Comparative Evaluation of Fifteen Anti-Sickling Agents

By Henry Chang, Sandra M. Ewert, Robert M. Bookchin, and Ronald L. Nagel

Fifteen compounds reported to be inhibitors of gelation or sickling were studied by standard methods. These tests included (1) the determination of the solubility of deoxyhemoglobin S or Csat, (2) evaluation of sickling in whole SS blood at various p02's, (3) measurement of the oxygen affinity of hemoglobin and blood, and (4) examination of red cell indices and morphology. Among the 4 noncovalent agents tested, buty lurea was the most potent inhibitor of gelation and sickling in vitro; however, relatively high concentrations were required compared to the covalent agents. In the latter group, bis-(3,5 dibromosalicyl)-fumarate, nitrogen mustard, and dimethyladipimidate were especially effective inhibitors of gelation and/or sickling. All of these compounds require further development before they can be considered for clinical use.

O V E R T H E Y E A R S, numerous therapies have been proposed for sickle cell anemia. Although most have had a rational basis, independent verification of the antischickling properties of many compounds has been lacking. Furthermore, since the methods used by different researchers varied, uncertainty arose about the relative potency of various compounds and the choice of agents worthy of further investigation.

We report the results on 15 antisickling agents that were studied by a uniform set of procedures devised to examine hemoglobin function and specific aspects of the sickling process. The tests included (1) a measure of the inhibition of HbS gelation (determined by the solubility of concentrated deoxyHbS after ultracentrifugation of the gel, or Csat), (2) evaluation of cellular sickling under more physiologic conditions in vitro, using whole blood at various oxygen tensions and levels of O2 saturation, (3) assessment of the compounds' effects on oxygen affinity per se using dilute HbS solutions and AA red cell suspensions, and (4) calculation of the red cell indices, plus microscopic examination to detect any changes in cell size or shape due to the agents.

MATERIALS AND METHODS

Venous blood was collected in heparin from donors with sickle cell (SS) disease who had less than 5% HbF and was used within 48 hr. Hemolysates were prepared by Drabkins' technique,1 and HbS was purified by chromatography on DEAE-cellulose (Whatman, Clifton, N.J.).2 Drug samples were prepared under physiologic conditions that are rapidly hydrolyzed (e.g., mechlorethamine, mono- and bifunctional imidoesters); preparation times did not vary by more than 1–2 min. Finally, if compounds were known not to cross membranes easily, isotonic solutions of buffer and drug were mixed to obtain the desired concentrations to prevent hyperosmolarity in cellular studies.

The covalent agents all tended to enhance methemoglobin S formation in Hb solutions after a 1-hr incubation at 37°C. Subsequently, the Hb samples were dialyzed to stop the reaction and remove by-products. If more than 5% methemoglobin was detectable spectrally,3 it was reduced with phenazine methosulfate and NADH,4 followed by purification on a Sephadex G-25 column before oxygen affinity measurements. There was generally little oxidation of hemoglobin within red cells because of their intrinsic reduction system. Treatment of HbS and red cells with the imidoesters was carried out at pH 8.2, and restoration of assay conditions was accomplished by dialysis, extensive washing in the appropriate buffer, or resuspension of the red cells in plasma. In the case of cystamine, after reaction for 1 hr at 37°C with hemoglobin, dialysis was not performed because the covalent modification is reversible. For oxygen affinity determination on HbS solutions treated with this compound, the incubation time was shortened to 15 min in order to minimize methemoglobin formation. The following tests were performed:

Solubility of DeoxyHbS (Csaf)

The method used was adapted from Hofrichter et al.3 and Noguchi and Schechter.6 Purified HbS was concentrated by ultrafiltration during dialysis against 0.1 M KPO4 buffer, pH 7.35. This solvent was chosen because its ionic strength is only moderately higher than that inside the red cell, while it provides sufficient buffer capacity. Calculated amounts of Hb were placed in small vessels for reaction at known molar ratios with the compounds dissolved in the same buffer. The samples were flushed with nitrogen and chilled on ice before a fresh solution of sodium dithionite (G. Frederick Smith Chemicals, Columbus, Ohio), prepared in deoxygenated buffer, was added anaerobically as a 3:1 molar ratio per heme to deoxygenate the hemoglobin solutions. The mixtures (final concentration 20–25 g Hb/dl) were kept cold and transferred into quartz EPR tubes (no. PQ701, Wilmad Glass, Buena, N.J.) by introducing them under paraffin oil with gas-tight syringes. After the samples were allowed to gel overnight at 25°C, they were spun in a Beckman ultracentrifuge Model L-2-65B, Palo Alto, Calif.) at 140,000 g for 2 hr at the same temperature. Complete deoxygenation of the liquid phase was verified first by infra-red spectroscopy,3 and the entire supernatant

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was removed with a syringe. Its Hb concentration was determined after conversion of aliquots to cyanmethemoglobin with Drabkins' reagent, and the pH was measured with a Radiometer microelectrode (model G297/G2, Copenhagen, Denmark). Each result was expressed as a relative solubility ratio, i.e., the supernatant concentration of the treated sample divided by that of the control.

**Oxygen Equilibrium Studies**

The same samples of HbS as used for the Cm experiments were diluted to under 10 g/dl with 0.15 M KPO₄, pH 7.35. Measurements were carried out in this buffer rather than with stripped hemoglobin because leftward shifts were found easier to detect when compared to control curves situated relatively toward the right (data not shown). In the case of noncovalent agents, the diluent contained an appropriate concentration of the test drug.

Whole AA blood was treated in a 1:1 (v/v) ratio with solution of the drug in isotonic NaPO₄, pH 7.35, followed by incubation if required. For the study of red cell suspensions, a few drops of this described. From these data, a 15 oxygen dissociation curve was calculated from standard tables) were <0.05.

**Red Cell Parameters**

Normal AA blood was used because of its uniform cell population and prepared as described above. The hemoglobin concentration was determined by the use of Drabkins' reagent, the microhematocrit by centrifugation, and the red cell count with a Coulter counter (Model ZBI, Coulter Electronics, Hialeah, Fla.). From these measurements, the red cell indices (MCV, MCH, MCHC) could be computed manually. The percentage of methemoglobin produced was measured on a CO-Oximeter (model 282, Instrumentation Laboratories, Lexington, Mass.) and the cell were examined under an interference contrast microscope (Nomarski optics) for morphological changes.

**Sickling Curves**

The effect of each drug was assessed in whole sickle blood because plasma binding could alter its solubility and/or distribution and thus influence its potency. For these studies, the concentrations of drug attainable were limited by restrictions in solubility and osmolarity. In addition, no more than 1:3 (v/v) ratio of drug solution to SS blood was used, so that conditions similar to whole blood could be maintained. After incubation, if necessary, the blood was placed in an IL237 tonometer at 37°C and progressively deoxygenated with varying proportions of O₂, N₂, and 5.6% CO₂. After 10 min equilibration with each gas mixture, the pO₂, percent O₂ saturation, and pH of the sample were measured, and an aliquot was transferred into buffered formalin for examination of cell morphology as previously described. From these data, an oxygen dissociation curve was plotted. In addition, the percentage of newly sickled cells was calculated from the equation:

\[
\text{Percent sickled cells} = \frac{\text{Number of sickled cells} - \text{ISCs}}{\text{Total cells counted} - \text{ISCs}} \times 100
\]

and graphed as a function of pO₂ or percent O₂ saturation. Deformed cells (with membrane irregularities but no spiculation) were not included in this figure.

**Statistical Analyses**

A one-tailed t test was carried out for matched samples. The results were considered to be significant if the p values (obtained from standard tables) were <0.05.

**RESULTS**

**Statistical Analysis of Controls**

**Solubility of deoxyhemoglobin S.** The results of a series of 10 Cₒ₅₅₃ in the absence of drugs showed a mean ± SD of 17.4 ± 0.29 g/dl at 25°C with a final pH range of 6.80–6.90 after the addition of dithionite.

**Oxygen equilibrium studies.** A series of 10 oxygen dissociation curves for dilute solutions of HbS in 0.15 M KPO₄ buffer, pH 7.35, at 37°C showed a reproducible P₅₀ of 15.8 ± 0.3 torr (1 SD). For 10 samples of fresh AA cells (with under 0.5% methemoglobin) in 0.15 M NaPO₄ buffer, pH 7.35, at 37°C, the average P₅₀ was 26.0 ± 1.0 torr.

**Red cell parameters.** Specimens of blood from 5 normal donors, used as controls, showed the following range of red cell indices: MCV = 82–92 fl, MCH = 27–31 pg, MCHC = 32–35 g/dl.

**Values for whole SS blood.** Since oxygen affinity and the propensity to sickle are inherently variable for the blood of different SS donors, statistical comparisons of values in the presence or absence of drug must be made with portions of a sample taken from a single individual. A series of 4 patients with SS disease were studied repeatedly to establish the error of the methods. For each individual, the standard error for the determination of P₅₀ was ±1.5 torr; for the pO₂ at which 50% of the cells became sickled, ±2.7 torr; and for the percentage of newly sickled cells at 50% saturation, ±3.5%.

**Compounds Studied**

For conciseness, an interpretation of the results for each agent is given and followed by comparison with the literature. A summary of all the agents is presented with average values in Tables 1 and 2; however, a full data set can be obtained from the authors.

**Noncovalent Agents**

**Urea**

This compound was suggested originally because of its ability to disrupt hydrophobic bonds. Measurements of Cₛₑₐ in the presence of urea showed a small,
Table 1. Noncovalent Agents

<table>
<thead>
<tr>
<th>Noncovalent Agents</th>
<th>C&lt;sub&gt;m&lt;/sub&gt;</th>
<th>O&lt;sub&gt;2&lt;/sub&gt; Affinity</th>
<th>Whole SS Blood (paired samples)</th>
<th>RBC Indices and Morphology</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Concentration</td>
<td>Molar Ratio (Drug: Heme)</td>
<td>Relative Solubility</td>
<td>P&lt;sub&gt;50&lt;/sub&gt;</td>
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</tr>
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<td>6.45</td>
<td>1.08‡</td>
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<td>200mM</td>
<td>12.90</td>
<td>1.14‡</td>
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<td>31 31 2.9 26 28 35 30</td>
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<tr>
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<td></td>
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<td>28 30 2.6 27 28 35 39</td>
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<tr>
<td>50mM</td>
<td>3.22</td>
<td>1.19‡</td>
<td>+0.2 2.5 +0.3 2.7</td>
<td>26‡ 28‡ 2.6 23‡ 18‡ 39‡ 24‡</td>
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</tr>
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<td>1.00</td>
<td>0.2 2.5 -0.5 2.6</td>
</tr>
<tr>
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<td>1.01</td>
<td></td>
<td>32 32 2.8 25 26 37 35</td>
</tr>
</tbody>
</table>

Numbers are the averages of at least 2 determinations.

‡pO<sub>2</sub>(50%S) is the pO<sub>2</sub> in torr at which 50% sickling is observed.

†%S(P<sub>50</sub>) is the percentage of sickled cells at half oxygen saturation.

‡Significant at the p < 0.05 level.

NE, not evaluable.

but significant elevation in the solubility of deoxyhemoglobin S (Fig. 1A), in agreement with previous findings.\(^5,10\) The oxygen affinity of dilute HbS solutions and AA red cells showed small changes that were not significant but were similar to observations by May and Huehns\(^11\) and Segel et al.\(^12\) The red cell volume was slightly increased and the MCHC slightly lowered at urea concentrations of 200 mM. The P<sub>50</sub> of whole SS blood was reduced by 2–3 torr but there was no significant change in the pO<sub>2</sub> for 50% sickling or in the percent sickled cells at P<sub>50</sub> (Table 1). Thus, as also found by others,\(^5,10,12\) urea does not appear to be effective at concentrations of 100–200 mM. Clinical trials of this agent have not proven it to be useful in the treatment or prevention of painful crisis.\(^13\)

**Butylurea**

This compound was the most potent of the alkylureas reported by Elbaum et al.\(^14\) to inhibit gelation. The authors showed that a 0.1 M concentration of butylurea increased the minimum gelling concentration of HbS from 24 to 34 g/dl. There was a 1 torr drop in the P<sub>50</sub> of dilute HbA solutions in 0.15 M KPO<sub>4</sub> buffer, pH 7.35 at 23°C. Treatment reduced the percentage of sickled and deformed cells found in SS blood with progressive deoxygenation, and later it was reported that the rate of sickling was slowed 5–6-fold by 50 mM butylurea at 23°C.\(^8\)

We have verified that butylurea increases the solubility of deoxy HbS by a relative C<sub>m</sub> ratio of 1.18–1.20 at 50 mM (Fig. 1A). These results are comparable to the values of 1.21 reported by Behe and Englander\(^15\) and 1.17 by Poillon.\(^16\) There is no change in the oxygen affinity of dilute HbS solutions or AA red cells. The MCV of normal cells is increased and the MCHC slightly reduced by 20–50 mM butylurea, but their shapes appear normal. The former alterations could contribute to the effects seen in whole SS blood, where there are detectable decreases in the P<sub>50</sub>, pO<sub>2</sub> for 50% sickling, and percent sickling at P<sub>50</sub> with a 20-mM concentration, but larger, more significant decrements at 50 mM (Table 1). Thus, butylurea appears to be an effective antisickling agent.

**L-Phenylalanine**

Amino acids and oligopeptides have been proposed as stereospecific inhibitors of gelation.\(^17,19\) We have confirmed that L-phenylalanine enhances the solubility of deoxy HbS linearly with increasing drug concentration (Fig. 1A); these data are similar to the results of Noguchi and Schechter\(^6\) and Sunshine et al.\(^18\) In addition, we demonstrated no change in the oxygen affinity of dilute HbS solutions or AA red cells, nor in erythrocyte indices or morphology. For whole SS blood, there was no significant alteration in P<sub>50</sub> or sickling with the agent, findings compatible with evi-
Table 2. Covalent Agents

<table>
<thead>
<tr>
<th>C&lt;sub&gt;m&lt;/sub&gt;</th>
<th>Molar Ratio</th>
<th>Relative Solubility (Drug/Host)</th>
<th>HbS Solution</th>
<th>AA Cells</th>
<th>Whole SS Blood (paired samples)</th>
<th>RBC Indices and Morphology</th>
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</thead>
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<td>Concentration</td>
<td>(Drug/Host)</td>
<td>∆P&lt;sub&gt;50&lt;/sub&gt;</td>
<td>∆P&lt;sub&gt;50&lt;/sub&gt;</td>
<td>P&lt;sub&gt;50&lt;/sub&gt;</td>
<td>∆P&lt;sub&gt;50&lt;/sub&gt;</td>
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<td>Potassium cyanate</td>
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<td>—</td>
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<tr>
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<td>2.2</td>
<td>-9.0</td>
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<tr>
<td>Bis (3, 5 dibromosalicylic) succinate</td>
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<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
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<td>-16.5</td>
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<td>Nitrogen mustard (methylnitrosourea)</td>
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<td>—</td>
<td>—</td>
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</tr>
<tr>
<td>5mM</td>
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<td>-2.6</td>
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</tr>
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<td>1.39</td>
<td>-3.4</td>
<td>2.3</td>
<td>-2.0</td>
<td>2.5</td>
</tr>
</tbody>
</table>

Footnotes as in Table 1.

dence that the substance does not enter erythrocytes readily. Thus, this compound is an effective inhibitor of gelation but does not prevent cellular sickling.

**Cetiedil Citrate Monohydrate**

This drug has been reported to reduce sickling and improve red cell filterability, however, our tests showed that it did not inhibit gelation (Fig. 1A). Moreover, it did not affect the oxygen affinity of HbS or SS cells, and there was no significant change in red cell indices or morphology. A decrease in sickling was described originally by Benjamin et al. and Asakura et al., but their results differed: the former noted a maximal effect at 100 μM, which diminished as the drug concentration was raised, but the latter found no limiting dose up to several hundred micromolar. Both groups observed improvement in red cell filterability or deformability, properties which we did not test.

Our studies show that 500μM - 1mM cetiedil has little effect on the P<sub>50</sub> and sickling of whole SS blood.
Fig. 1. The comparative effect on HbS solubility of the non-covalent agents studied: urea, butylurea (Bur), 1-phenylalanine (Phe), and cetirolid (Cet). The points correspond to average values of 2 or 3 determinations.

Ekvaluation of anti-sickling agents

Non-covalent Agents

(A) Mildly effective

- Urea
- Cystamine (Cys)
- Pyridoxal (Pyr)
- Cyanate (KNCO)
- Carbamyl phosphate (Carb.-P)

(B) Moderately effective

- Methylacetimidate (MeAc)
- Dimeinthyladipimidate (DMA)
- Glyceraldehyde (Gly)

(C) Very effective

- Nitrogen mustard (HN2)
- Bis(3,5-dibromosalicyl) fumarate (bisBr2 sal.-fum.)
- Succinate (bisBr2 sal.-suc.)
- Dibromoacetyl-saliclylic acid (Br2 ASA)

Fig. 2. The comparative effect on HbS solubility of the covalent agents studied.

(A) The weaker agents: cystamine (Cys), pyridoxal (Pyr), cyanate (KNCO), carbamyl phosphate (Carb.-P).

(B) Moderately effective agents: methylacetimidate (MeAc), dimethyladipimidate (DMA), glyceraldehyde (Gly).

(C) Stronger agents: nitrogen mustard (HN2), bis(3,5-dibromosalicyl) fumarate (bisBr2 sal.-fum.) and succinate (bisBr2 sal.-suc.). Dibromoacetyl-saliclylic acid (Br2 ASA) is added for comparison. In most experiments, the drug was reacted with a standard hemoglobin solution to produce a final heme concentration of 5 mM, so the effects due to drug concentration correspond to those for molar ratios of drug to heme. Some studies with HN2 (open circles) were carried out at a higher heme concentration (15 mM), so the effect appears to be greater when molar ratio (O-O) is considered instead of absolute drug concentration (O—O).
tion (Table 2). The latter finding agrees with the results of May et al.,30 Diederich,32 and Jensen et al.,33 which indicate that small amounts of cyanate (<50 mM) inhibit sickling by an oxygen affinity-dependent mechanism, but greater quantities block intracellular polymerization more directly. Thus, this agent seems to be effective by two different mechanisms.

Carbamyl Phosphate

This agent was proposed by Wood and Kraus et al.34 as an alternative to cyanate. It appeared to inhibit sickling up to 80% at 4–8 mole drug/mole Hb when incubated from 45 to 125 min35 and increased the survival of a 51Cr-labeled aliquot of blood. We have confirmed that there is a gradual inhibition of deoxy-HbS gelation with increasing drug concentration (Fig. 2A). The compound raises the oxygen affinity of dilute HbS solutions and AA cells, but the red cell indices and morphology remain normal. Since the agent is a disodium salt, drug concentrations were limited by osmotic restrictions in the studies on whole SS blood. At 50 mM, the cells were treated, centrifuged, and suspended in plasma to reconstitute whole blood. Although the P50 was clearly lowered by the compound, the PO2 for 50% sickling was significantly lowered only at 50 mM. Sickling as a function of oxygen saturation was not inhibited, which supports the idea that the agent works primarily by altering oxygen affinity (Table 2). It should be kept in mind that the drug carbamylates Hb only 60%–70% as readily as cyanate,36 may not get into cells as readily as its metabolite cyanate, and is detoxified by phosphatases in tissues, which could provide a margin of safety. The breakdown products from the latter process, ammonia and CO2, also could influence red cell function in vitro. Thus, carbamyl phosphate is a mild antisickling agent that operates by increasing oxygen affinity.

Cystamine

This agent reacts reversibly with sulphydryl groups and has been shown by Hassan et al. to inhibit sickling.37 We confirmed their observation that it increases the oxygen affinity of HbS and SS cells and demonstrated a similar behavior with AA cells. In whole SS blood, the reduction in sickling appears to be dependent only on changes in oxygen affinity (Table 2). This result is supported by the lack of antipolymerization activity as measured by Csat assay (Fig. 2A). The above authors also found a 5%–10% decrease in the MCHC of cystamine-treated red cells; however, they employed dilute cell suspensions with longer incubation times. Under such conditions of high drug-to-hemoglobin ratios, membrane sulphydryl groups also could be affected. In this circumstance, we noted a small increase in MCV, but it was accompanied by an elevation in methemoglobin content. Thus, this anti-sickling agent is effective, insofar as it alters oxygen affinity.

Pyridoxal HCl

Pyridoxal phosphate was studied first by the Ben-esches38 as a modifier of the β-NH2 terminus of hemoglobin, but it traverses the red cell membrane with difficulty. This was not a problem with pyridoxal, which was shown by Zaugg et al.39 to form a Schiff base adduct with hemoglobin. Kark et al.40 found that the compound could inhibit sickling at 20 mM concentration, primarily by increasing oxygen affinity. We have determined that pyridoxal is a weak inhibitor of gelation (Fig. 2A) and increases the oxygen affinity of HbS, AA, and SS cells. Sickling is inhibited as a function of PO2 but not of oxygen saturation (Table 2), which confirms the observations of the latter authors. More recent evidence suggests that pyridoxal may be rapidly eluted from red cells41 and that it may fall into the class of reversible covalent agents.

Methylacetimidate HCl

This monofunctional imidoester has been reported to inhibit gelation and sickling.42 In our preliminary experiments, this agent appeared to be inactive at physiologic pH, so reactions were carried out in alkaline buffers. Readjustment to conditions for the assays was usually possible, except that the difference between intra- and extracellular pH remained about 0.05 units less than normal. Our Csat results show a progressive increase with drug concentration (Fig. 2B), which parallels the rise in minimum gelling concentration observed by Chao et al.43 Confirming the work of these authors, we found that the oxygen affinities of HbS, AA, and SS cells are also elevated by this compound. In whole SS blood, which had been reconstituted with plasma after drug treatment of the red cells, significant inhibition of sickling was oxygen affinity-dependent and observed only at the higher concentration of 10 mM; however, sickling as a function of O2 saturation was decreased only marginally (Table 2). Thus, this compound inhibits gelation, but sickling in whole SS blood is reduced primarily by an increase in oxygen affinity and perhaps by a small effect on polymerization. The unusual reaction conditions (high pH) suggest that this agent can be used only for the extracorporeal treatment of blood.

Dimethyladipimidate

This agent was described by Lubin et al.44 to cross-link amino groups and inhibit red cell sickling, potas-
sium loss, and a rise in SS blood viscosity upon deoxygenation. At a concentration of 5 mM, the oxygen affinity of SS blood was increased from a P50 of 33 to 24.1 torr and AA blood from 28.7 to 23 torr, largely due to modification of hemoglobin α-chains. High molecular weight species of crosslinked tetramer were described by these authors and Waterman et al.45

More recent work by Lubin et al.46 revealed that the deoxyHbS solubility was increased from 16 g/dl to 19.5 g/dl at 5 mM and 22.5 g/dl at 9 mM (relative Csat of 1.21 and 1.41). These samples were reacted with the compound in the dry state to prevent hydrolysis during preparation. However, we prefer to predissolve the drug for better pH control and to minimize protein denaturation, so we found more modest increases in relative solubility of 1.06 and 1.12 at these concentrations (Fig. 2B). The oxygen affinities of HbS, AA, and SS cells were increased unequivocally, but sickling was reduced only at the higher concentration of 10 mM (Table 2). Thus, this compound is an effective antisickling agent; membrane crosslinking also may have helped to retard the formation of sickle cells.

Glyceraldehyde

This compound may bind to HbS as a Schiff base and inhibit gelation and sickling.47,48 In our assays, the solubility of deoxyHbS was raised substantially (Fig. 2B) in accord with elevations in minimum gelling concentrations found by Nigen and Manning. These authors, under different conditions, also found that the oxygen affinity curve of HbS in cell lysates was shifted to the left by 20 mM glyceraldehyde. At this concentration, the oxygen affinity of AA cells was increased slightly, and for whole SS blood, the P50 and the percentage sickling as a function of both pO2 and O2 saturation were all significantly reduced (Table 2). Thus, glyceraldehyde appears to be a moderately effective antisickling agent in vitro.

Dibromoacetylsalicylic Acid

This aspirin analogue has been reported to have no effect by Kokkini et al.49 or an inhibitory one on sickling by Walder et al.50 Our studies showed that it increases the solubility of deoxyHbS at 5 and 10 mM concentrations (Fig. 2C), which tends to support the findings of the latter authors. There was no increase in the oxygen affinity of HbS with 5 mM drug compared to the control, as the pO2 was lowered, but the difference only bordered on significance. With 10 mM drug, the cells were initially deformed and could not be counted, even though the continued fall in P50 of the blood suggested a further inhibition of sickling. Thus, this compound appears moderately effective, but its interaction with the red cell membrane may limit its usefulness in vivo.

Bis(3,5-Dibromosalicylic) Fumarate and Succinate

These bifunctional aspirins were proposed and shown by Walder, Klotz, and colleagues51,52 to crosslink the two beta chains of hemoglobin between β82 lys and β82 lys. DeoxyHbS was modified more extensively by the fumarate derivative, and its solubility was 35% greater than the control. The oxygen affinity of Hb and red cells, however, was increased due to blockade of the 2,3-DPG binding site and noncovalent binding of the 3,5 dibromosalicylic acid moiety. The P50 of a 20% normal erythrocyte suspension treated with 1 mM for 2 doses over 2 hr at 37°C was lowered from 30 to 20 torr. In the first paper, the authors mentioned that sickling was not inhibited "to any greater extent than could be accounted for by 3,5 dibromosalicylic acid released upon the transacylation reaction," but there were abnormal morphological changes attributed to cell washing.

We have confirmed that gelation is substantially inhibited, with the fumarate more potent than the succinate derivative at concentrations of 2–5 mM (Fig. 2C). In addition, the oxygen affinity increases correspondingly for HbS, but with AA cells, this functional alteration occurs in reverse order for the two analogues (Table 2). Such a result could be explained by the greater potency of the fumarate derivative to modify Hb but inferior capacity to penetrate cells. The oxygen equilibrium curves of SS blood were significantly shifted to the left, but it was not possible to evaluate cell sickling accurately because of moderate to severe echinocytosis induced by the compounds.
Nitrogen Mustard (Mechloretamine)

This compound was reported by Roth et al\textsuperscript{13} to inhibit sickling and increase the minimum gelling concentration of HbS. The effect was accomplished with very small changes in the oxygen affinity of Hb or red cells and was probably due to the modification of $\beta$2 histidine. We have confirmed that this drug is indeed a very potent inhibitor of gelation, with relative solubilities averaging 1.22–1.39 at 5 and 10 mM, respectively (Fig. 2C). These values represent rapid reaction conditions with hemoglobin, since the drug is quickly hydrolyzed while being prepared. The oxygen affinities of dilute HbS solutions and AA cells are moderately increased, the latter less so on a percentage basis. This result may be explained by consumption of drug due to alkylation of membrane of plasma proteins, but furthermore, the reaction by-product, HCl, could lower intracellular pH. With the in vitro system using SS blood, the cells had to be washed and resuspended in plasma before assay; nevertheless, the intracellular pH tended to be about 0.05 units lower than normal. This could mitigate excessive increases in oxygen affinity. The $P_{50}$ for 50% sickling and the percent sickling at $P_{50}$ were significantly lower at both 5 and 10 mM. Thus, this compound is a potent antischisking agent, but because it is toxic upon systemic administration, can be used only for the extracorporeal treatment of blood.

Comparison of the Compounds

Among the noncovalent inhibitors, urea is a weak agent in vitro (Fig. 1), which at a level of 0.2 M or less, is unable to prevent sickling. Butylicurea is more potent and may be effective at 25–50 mM. On the other hand, L-phenylalanine also inhibits gelation, but does not block sickling, probably because it cannot enter erythrocytes readily. For cetiedil citrate, the results differ from previous reports because we found no significant inhibition of gelation or sickling under our test conditions.

The covalent agents may be divided into weak, moderate, and strong inhibitors of gelation (Fig. 2, A–C). The most potent compounds appear to be the bifunctional dibromosalicylates, followed closely by nitrogen mustard, particularly when compared at the same molar ratios of drug to heme (Fig. 2C). Similarly, the order of the moderately effective agents shows that dibromocetisalicylic acid and the imidoesters are comparable and slightly more potent than glyceraldehyde on a concentration basis, but the latter is actually more active at a given drug:heme ratio. This behavior could be due to differences in the reactivity of the compounds. The remainder of the agents are weaker and require higher concentrations to be effective.

An increase in oxygen affinity seems to be a common feature after treatment of hemoglobin or blood with covalent agents (Fig. 3). This functional alteration can inhibit sickling but also may contribute to a rise in hematocrit that increases blood viscosity. Although the ideal antisickling agent should maximally inhibit polymerization with the least change in the intrinsic oxygen affinity of the hemoglobin molecule, a simple ratio between the two parameters could be hard to interpret because small changes in $P_{50}$ may not be significant and the absolute potency of each agent should be considered. The strongest inhibitors of gelation, the bifunctional dibromosalicylates, are specific for the 2,3-DPG pocket and significantly raise oxygen affinity in phosphate buffer. This increase is somewhat less for nitrogen mustard. The agent with the greatest influence on oxygen affinity with the least effect on gelation appears to be cystamine.

In oxygen affinity experiments on cells, the molar ratio of drug to heme was kept uniform, as stock solutions were mixed 1:1 with whole AA blood or 1:3–4 with SS blood to achieve approximately the same final hematocrits. Since each compound behaved similarly with the two types of blood, only the results with SS samples are plotted (Fig. 4A). The factors governing the oxygen equilibrium curve are complex in SS patients and the baseline $P_{50}$s vary, so the shift in oxygen affinity is expressed as a percentage of control values. When this is plotted as a function of drug concentration, the largest changes are seen with pyridoxal and the mono- and bifunctional dibromosalicylates. Significant changes also occur with dibromocetisalicylic acid and cystamine, which produce a larger change in oxygen affinity than the other compounds at equivalent concentrations. Compared to the results with HbS, the agents differ somewhat in their abilities to alter oxygen affinity. The reasons for this may include the ease with which the compound can penetrate the membrane, and the effects of reaction byproducts on cell function (e.g., changes in intracellular pH or 2,3-DPG binding).

Studies on whole sickle blood can yield two other types of information. Changes in the fraction of newly sickled cells may be observed upon a drop in $P_{50}$. Generally, these results tend to parallel the shift in $P_{50}$. Of greater interest, however, is the fall in the proportion of newly sickled cells at a given state of oxygen saturation and R-T conformation (e.g., at $P_{50}$), which reflects antipolymerization activity independent from oxygen-affinity effects. When these numbers also are expressed as a percentage decrease relative to concentration of drug (Fig. 4B), it should be kept in mind that the method requires a large reduction in sickling to be significant (about 20% at $P_{50}$). Agents that do not meet this criterion still may have a small effect on sickling,
Fig. 3. (A) The change in $P_{50}$ of HbS solutions (ordinate) versus the concentration of drug or molar ratio of drug to heme (abscissa). Numbers used are averages of 2 determinations. Slightly greater increases in oxygen affinity on the molar ratio scale for Gly, Pyr, KNCO, and Carb.-P were seen but omitted for the sake of clarity. A larger change in $P_{50}$ was evident for nitrogen mustard when the molar ratio is considered (see explanation for dashed line in legend to Fig. 2C). (B) The percentage change in $P_{50}$ of SS blood (ordinate) with drug concentration (abscissa).

Fig. 4. The percent change in sickling at $P_{50}$ (ordinate) versus the covalent drug concentration (abscissa). Numbers used are averages of 2 determinations. Note that the percent change in $P_{50}$ is plotted because of inherent variation among controls.

which could be verified by more extensive testing. Unfortunately, the dibromosalicylates caused membrane changes that precluded morphological evaluation, so the most potent agent appeared to be nitrogen mustard, followed by dimethyladipimidate. Glyceraldehyde and potassium cyanate may be effective at higher concentrations. As for methylacetimidate, the percentage reduction in sickling bordered on significance at 10 mM, and it is possible that the change would have been greater at higher doses. However, because of the manipulations at alkaline pH required for the compound to react, control values for the percent sickling at $P_{50}$ were lowered and could have resulted in an artifactually high percentage change in sickling of treated cells.

DISCUSSION

Various therapeutic strategies for sickle cell anemia have been reviewed.\textsuperscript{54-56} This work facilitates the evaluation of the agents, since similar conditions were employed. The tests were chosen not only to compare
certain characteristics of the compounds, but also to reveal their mechanism of action (e.g., inhibition of gelation, increase in oxygen affinity, change in MCHC, etc.). Although these results may be useful for predicting the eventual behavior of antisickling agents in vivo, it should be remembered that other aspects of the disease process, such as red cell deformability and blood viscosity, were not studied and could be important for the generation of crises and red cell survival. Thus, the terms “efficacy” and “potency” used here refer only to the in vitro activity of the agents. Further caution should be exercised in comparing our data with those of various investigators who used different conditions for reactions and testing.

For the noncovalent agents studied, butylurea is the most potent in vitro, yet no extensive pharmacokinetic or toxicologic information is available. With ceteddil citrate under our conditions, we found no significant inhibition of gelation or sickling, although this compound could be effective under circumstances described by other investigators. Toxicity data indicate that there are few side effects of this drug, so clinical trials are currently in progress. The other two noncovalent agents are unlikely to be useful clinically in their present form. To be effective, urea requires a concentration that is too high to be reached safely in vivo. Phenylalanine can inhibit gelation but does not enter erythrocytes readily, so derivatives such as the benzyl ester are currently under investigation.

Most of the covalent modifiers of hemoglobin inhibit gelation and sickling at lower concentrations. Currently, the strongest inhibitors of gelation tested are the bifunctional dibromosalicylates, followed closely by nitrogen mustard. The former compounds appear to be specific for the 2,3-DPG binding site of hemoglobin, but consequently, increase oxygen affinity, an effect that may not be desirable. Furthermore, they cause echinocytic shape changes, and red cell survival studies should be carried out. This finding suggests that the agents may interact with membrane components, so the toxicity of these compounds also needs to be established. As for nitrogen mustard, its toxicity is known to be high, so the use of this agent is limited to the extracorporeal treatment of blood. Its effect on the oxygen affinity of red cells appears significantly less than that of the dibromosalicylates, and preliminary studies indicate that it prolongs the survival of sickle cells.

In the moderately effective group, dimethylidipimi-
date and glyceraldehyde also merit red cell survival studies, since they can crosslink proteins. For methylaceticmide, this work has been reported: the red cell lifespan initially increases but falls later in some patients, probably due to altered membrane antigenicity. This problem may occur with other covalent agents and will require additional precautions, such as the blocking of membrane amino groups with pyridoxal-5'-phosphate to prevent their reaction. The imidoesters, because of their special reaction conditions requiring high pH, are suited only for extracorporeal use with the current modes of drug delivery.

Although relatively less effective in vitro, cyanate has reached the furthest level of development. It has been administered to patients with an improvement in hematologic parameters, but no amelioration of sickle cell crises. Chronic ingestion can produce cataracts and peripheral neuropathy, so blood now is being treated extracorporeally with high concentrations of cyanate and then returned to patients. The experience with small oral doses, which increased oxygen affinity without preventing crises, suggests that pyridoxal and cystamine, which work largely by shifting the oxygen equilibrium curve, probably will not be useful alone (although this is a controversial point). The usefulness of the other agents remains uncertain.

Thus, none of the compounds studied appear to be the optimal agent for the treatment of sickle cell anemia, yet some of them might find their way to practical application. In any case, the cumulative knowledge that the various investigators have contributed to the development of these agents has greatly improved the likelihood of arriving at a therapy in the foreseeable future.

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Comparative evaluation of fifteen anti-sickling agents

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