MOLECULAR PHARMACOLOGY, 23:731-734

Identification of 2-Imidazolines as Anti-Sickling Agents

HENRY CHANG, SANDRA M. EWERT, AND RONALD L. NAGEL

Division of Hematology, Department of Medicine, Albert Einstein College of Medicine, Bronx, New York 10461

Received August 23, 1982; Accepted December 2, 1982

SUMMARY

The drugs tolazoline, clonidine, lofexidine, and fenmetozole were found to inhibit the gelation of hemoglobin S in the order of increasing effectiveness. Only the latter, however, reduced the sickling of red cells significantly and normalized the oxygen affinity of SS blood at 5–10 mM concentrations. Since this level of drug is lower than those reported for many other anti-sickling agents to achieve comparable effects, the 2-imidazoline class of compounds may provide important clues for the development of therapy for sickle cell anemia.

INTRODUCTION

One approach to the detection of therapeutic agents for sickle cell anemia is random screening. Over the past 8 years, approximately 5000 compounds were tested by the Dow Chemical Company (Indianapolis, Ind.) for their ability to inhibit morphological sickling induced by sodium metabisulfite. Of these, eight likely candidates were found and referred to us for evaluation, but only two proved to be effective, belonging to a class of compounds called 2-imidazolines. A third drug in the group, fenmetozole [2-(3,4-dichlorophenoxy-)methyl-2-imidazoline HCl, or DL-524; Table 1], was of particular interest because it had undergone clinical trials as an analeptic agent and it was hoped that its development could be expedited. To study this compound, we used low concentrations of 5–10 mM, which are almost stoichiometric with the quantity of β^S globin in blood, and current assay methods which determine (a) inhibition of Hb[1] S gelation by an increase in the concentration of deoxy-Hb S required for polymerization (C_{sat}), (b) the ability of the drug to block cellular sickling, and (c) whether the agent affects oxygen transport by the blood.

We have found that fenmetozole inhibited the gelation of deoxy-Hb S and cellular sickling, and restored the oxygen affinity of SS blood toward normal at drug concentrations which were significantly below those of many other agents described, possibly by a unique mechanism. The compound bears structural similarities to the antihypertensive medications clonidine [2-(2,6-dichloroanilino-2-imidazoline], tolazoline [2-benzyl-2-imidazoline], and lofexidine [2-[α-(2,6-dichlorophenoxy)ethyl]-Δ^2-imidazoline], which inhibit Hb S gelation also, although to lesser degrees.

This research was supported by Grant HL-21016-03 from the National Heart, Lung and Blood Institute, National Institutes of Health.

[1] The abbreviations used are: Hb, hemoglobin; C_{sat}, concentration of deoxy-Hb S required for polymerization; ODC, oxygen dissociation curve; ISC, irreversibly sickled cell; MCV, mean corpuscular volume; MCH, mean corpuscular Hb; MCHC, mean corpuscular Hb concentration.

METHODS

Blood samples were collected in heparin from donors with SS, AS, and AA blood after informed consent. Fenmetozole was kindly supplied by the Dow Chemical Company; lofexidine by Merrell Dow Pharmaceuticals Inc. (Cincinnati, Ohio); clonidine by Boehringer Ingelheim Ltd. (Ridgefield, Conn.); and tolazoline by Ciba Pharmaceutical Company (Summit, N. J.).

Gelation studies. Hb S was purified on DEAE-cellulose columns (DE-52, Whatman, Clifton, N. J.) (1) and dialyzed against 0.1 M KPO_4 buffer at pH 7.35. To evaluate the solubility of deoxy-Hb S, samples were prepared anaerobically in quartz EPR tubes by admixture of Hb S (final concentration 25 g/dl), buffer with or without drug, and a 3:1 molar ratio of sodium dithionite to heme (2, 3). After gelation at 25°, the solid and liquid phases were separated by ultracentrifugation at 140,000 × *g* for 2 hr and the supernatant concentrations (C_{sat}) determined by infrared spectroscopy and Drabkin's reagent (4). The relative solubility is given as a value for the treated sample divided by that of the control.

Oxygen affinity measurements. Approximately 0.1 ml of SS or AA blood was diluted in 10 ml of 0.15 M $NaPO_4$ buffer with or without drug. The cell suspension was placed in an Imai cell at pH 7.35, 37° (5), and the ODC was recorded. With SS blood, samples also could be collected in formalin at different P_{O_2} values for sickle cell counts. At each point, 300 cells were examined by interference contrast microscopy (Nomarski optics) and classified as sickled if they had sharp projections on the surface. The percentage of newly sickled cells was calculated by the formula

$$\frac{\text{No. of sickled cells} - \text{ISCs}}{\text{Total cells counted} - \text{ISCs}}$$

in order to subtract the background of ISCs. Finally, these results were plotted against the P_{O_2} and O_2 saturation.

Kinetics of sickling. The rate of sickling of AS cells was measured using two syringes connected by a three-way stopcock. One syringe contained a sodium dithionite solution (20 mg/ml of water and the other, a 50-fold dilution of AS blood in 0.15 M $NaPO_4$ buffer (pH 7.35) with and without drug. A portion (3 ml) from the former was injected into 12 ml of cell suspension in the latter, and at various time intervals aliquots were extruded into 10% buffered formalin for cell counts (6).

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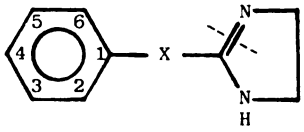
Red cell parameters. Whole AA blood was mixed 1:1 with 0.15 M $NaPO_4$ buffer (pH 7.4) with and without drug. The manually determined Hb and hematocrit, and the red blood cell count by electronic cell counter (Coulter Electronics, Hialeah, Fla.), were used to calculate the red cell indices (MCV, MCH, and MCHC). The percentage of met-Hb was measured on a CO-Oximeter (No. 282, Instrumentation Laboratories, Lexington, Mass.). The intracellular pH was obtained on freeze-thaw lysates of packed red cells by a microelectrode (No. G2971G2 Radiometer, Copenhagen, Denmark). Hb electrophoresis was carried out on starch gel with Tris-EDTA borate buffers at pH 8.6 (7).

RESULTS

Our average normal value in the gelation assay is a saturation concentration for deoxy-Hb S of 17.4 ± 0.3 g/dl (1 SD) at 25°, pH 6.8–6.9. In individual experiments, fenmetozole increased this solubility from control values of 16.9–17.0 to 18.5–18.7 g/dl at 5 mM drug and from 17.4–17.5 to 20.8–21.1 g/dl at 10 mM. This represents C_{sat} ratios of 1.09–1.11 and 1.19–1.21, respectively (Table 1). When Hb S was treated with 10 mM fenmetozole and then dialyzed for 24 hr, the relative solubility was lowered from 1.19 to 1.05. The hydrolysis product of this compound increased the C_{sat} by only 7% at 10 mM. Lofexidine was almost as potent as fenmetozole and enhanced the solubility of deoxy-Hb S by a factor of 1.17 at 10 mM, but clonidine and tolazoline had smaller effects, with C_{sat} ratios of 1.07–1.09.

TABLE 1

Solubility of deoxy-Hb S in the presence of imidazolines and other inhibitors



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Drug	Concentration	Molar ratio, drug/heme	Relative solubility	Reference
	mM			
Fenmetozole	5	0.32	1.09–1.11	This paper
3,4-Dichloro-, *X* = —OCH_2—	10	0.64	1.19–1.21	This paper
3,4-Dichloro-, *X* = —OCH_2—	10[a]		1.05	This paper
Fenmetozole (hydrolysis product)	10	0.64	1.07	This paper
Lofexidine				
2,6-Dichloro-, *X* = —OCH(—CH_3)—	10	0.64	1.17	This paper
Clonidine				
2,6-Dichloro-, *X* = —NH—	10	0.64	1.09	This paper
Tolazoline				
X = —CH_2—	10	0.64	1.07	This paper
Urea	10	0.64	1.01	—[b]
Tryptophan	10	0.64	1.10	3
Amphetamine	10	0.64	1.06	3
Potassium cyanate	10	0.67[c]	1.04	8
Glyceraldehyde	10	0.67[c]	1.11	—[b]

[a] Followed by dialysis for 24 hr.
[b] H. Chang, S. Ewert, and R. L. Nagel, unpublished data.
[c] At 37° for 1 hr.

The ODC of SS blood was measured in an Imai cell, which showed that a deoxygenation curve of a control red cell suspension had a P_{50} of 32 torr at 37°, pH 7.35, whereas its P_{50} in the presence of 5 mM fenmetozole was 24 torr. At 1 mM, however, the drug reduced the P_{50} only 1 torr. With lofexidine at a level of 5 mM, the oxygen affinity curve was shifted leftward by 7 torr and with clonidine by 5 torr, but tolazoline lowered the P_{50} only 1 torr. The oxygen affinities of normal AA cells or dilute Hb S solutions were not changed by 5–10 mM fenmetozole (Table 2).

Sickling of SS cells also was inhibited by fenmetozole when examined as a function of P_{O_2} or O_2 saturation (Fig. 1A and B). Fifty per cent newly sickled cells were detected at a P_{O_2} of 30 torr in a control sample but did not appear until 20 torr in the presence of 5 mM fenmetozole. (The standard error for this determination was ± 2.7 torr.) At 50% oxygen saturation, there were 47% newly sickled control cells and 30% sickle cells with the drug and a standard error of ± 3.5%. When SS cells were treated with 5 mM fenmetozole and then washed, there was no difference from the control. Inhibition of morphological sickling was not obvious with 5 mM of any of the other compounds. This result was surprising for lofexidine, since it apparently could enter cells and change the oxygen affinity of SS cells but not of AA cells. Clonidine made the red cell membranes appear diaphanous, although no overt lysis occurred.

Kinetic studies with AS red cells showed a significant

TABLE 2

Oxygen affinity and red cell parameters

	Oxygen affinity	
	P_{50}	Sickled cells at P_{50}
	torr	%
SS cells		
Control	32	47
With 5 mM drug		
Fenmetozole	24	30
Lofexidine	25	40
Clonidine	27	NE[a]
Tolazoline	31	45
AA cells		
Control	26	—
With 5 mM drug		
Fenmetozole	28	—
Lofexidine	27	—

	Red cell parameters						
	MCV	MCH	MCHC	met-Hb	pH		
					Extracellular	Intracellular	Δ
	fl	*pg*	*g/dl*	%			
SS cells							
Control	83	29	35	—	7.50	7.22	0.28
With 10 mM fenmetozole	85	28	33	—	7.45	7.27	0.18
AA cells							
Control	86	31	36	0	7.49	7.33	0.16
With 10 mM fenmetozole	91	31	34	0.3	7.45	7.33	0.12

[a] NE, Not evaluable.

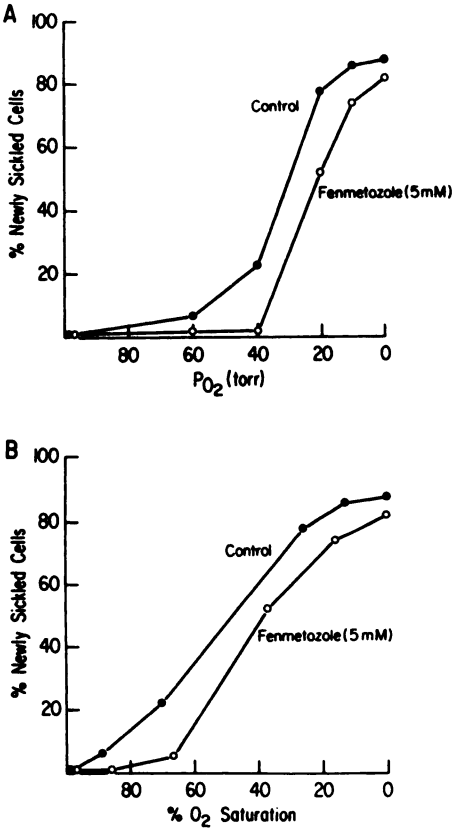

FIG. 1. *Percentage of newly sickled cells*
A. Percentage of newly sickled cells expressed as a function of P_{O_2}.
B. Percentage of newly sickled cells expressed as a function of oxygen saturation. ●, Control; ○, in the presence of 5 mM fenmetozole.

decrease in the rate of sickling due to 5 mM fenmetozole. The compound prolonged the time for appearance of 50% morphological sickling from a control value of 2 min to 8 min. The difference between intra- and extracellular pH was lowered by 10 mM fenmetozole, the MCV was slightly increased, and the MCHC was slightly reduced. The met-Hb content and electrophoretic mobility of Hb, however, were not altered (Table 2).

DISCUSSION

This report establishes the ability of 2-imidazolines to inhibit the gelation of deoxy-Hb S. In an ultracentrifugation assay of gelation, the solubility of deoxy-Hb S was augmented most by fenmetozole and lofexidine, but to a lesser extent by clonidine and tolazoline. Their effect on intact red cells was assessed objectively by measurement of the ODC. Fenmetozole normalized the P_{50} of SS blood more effectively than its congeners. The P_{50} varied in a sigmoidal fashion, with drug concentration reaching a plateau at 5–10 mM. This behavior is due to complex factors which promote Hb aggregation and impair oxygen binding in sickle cells. Both antipolymerization properties of the drug and small changes in MCHC seem to contribute equally to the shift in the ODC as estimated from the data of Sunshine *et al.* (9). Other factors which lower the P_{50}, such as an increase in Hb oxygen affinity per se, generation of met-Hb, elevation of pH, or interference with 2,3-diphosphoglycerate binding, were excluded by the lack of demonstrable change in the ODC of AA cells.

Analysis of kinetic curves of sickling with AS cells revealed a distinct inhibition of the rate of sickling due to 5 mM drug. When morphological changes are induced by a more gradual reduction in P_{O_2} and O_2 saturation, a similar decrease in the percentage of sickling was noted. At low concentrations, lofexidine, clonidine, and tolazoline had little effect. For lofexidine, the discrepancy between changes in P_{50} and the lack of significant inhibition of morphological sickling could not be explained, apart from the error of the methods. Since these agents did not decrease sickling, further investigation was not pursued.

In comparison with other anti-sickling agents (10, 11), fenmetozole is substantially potent. Since it is easily dialyzable, does not alter Hb electrophoretic mobility, and can be washed from red cells, it appears to work by either a noncovalent or reversibly covalent mechanism. That the latter might be the case was proposed by Dr. Gail H. Hurst, of the Dow Chemical Company (Fig. 2). In this scheme, a nucleophile of the protein can form a covalent bond by breaking the five-membered ring but the step can be reversed upon ring closure. The significance of this mechanism is that the first part of the reaction would permit a longer residence time on Hb, whereas the second step, facilitated by an *internal* nucleophile, might allow toxicity from binding to other proteins (e.g., eye or nerve) to be more rapidly reversed. Evidence to be reported elsewhere suggests that such a unique mechanism, although hard to prove, may indeed be operative.

The increase in Hb S solubility due to 5–10 mM fen-

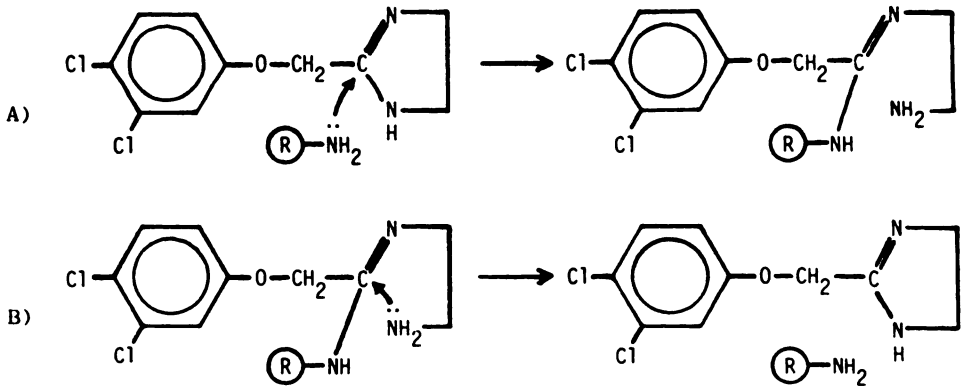

FIG. 2. *Possible reversibly covalent reaction mechanism*
A, The forward reaction; B, the reverse reaction, where *R* represents either Hb or other protein with a nucleophile such as an amino group.

metozole is well above that for other noncovalent inhibitors of gelation (12) and even some covalent modifiers of Hb at similar concentrations (Table 1). It and the three related compounds have been administered to man, albeit at lower doses. Other drugs which have been used clinically are probably toxic *in vivo* at levels required to inhibit sickling (e.g., amphetamine). Some agents, such as cetiedil (13) and the benzyl ester of phenylalanine (14), have been reported to be active at lower concentrations, but probably due to membrane effects. It should be noted that the aromatic nature of the 2-imidazolines is not a sufficient condition for inhibition of gelation, since there is a wide range of activity among these and other ring structures.

Since fenmetozole is effective at low concentrations, it may be more specific and less toxic than many drugs yet described. Still, the drug level of 5–10 mM (1.4–2.8 mg/ml) required may be difficult to achieve *in vivo*. Prior to this work, acute tolerance studies had been carried out in humans with bolus injections of up to 20 mg drug/ml i.v. over 5 min. Most patients exhibited mild hypertension and bradycardia with some postural dizziness. The mean lethal dose is approximately 17 mg/kg i.p. in rats and 27–40 mg/kg in monkeys[2]; however, a note of optimism which should be mentioned is that studies with [^{14}C]fenmetozole have shown that it is concentrated 2.5–3 times more in red cells than in plasma (which serves as a reservoir for the drug).[2] Whether it is binding to the membrane or intracellular components remains to be established. By comparison, the LD_{50} values for the less effective agents in rats are similar or higher. For lofexidine, the values range from 5 to 9 mg/kg i.v.[3]; with clonidine, from 69 mg/kg i.v. to 290 mg/kg s.c.[4]; and for tolazoline, about 100–125 mg/kg i.p.[5]

In this report, we have identified significant anti-sickling activity within the class of 2-imidazolines. They exemplify numerous drugs currently being used or developed which may be effective in disorders different from those for which they were originally designed. Thus, many agents of interest to pharmaceutical companies for other reasons should be screened for anti-sickling efficacy at nontoxic levels, and this approach may be economically viable. The continued pursuit of the reversibly covalent mechanism proposed may provide new strategies for the development of therapy for sickle cell anemia.

[2] Information furnished by the Dow Chemical Company.
[3] Information furnished by Merrell Dow Pharmaceuticals, Inc.
[4] Information furnished by Boehringer-Ingelheim Ltd.
[5] Information furnished by Ciba Pharmaceutical Company.

ACKNOWLEDGMENTS

We wish to acknowledge the helpful advice of Dr. Robert Broersma and thank Theresa Moccia and Elizabeth Ezzone for preparation of the manuscript.

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Send reprint requests to: Dr. Henry Chang, Division of Hematology, Department of Medicine, Albert Einstein College of Medicine, Bronx, N. Y. 10461.