A new antisickling agent: In vitro studies of its effect on S/S erythrocytes and on hemoglobin S[1]

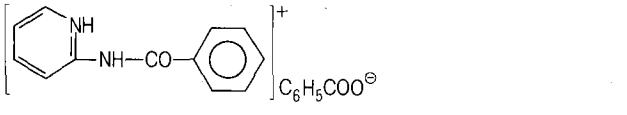
P.K. Adhikary, J.K. Haynes, H.L. Patthey and R.S. Rhodes

Division of Genetics & Molecular Medicine and Department of Pathology, School of Graduate Studies and Research and School of Medicine, Meharry Medical College, 1005 18th Ave. N., Nashville (Tennessee 37208, USA), 3 October 1977

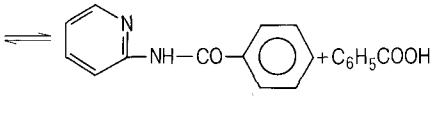
Summary. 2-{benzoylamino} pyridinium benzoate (BAPB) has exhibited an antisickling effect with homozygous S/S erythrocytes in vitro. This study suggests that BAPB prevents sickling by inhibiting the gelation of hemoglobin S.

A new compound 2-{benzoylamino} pyridinium benzoate (BAPB)[2] has been tested as a potential antisickling agent. In vitro studies show that this compound inhibits sickling of S/S erythrocytes at a much lower concentration than potassium cyanate. Previous studies indicate that the antisickling properties of cyanates are due to carbamylation of the alpha-amino groups of hemoglobin S by the isocyanic acid released from the cyanates[3,4]. Isocyanic acid is non-specific and is capable of participating in carbamylation reactions with various other cell proteins. Therefore, those compounds which generate free isocyanic acid may be expected to cause untoward side effects. Cyanate treatment causes serious side effects among sickle cell patients[5]. Methylisocyanate overcomes some of the disadvantages of cyanate as an antisickling agent since it does not generate free isocyanic acid. However, alkylisocyanates are rapidly hydrolyzed by water and methylisocyanate is toxic in nature and is not recommended for in vivo use[4]. BAPB contains the reactive carbamylating moiety, does not generate free isocyanic acid and is stable between pH 6.8 and 7.5[2]. These characteristics distinguish BAPB from the cyanates and alkylcyanates as a potential antisickling agent.

The molecular structure of BAPB has several potential advantages over the cyanates as an antisickling agent, such as: a) increased specificity because the large molecular size of BAPB may cause steric hindrance and thereby limit its interaction with other cell proteins; b) steric hindrance could augment the antisickling effect by carbamylation of NH_2-termini of hemoglobin S thereby inhibiting the aggregation of these molecules; c) since BAPB contains both amino and carbonyl groups, it has the capability of reacting with either carbonyl or amino groups of hemoglobin; d) the aromatic groups present in BAPB and its complex salt structure (figure 1) make it very lipophilic possibly allowing easy entry into the cell; and e) BAPB is reactive in its present form while cyanates have to be converted into their reactive form of isocyanic acid. This may be the reason why BAPB is about 100 times more effective than potassium cyanate[3] (table 1).



BAPB



2-{Benzoylamino} pyridine benzoic acid

$KCNO + H_2O \rightarrow KOH + HCNO \rightleftharpoons HN{=}C{=}O$

Isocyanic acid

Fig. 1. The structure of 2-{benzoylamino} pyridinium benzoate (BAPB), its breakdown products, and the conversion of potassium cyanate to isocyanic acid.

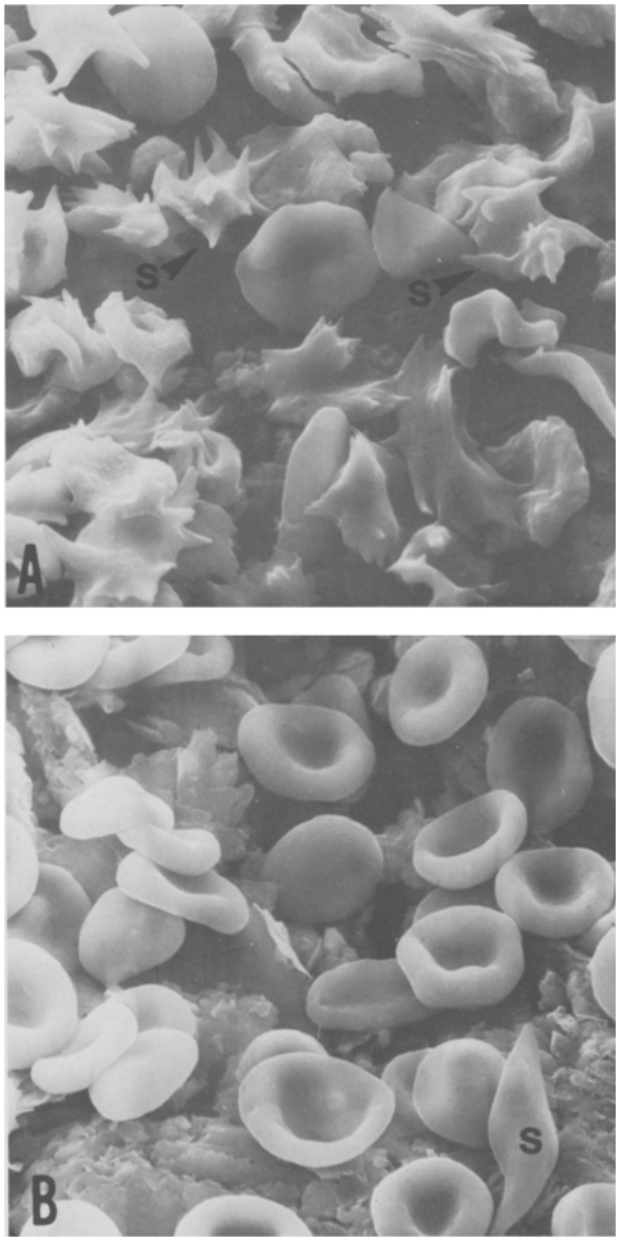


Fig. 2. Scanning electron micrographs of S/S erythrocytes (× 2400). *a* S/S erythrocytes after deoxygenation (untreated). Note that extreme sickling has taken place (s). *b* S/S erythrocytes after deoxygenation (BAPB treated). Irreversibly sickled cells (s) remain unchanged, but reversibly sickled cells have been restored to the normal biconcave shape by BAPB treatment.

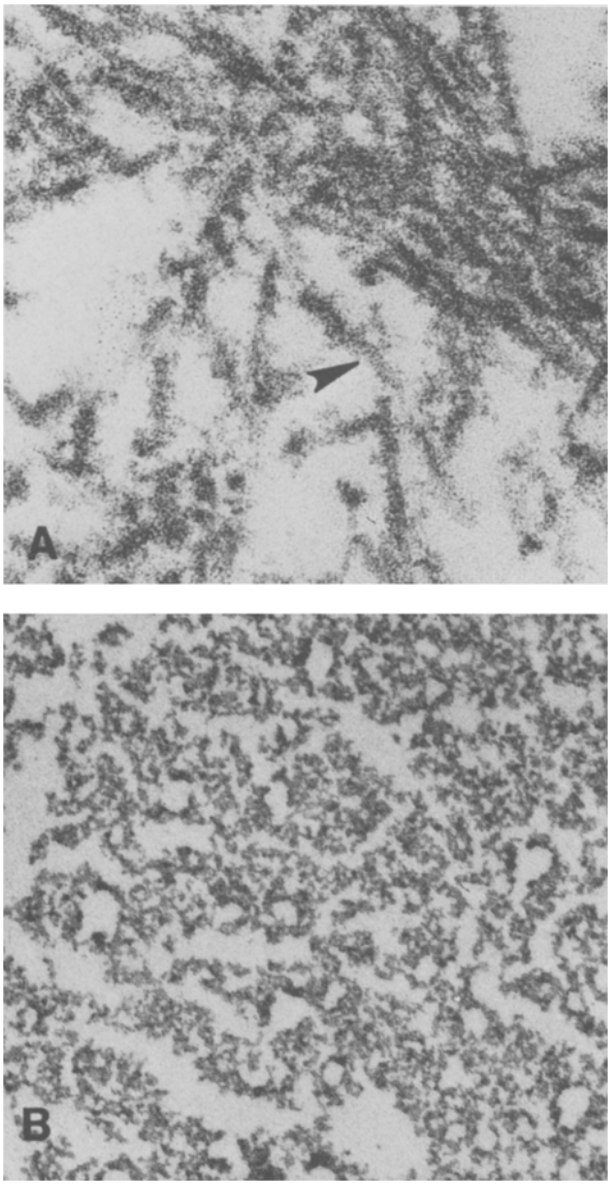


Fig. 3. Transmission electron micrographs of deoxygenated BAPB-treated and untreated gels of S/S hemoglobin (× 115,200). *a* The untreated gel shows the presence of characteristic rod-like hemoglobin fibers (arrow). *b* Treatment with 10^{-2} M BAPB results in a loss of the rod-like crystalline structure of the S/S hemoglobin.

Materials and methods. Blood was obtained by venupuncture (citric acid/citrate buffer as anticoagulant) from 4 male and 2 female individuals, 18–45 years of age, with sickle cell disease. 5 individuals were S/S and 1 was S/C. Fetal hemoglobin was less than 7% in all cases, when measured by the Singer alkali denaturation method[6]. All individuals were in a steady state and none had undergone blood transfusion within 6 months prior to blood sampling. Blood samples were used immediately or within 24 h after collection and storage at 4 °C. Erythrocytes were washed with Dulbecco's phosphate buffered saline (PBS), pH 7.4, for antisickling studies and prior to preparation of hemolysates. (BAPB) was dissolved in DMSO or toluene and stored tightly stoppered at room temperature.

A $^1/_{100}$ volume of DMSO/BAPB solution of varying molarity was added to a 10% suspension of test packed cells. Pure DMSO was used as a control. Suspensions were incubated at 37 °C for 15 min or at room temperature for 30 min with occasional shaking. Cells were then deoxygenated by evacuation with a water aspirator for 7–10 min at 30 °C. Cells were fixed under deoxygenated conditions by injecting 3 vol. of 3% glutaraldehyde in Sörensen's buffer through the rubber stopper, without discontinuing the aspiration. 1000 cells were scored from each preparation with the phase contrast microscope to determine the percentage of sickled cells. Calculations of the percentage inhibition of sickling by BAPB were corrected for the percentage of irreversibly sickled cells (ISC's) in the sample.

For scanning electron microscopic (SEM) studies, the fixed cells were kept in fixative for 10–12 h at 4 °C, washed and resuspended in distilled water. Specimens were sputter-coated with gold and observed with the JEOL JSM 35 Scanning Microscope.

Hemolysates for gelation studies were prepared by adding and equal volume of distilled water to packed erythrocytes and either 0.4 vol of toluene (control) or 0.4 vol. of toluene-BAPB solution. Samples were kept at room temperature for 30 min and then 12 h at 4 °C with occasional vortex mixing. After centrifugation at 30,000 × g at 4 °C for 30 min, the membranes and toluene supernatant were removed by pipette. Hemoglobin concentrations varied between 9.8 and 13.2 g/100 ml, hemolysates were further concentrated to 14–22 g/100 ml by passing dry nitrogen gas over the surface of the samples. A hydrated gas mixture of $N_2 + CO_2$ (95:5) was passed over 1.5 ml of concentrated hemolysate with shaking at 37 °C. The time was recorded from the start of gas flow until gelation occurred as noted by tilting the culture tubes[7]. After gelation, each tube was placed in an ice bath to resolubilize the hemoglobin and concentrations measured for each sample.

For transmission electron microscopic (TEM) studies, hemolysates were prepared as described above but were not concentrated. Gelation was carried out as described by White et al.[8]. Samples were deoxygenated by adding 2.5 ml of 1% sodium metabisulfite in 2.8 M potassium phosphate buffer, pH 7.2 dropwise to each 0.5 ml of control and test hemolysate. Each sample was stirred at room temperature for 30 min and left at 4 °C for 72 h. The resulting gels were

Table 1. Dose relationship of BAPB to its antisickling effect

% Normal cells* before deoxygenation	Molar concentration of BAPB	% Normal cells* after deoxygenation
83** ± 2.0***	None	5** ± 3.0***
85 ± 3.0	10^{-3} M	74 ± 3.0
82 ± 2.0	2×10^{-3} M	77 ± 1.0
85 ± 2.0	3×10^{-3} M	80 ± 2.0
87 ± 1.0	5×10^{-3} M	96 ± 2.0
83 ± 2.0	10^{-2} M	95 ± 3.0

* Normal cells are defined as those with biconcave disc shapes.
** % normal cells in a field of 500 cell ± SD of 3 samples.
*** SD between the counts of 3 samples.

Table 2. Gelation of BAPB-treated and un-treated hemoglobin S

Sample No.	Hemolysate	Hb concentration before gelation (g/100 ml)**	Hb concentration after gelation (g/100 ml)**	Gelation time
1	BAPB* treated	14.8 ± 0.1	16.8 ± 0.1	55 min
	Untreated	14.8 ± 0.1	16.2 ± 0.1	40 min
2	BAPB* treated	21.4 ± 0.1	22.2 ± 0.1	20 min
	Untreated	21.4 ± 0.1	21.6 ± 0.1	13 min

* Concentration of BAPB in each hemolysate was 10^{-2} M.
** Mean Hb concentration ± SD of 3 aliquots of the same sample.

processed for EM by the method of White[9]. Samples were sectioned and then stained with lead citrate and uranyl acetate.

Results. Antisickling effect of BAPB: Phase-contrast microscopy showed that 95% of the cells treated with 5×10^{-3} M BAPB, after deoxygenation, were not sickled. In contrast, 95% of the untreated cells were sickled after deoxygenation. SEM studies confirmed this finding and representative micrographs of treated and untreated cells are shown in figure 2.

Table 1 shows that the maximum antisickling effect of BAPB was achieved at a concentration of 5×10^{-3} M or higher. Table 2 shows that BAPB-treated hemolysates gel at higher hemoglobin concentrations and have longer delay times of gelation than those of untreated hemolysates.

Ultrastructure: TEM micrographs of gels of untreated deoxygenated hemolysates of S/S blood were similar to each other with masses of filamentous rodlike structures present. Gels of BAPB-treated, deoxygenated hemolysates of S/S blood were distinctly different and showed a lack of discernable filamentous structure. Representative micrographs of BAPB-treated gels (A) and untreated gels (B) are shown in figure 3.

Discussion. The studies reported here indicate that BAPB is a potent antisickling agent and possibly acts by preventing the polymerization of hemoglobin S. BAPB inhibits in vitro sickling of S/S Erythrocytes and inhibits gelation of hemoglobin. BAPB-treated gels of hemoglobin S do not show the presence of rod-like hemoglobin fibres and resemble the pictures of gels of oxygenated hemoglobin S[8,10]. The increase in delay time of gelation and ultrastructural appearance of gels of BAPB-treated hemoglobin indicate that BAPB reacts with hemoglobin.

The breakdown products of BAPB are 2-{benzoylamino} pyridine and benzoic acid[11] (figure 1). Neither of these products have shown any antisickling activity in in vitro studies[2]. It has been found that BAPB is far more lipophilic than either of its 2 component parts and we presume that the lipophilic nature of BAPB allows it to pass through the membrane into the cell where it or its breakdown products reacts with hemoglobin S by carbamylation. Studies are presently being conducted to further define its mechanism of action.

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2 P.K. Adhikary, A.K. Bhattacharya and A.M. Isa, manuscript in preparation.
3 A. Cerami and J.M. Manning, Proc. natl Acad. Sci. USA *68*, 1180 (1971).
4 C.K. Lee, J. biol. Chem. *25*, 6226 (1976).
5 D.R. Harkness and S. Roth, in: Progress in Hematology, vol. IX, p. 157. E. Grune Stratton Inc., 1975.
6 K. Singer, I.C. Chernoff and L. Singer, Blood *6*, 413 (1951).
7 R.M. Nalbandian, Molecular aspects of sickle cell hemoglobin; American Lecture series, Pub. No. 818:31, Charles C. Thomas. Springfield 1971.
8 J.G. White and B. Heagan, J. exp. Med. *131*, 1079 (1970).
9 J.B. White, Blood *31*, 561 (1968).
10 J.B. White and B. Heagan, Am. J. Path. *58*, 1 (1970).
11 E.H. Huntress and H.C. Walter, J. org. Chem. *13*, 735 (1948).

The environment in which lymphocytes differentiate influences their ability to cooperate in vivo

M. Marušić[1]

The University of Tennessee, Oak Ridge Graduate School of Biomedical Sciences and Biology Division, Oak Ridge National Laboratory[2], Oak Ridge (Tennessee 37830, USA), 19 December 1977

Summary. Whether or not parental B lymphocytes cooperate with F1 T lymphocytes depends upon the environment in which parental bone marrow cells differentiate. Only those parental B lymphocytes that have differentiated in the F1 environment are able to cooperate with F1 T lymphocytes.

The cooperation of T and B lymphocytes in the process of antibody production represents not only an interesting immunological phenomenon but may also serve as a general model of cell interactions and differentiation. It is now well established that cooperation of T and B lymphocytes is controlled by the major histocompatibility complex (MHC) genes[3]. It has been proposed that, in order to cooperate, T and B lymphocytes have to share or complement cell membrane determinants coded by certain MHC genes[3]. However, recent studies suggest that the genetic restriction of T-B cooperation may be a dynamic process dependent on the environment in which the lymphocytes are exposed to antigen. F1 lymphocytes primed in irradiated parental recipients were shown to interact preferentially with the cells of the respective parental haplotype[3,4]. The existence of 'adaptive differentiation' was thus suggested[3,4] proposing that antigen-driven differentiation in a semi-syngeneic environment allows the lymphocytes to interact preferentially with host-type cells.

The present experiment was designed to investigate the concept of adaptive differentiation in a model of immune response of mice to rat Yoshida ascites sarcoma (YAS). YAS grows well in the abdominal cavity of T-cell deficient thymectomized lethally irradiated bone marrow reconstituted (TIR) mice and kills the recipients[5]. Tumor growth can be inhibited by a single injection of normal syngeneic T lymphocytes[6]. Tumor rejection was shown to be due to antibody production involving interaction of transferred T and host B lymphocytes[6]. The failure of allogeneic cells to cooperate has been demonstrated in this model for normal lymphocytes[6,7] as well as for lymphocytes from mutually tolerant long-term radiation chimeras[7]. Both P→F1 and normal parental T lymphocytes cooperated with F1 B lymphocytes. However, F1 T lymphocytes failed to cooperate with parental B lymphocytes but did cooperate with parental B lymphocytes from P→F1 radiation chimeras[7]. This difference in the ability of P→F1 chimeric and normal parental B lymphocytes to cooperate with F1 T cells[7] constituted a basis for the present study. TIR mice of P→P, P→F1, P→F1→P, P→F1→F1, and F1→F1 constitutions were injected with YAS cells and with F1 T lymphocytes. The rejection of the tumor indicated a suc-