organs were thawed, weighed, and homogenized in 0.014 M NaCl made up in iron-free water. The total volume of homogenate for each organ was recorded and the supernatant fraction was analyzed for iron by atomic absorption spectroscopy. Portions of the supernatant fractions of hearts and livers were also analyzed for heme iron employing benzidine as a reagent.[18] Less than 5% of the iron in these samples was measurable as heme iron. Data from all groups were compared with the iron-loaded vehicle-treated control C_2 using the Student's t test.

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References and Notes

(1) D. J. Weatherall, and J. B. Clegg, "The Thalassemia Syndromes", Blackwell Scientific Publications, Oxford, 1972.
(2) H. Pearson and R. O'Brien, *Semin. Hematol.*, **12**, 255 (1975).
(3) B. Modell, *Br. Med. Bull.*, **32**, 270 (1976).
(4) A. W. Nienhuis, *J. Am. Med. Assoc.*, **237**, 1926 (1977).
(5) R. C. DeConti, B. R. Toftness, K. C. Agrawal, R. Tomchick, J. A. R. Mead, J. R. Bertino, A. C. Sartorelli, and W. A. Creasey, *Cancer Res.*, **32**, 1455 (1972); I. H. Krakoff, E. Etcubanas, C. Tan, K. Mayer, V. Bethune, and J. H. Burchenal, *Cancer Chemother. Rep.*, **58**, 207 (1974); C. B. Hyman, J. A. Ortega, G. Costin, B. Landing, J. Lazerson, S. Leimbrock, and P. Lurie, *Birth Defects, Orig. Artic. Ser.*, 12(8), 43 (1976).
(6) F. A. French, A. E. Lewis, E. J. Blanz, Jr., and A. H. Sheena, *Fed. Proc., Fed. Am. Soc. Exp. Biol.*, **24**, 402 (1965); J. M. Cano, D. P. Benito, and E. Pino, *Quimica*, **67**, 299 (1971); D. J. Leggett and W. A. E. McBryde, *Talanta*, **21**, 1005 (1974); W. Antholine, J. Knight, H. Whelan, and D. H. Petering, *Mol. Pharmacol.*, **13**, 89 (1977); K. C. Agrawal, B. A. Booth, R. L. Michaud, E. C. Moore, and A. C. Sartorelli, *Biochem. Pharmacol.*, **23**, 2421 (1974).
(7) N. E. Spingarn, Ph.D. Thesis, Yale University (1978).
(8) E. S. Scott and L. F. Audrieth, *J. Org. Chem.*, **19**, 742 (1954).
(9) B. Budesinsky, *J. Inorg. Nucl. Chem.*, **31**, 1345 (1969).
(10) G. P. White, A. Jacobs, R. W. Grady, and A. Cerami, *Blood*, **48**, 923 (1976).
(11) E. J. Gralla, in "Proceedings of Symposium on the Development of Iron Chelators for Clinical Use", W. F. Anderson and M. C. Hiller, Eds., DHEW Publication No. (NIH) 77-994, Bethesda, Md., 1975, pp 229–254; C. G. Pitt, G. Gupta, W. E. Estes, H. Rosenkrantz, J. J. Metterville, A. L. Crumbliss, R. A. Palmer, K. W. Nordquest, K. A. Sprinkle Hardy, D. R. Whitcomb, B. R. Byers, J. E. L. Arceneaux, C. G. Gaines, and C. V. Sciortino, *J. Pharmacol. Exp. Ther.*, **208**, 12 (1979).
(12) C. Hershko, *J. Lab. Clin. Med.*, **85**, 913 (1975).
(13) H. Rutner and P. E. Spoerri, *J. Org. Chem.*, **28**, 1898 (1963).
(14) S. Kushner, H. Dalalian, J. L. Sanjurjo, F. L. Bach, Jr., S. R. Safir, V. K. Smith, Jr., and J. H. Williams, *J. Am. Chem. Soc.*, **74**, 3617 (1952).
(15) I. Cavill, *J. Clin. Pathol.*, **24**, 472 (1971).
(16) G. Schwarzenbach and K. Schwarzenbach, *Helv. Chim. Acta*, **46**, 1390 (1963).
(17) A. E. Martell and R. M. Smith "Critical Stability Constants", Vol. 3, Plenum Press, New York, 1977.
(18) P. L. Wolf, P. Ferguson, I. T. Mills, E. Vonder Muehll, and M. Thompson, "Practical Clinical Hematology: Interpretations and Techniques", Wiley, New York, 1973, pp 164–165.