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LAWSONE (2-OH-1,4-NAPHTHOQUINONE) DERIVED FROM THE HENNA PLANT INCREASES THE OXYGEN AFFINITY OF SICKLE CELL BLOOD

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SUMMARY: We examined an extract of the henna plant for the ability to inhibit the sickling of hemoglobin S-containing red cells upon deoxygenation in vitro and found that it could do so. Further investigation revealed that the active principle was lawsone, 2-OH-1,4-naphthoquinone, which produced an increase in the oxygen affinity of SS blood at levels as low as 1 mM of the compound but with little change in the characteristics of normal blood at this concentration.

In screening for potentially useful agents in the treatment of sickle cell anemia, we tested substances which are known to bind to proteins. One of these was an extract from the henna plant (Lawsonia alba) which is used as a hair conditioner and for a variety of medicinal purposes. We discovered that it could increase the oxygen affinity of SS blood and inhibit sickling. From the literature, we obtained information about the composition of the leaves and tested two of the constituents, gallic acid and 2-OH-1,4-naphthoquinone (lawsone). This report provides evidence that the latter ingredient is responsible for the effects observed.

METHODS

Venous blood was collected in heparin from donors with sickle cell (SS) disease who had less than 5% Hb F, and was used within 2 days of venipuncture. Hemolysates were prepared by Drabkins' technique (1) and Hb S was purified by chromatography on DEAE-cellulose (Whatman, Clifton, NJ) (2). Henna was obtained as a powder from dried leaves (Meta Henna International, Inc., Elk Grove, IL), dissolved in the appropriate buffer, and the mixture was centrifuged to remove undissolved material. Lawsone and gallic acid were obtained from Sigma (St. Louis, MO).

(1) Measurements of Oxygen Dissociation and Sickling - SS and AA red cells were suspended in 0.15 M $NaPO_4$, pH 7.35 in the presence or

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absence of henna extracts or lawsone. The preparations were placed in an Imai cell (3) and slowly deoxygenated with nitrogen at 37°C while being monitored spectrophotometrically (Cary 17D, Varian, Palo Alto, CA). Continuous oxygen dissociation curves were recorded. For SS cells, aliquots could be removed at different pO_2's and fixed in buffered formalin for counting. The percentage of newly sickled cells was calculated from the formula:

$$\frac{\text{number of sickled cells - ISC's}}{\text{total cells counted - ISC's}} \times 100$$

These results were plotted against P_{O_2} and % oxygen saturation.

With the same apparatus, the oxygen affinity of Hb S was measured after it had been stripped of phosphate on a Sephadex G-25 column (Pharmacia, Uppsala, Sweden). The buffer used was 0.1 M NaCl with 0.05 M bis-Tris, pH 7.35, to which reagents such as the di-Tris salt of 2,3-diphosphoglycerate (Sigma, St. Louis, MO) could be added if required.

(2) Solubility of DeoxyHb S (C_{sat}) - The method used was adapted from Hofrichter et al. (4) and Noguchi and Schechter (5). Purified Hb S was concentrated by ultrafiltration during dialysis against 0.1 M KPO_4 buffer, pH 7.35. 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, OH) 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. PQ 701, Wilmad Glass, Buena, NJ) 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 L2-65B, Palo Alto, CA) at 140,000 x g for 2 hrs at the same temperature. Complete deoxygenation of the liquid phase was verified first by infra-red spectroscopy (4), and the entire supernatant 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.

(3) Red Cell Parameters - Normal AA blood was mixed with a 1:1 (v/v) solution of lawsone in 0.15 M $NaPO_4$ buffer. 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, FL). 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, MA) and the cells were examined under an interference contrast microscope for morphologic changes.

(4) Animal Studies - Solutions of lawsone in 0.15 M $NaPO_4$ buffer were injected into the peritoneum of C57 black mice (Jackson Laboratories, Bar Harbor, ME). After one hour, the red cell morphology was assessed and the hematocrit and P_{50} were compared to pre-treatment values. The latter determination was accomplished on whole blood with a Hem-O-Scan (Aminco, Silver Spring, MD).

RESULTS

Three main types of henna were tested for the ability to shift the oxygen dissociation curve (ODC) of SS blood and they varied considerably. Compared to a control P_{50} of 36 torr, SS cells suspended in an extract of red henna showed the largest change (P_{50} = 23), followed by neutral (P_{50} = 29), and black henna (P_{50} = 34) (Table I). From a literature search, we learned that two major components in the leaves were gallic acid and lawsone (6). We obtained them as purified reagents, but the former was difficult to test. It progressively reduced the P_{O_2} of the suspension buffer and therefore could not be studied in the oxygen affinity assay. Lawsone, on the other hand, was able to lower the P_{50} of SS blood from 36 to 24 torr at 1 mM concentration and decreased the P_{O_2} required to achieve 50% sickling from 19 to 12 torr (1 S.D. = 3 torr), effects which could be removed by cell washing. The percentage of newly sickled cells at P_{50}, however, was not significantly different from the control (Fig. 1). With 10 mM compound, the P_{50} fell to 14 torr, but at this concentration the cell membrane appeared dimpled and possibly damaged. The red cells remained intact at the lower level (1 mM) of the compound, however, and there was no significant increase in methemoglobin, elevation of intracellular pH, or change in MCHC sufficient to explain the results. Comparatively, there was a small effect on the oxygen affinity of AA cells at 1 mM (the P_{50} fell only 1 torr) but an equivalent one to SS cells as the concentration was raised to 5 mM (P_{50} = 13 torr).

To study the mode of its action, we examined the effect of the compound on purified hemoglobin. A 1 mM concentration had no effect on Hb electrophoretic mobility on cellulose acetate, pH 8.2, which suggested that the agent did not permanently modify the protein. Surprisingly, 5 mM lawsone shifted the ODC of stripped Hb S to the right (from 9.7 to 10.6 torr) and did not significantly lower the P_{50} of Hb S when present with phosphate or 2,3-DPG (Table II).

TABLE I

SS Red Cells	Concentration	P_{50}	P_{O_2} (50% sickling)	% sickling at P_{50}	MCV	MCH	MCHC	pH (intra/extra-cellular)
control		36 torr	19 torr	19%	86	30	35	7.34/7.44
red henna	17 mg/10ml	23						
neutral henna	" "	29						
black henna	" "	34						
lawsone	1 mM	24	12 torr	23%	86	29	34	7.33/7.42
	10 "	14						
	1 " (then washed)	33 (control was also 33 for this experiment)						
AA Red Cells								
control	1 mM	26 torr						
lawsone	5 "	13						

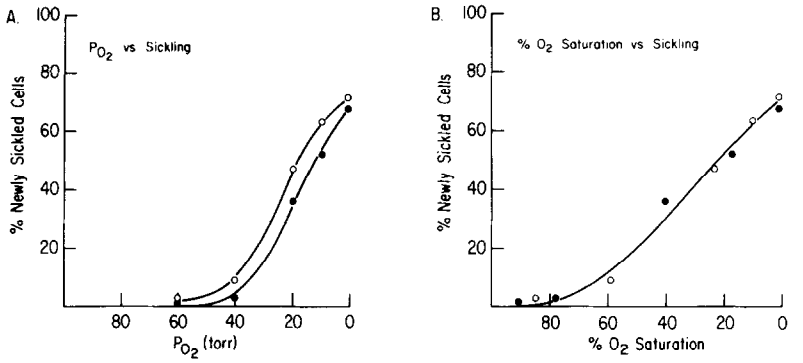

FIG. 1: Percentage of newly sickled cells as a function of P_{O_2} and O_2 saturation. Open circles: control SS cells. Solid circles: SS cells + 1mM lawsone.

Lawsone did not increase the solubility of deoxyHb S (Table III). The supernatant Hb concentration of an ultracentrifuged gel was not increased by 2-10 mM, which indicates that the compound does not inhibit aggregation directly. Finally, quantities of lawsone sufficient to achieve 1 mM in total body water were injected into the peritoneal cavities of two mice without adverse effect or an increase in the oxygen affinity of the blood, although 3 times this dose produced anemia and a 10-fold greater amount produced prostration.

DISCUSSION

This report describes a new, potent anti-sickling agent. It appears to work non-covalently by increasing the oxygen affinity of SS red cells. Efforts to establish its precise mechanism of action were unsuccessful: the compound actually raised the P_{50} of stripped Hb S and did not alter the oxygen dissociation curve in the presence of 2,3-DPG. Thus the basis for its effect still remains enigmatic.

TABLE II

	Lawsone moles/Hb	0	1	2	3	
2,3-DPG moles/Hb	0	9.7(15.9)	-	-	10.6(16.3)	values in parentheses indicate P_{50} in 0.15M KPO_4 buffer
	1	10.4	-	-	11.1	
	2	11.4	-	-	11.1	
	3	13.9	-	-	14.0	

TABLE III

Solubility of Deoxyhemoglobin S	Concentration	C_{sat}	Relative C_{sat}
control		17.6	0.96
lawsone	2mM	16.8	
control		17.4	0.78
lawsone	10mM	13.6	

There are several important features of this compound. First, it has a differential effect on SS and AA cells at low concentrations. One mM lawsone can normalize the oxygen affinity of SS cells but has little effect on the P_{50} of AA cells. This concentration potentially is achievable in humans as our studies with mice suggest. Reports from the Middle East and South America indicate that, when given systemically, lawsone or raw henna leaves may be useful in the treatment of intestinal moniliasis or amebiasis (7) and sarcoma 140 tumors in mice (8). Nevertheless, with high doses, adverse effects due to increased oxygen affinity could occur; industrial workers should be protected against inhalant exposure, even though this risk is remote.

Finally, the fact that a compound which enhances oxygen binding (lawsone) is found together with a reducing agent (gallic acid) in the same plant suggests that these substances may play a role in the oxidation-reduction system of this species. Further work will be required to elucidate such mechanisms and to establish the usefulness of lawsone as an anti-sickling agent.

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