Biochemical Pharmacology, Vol. 37, No. 6, pp. 1057–1060, 1988.
Printed in Great Britain.

0006-2952/88 $3.00 + 0.00
Pergamon Press plc

MECHANISM OF IMPROVED MAINTENANCE OF 2,3-DIPHOSPHOGLYCERATE IN STORED BLOOD BY THE XANTHONE COMPOUND 2-(2-HYDROXYETHOXY)-6-(1-*H*-TETRAZOLE-5-YL)XANTHEN-9-ONE (BW A440C)*†

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(*Received* 9 *February* 1987; *accepted* 6 *August* 1987)

Abstract—The effect of the xanthone derivative 2-(2-hydroxyethoxy)-6-(1-*H*-tetrazole-5-yl)xanthen-9-one (BW A440C) on red cells was studied. When added to stored red cells at a concentration of 6 mM, greatly improved preservation of 2,3-diphosphoglycerate (2,3-DPG) was observed. There was no effect on internal pH of the erythrocyte. At a concentration 0.500 mM, many red cell enzyme activities were inhibited completely. At a 0.050 mM concentration, however, inhibition of pyruvate kinase and diphosphoglycerate phosphatase was most striking. Inhibition of either of these enzymes could result in elevation of 2,3-DPG levels. BW A440C in concentrations which elevated 2,3-DPG levels in humans caused a decrease in 2,3-DPG levels in rabbits and markedly impaired the viability of 21-day stored rabbit erythrocytes.

Since the demonstration by Chanutin and Curnish [1] and Benesch and Benesch [2] that 2,3-diphosphoglycerate (2,3-DPG) is an important modulator of the oxygen dissociation curve, many investigators have attempted to develop strategies that might permit better preservation of this compound during liquid blood storage [3]. In 1984, Hyde *et al.* [4], investigating a compound [2-ethoxy-6-(5-tetrazolyl) xanthone (BW A827C)] that modulated the oxygen dissociation curve through a direct effect on the hemoglobin molecule, discovered that this agent also exerted a favorable effect on the preservation of 2,3-DPG. A closely related drug, 2-(2-hydroxyethoxy)-6-(1-*H*-tetrazole-5-yl) xanthen-9-one (BW A440C), also increases red cell 2,3-DPG levels during storage [5]. We have now investigated the mechanism by which the latter compound influences 2,3-DPG levels, and we have also tested its effect on the *in vivo* lifespan of rabbit erythrocytes.

MATERIALS AND METHODS

The compound BW A440C was a gift from Dr. James W. Crow of the Burroughs Wellcome Co. (Research Triangle Park, NC).

In investigations of the effect of BW A440C on red cell storage, blood from human volunteers or rabbits was collected into CPDA-1 (citrate-phosphate-dextrose-adenine) or CPD [6], respectively, in plastic blood collection bags. A portion of the plasma was removed after centrifugation, and BW A440C was dissolved in the plasma which was then returned to the bag. To measure the effect of the drug on red cell enzyme activities, erythrocytes from blood drawn from normal donors into EDTA were used.

Diphosphoglycerate phosphatase (DPGP) was partially purified from red cells, and its activity was assayed by a modification of the method described by Rose and Liebowitz [7]. Red cell DPGP was partially purified by applying a 1:50 destromatized hemolysate prepared in 1 mM EDTA–0.7 mM β-mercaptoethanol to a Whatman DE-52 column in 5 mM KH_2PO_4/K_2HPO_4 (0.534/0.466). After washing with 20 mM KH_2PO_4/K_2HPO_4 (0.534/0.466), the enzyme was eluted with a 100-ml linear gradient from 20 to 300 mM KH_2PO_4/K_2HPO_4 (0.534/0.466). A precipitate in 60% saturated ammonium sulfate was dissolved in the EDTA–β-mercaptoethanol solution and dialyzed against the same solution prior to assay.

The labeled substrate [^{32}P]2,3-DPG was prepared from outdated, 2,3-DPG-depleted red cells. The cells were washed four times at room temperature in 10 vol. of 0.05 M Tris, pH 7.7, in 0.104 M sodium chloride. Twenty milliliters of packed red cells were resuspended in 12.4 ml of a solution containing 27 mM KH_2PO_4/K_2HPO_4, pH 7.4, 32 mM sodium pyruvate, 0.24 mCi ^{32}P/ml, 43 mM Tris–chloride, pH 7.7, 90 mM sodium chloride and 16 mM glucose and were shaken for 30 min. Eight milliliters of 50 mM inosine in Tris–saline was then added, and the suspension was shaken for an additional 2 hr at 37°. A perchloric acid extract was prepared by the addition of 40 ml of 8% perchloric acid and the supernatant after percipitation of proteins neutralized with potassium hydroxide. A 1:20 dilution of the supernatant was applied to a Dowex-1-Cl column and eluted with a gradient of 0.0 to 0.15 N

* Publication No. BCR-4703 from the Research Institute of Scripps Clinic.

† Supported in part by Grant HL 25552 from the National Institutes of Health, Grant RR00833, U.S. Army Contract DAMD17-87-C-7074, and by a grant from the Burroughs Wellcome Co.

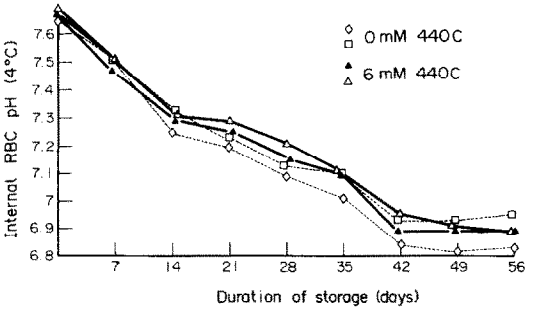

Fig. 1. Internal pH of blood cells in 4 units of normal human blood stored in CPDA-1 with or without BW A440C (440C).

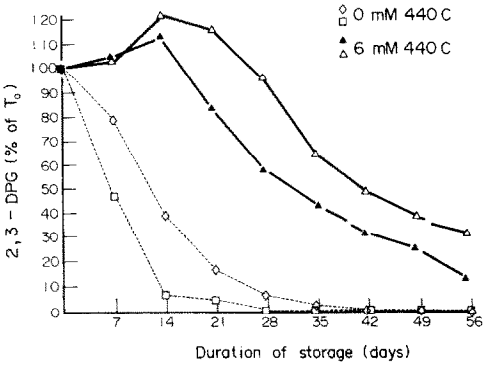

Fig. 2. 2,3-Diphosphoglycerate (2,3-DPG) in 4 units of normal human blood stored in CPDA-1 with or without BW A440C (440C). The initial 2,3-DPG levels of the samples were 11.6 (◆), 13.6 (■), 11.7 (◇) and 13.6 (□) μmol/g Hb.

HCl. The last major peak, containing the 2,3-DPG, was collected, neutralized with 2 M Tris and applied to a Dowex-1-bicarbonate column. The column was washed with 0.3 M triethylammonium bicarbonate (TEAB), pH 7.2, and then with 0.5 M TEAB. The column was eluted with a gradient of 0.5 M to 2.0 M TEAB, and the peak containing the radioactive 2,3-DPG was lyophilized. The assay system contained 50 mM glycylglycine buffer, pH 7.5, 100 mM potassium chloride, 5 mM β-mercaptoethanol, 1 mM KH_2PO_4/K_2HPO_4, pH 7.5, 0 or 10 μM 2-phosphoglycolate and 0.1 mM labeled 2,3-DPG. After 15 min at 37°, the reaction was stopped by the addition of trichloroacetic acid. Inorganic phosphate was extracted as the molybdate complex into isobutanol-benzene and counted [7]. Other enzyme assays and measurement of 2,3-DPG and ATP concentrations were performed as described previously [6].

The intracellular pH of red cells was determined directly by centrifuging the blood sample, removing the supernatant plasma, lysing the packed erythrocytes, and measuring their pH at 4° with a Corning model 150 pH meter. Red cell survival studies in rabbits were done using the combined $^{99m}Tc/^{51}Cr$ method [8].

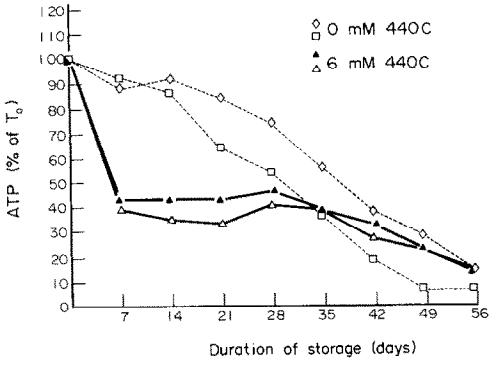

Fig. 3. ATP in 4 units of normal human blood stored in CPDA-1 with or without BW A440C (440C). The initial ATP levels of the samples were 4.7 (◆), 5.2 (■), 5.1 (◇), and 4.5 (□) μmol/g Hb.

RESULTS

Effect of BW A440C on the storage of red blood cells. Four hundred and fifty milliliters of human blood was collected into 63 ml of CPDA-1. Figures 1–3 illustrate the effect of BW A440C on red cell internal pH, 2,3-DPG and ATP during storage. BW A440C was found to have a favorable effect on 2,3-DPG levels with a corresponding decrease in the concentration of red cell ATP. The drug did not affect the internal pH of the red cells.

Effect of BW A440C on red cell enzymes. Sufficient BWA 440C was added to red cell enzyme assay systems to provide final concentrations of 0.50 and 0.050 mM. The effects of these concentrations on the activities of human and rabbit red cell enzymes are summarized in Table 1. The drug proved to be a potent inhibitor of many red cell enzymes. The enzymes that were most sensitive at the lower concentration of BW A440C were pyruvate kinase (PK) and diphosphoglycerate phosphatase (DPGP) when its activity was measured in the presence of 10 μM phosphoglycolate.

Effect of BW A440C on red cell lifespan in rabbits. Twenty milliliters of rabbit blood was collected into 2.8 ml of CPD solution and divided into bags to which no drug was added and those to which sufficient BW A440C was added to provide a final concentration of 3 mM. At the end of 21 days of storage, the survival of the red cells of one of the samples was measured in a normal recipient rabbit, and at the end of 22 days the other sample was labeled with a larger amount of ^{51}Cr and viability was again determined. The order of presentation was varied so that four of the rabbits received red cells that had been stored with drug first and the other four those that had been stored without drug. The results of these studies are presented in Table 2. Shown also is the effect of storage of rabbit blood on red cell ATP and 2,3-DPG levels. BW A440C did not have the same favorable effects on 2,3-DPG concentrations of rabbit erythrocytes as were observed with human cells, and the ATP levels fell sharply during storage, particularly in the presence of drug.

Table 1. Inhibition of enzyme activities by BW A440C

	% Inhibition		
	BW A440C (500 μM)	BW A440C (50 μM)	
	Human RBC (N = 1)	Human RBC (N = 4)	Rabbit RBC (N = 1)
Hexokinase	100.0	0.45 ± 0.45*	0
Glucose phosphate isomerase	25.0	0.0 ± 0.0	0.2
Phosphofructokinase	100.0	0.20 ± 0.67	−4.8
Aldolase	82.9	0.35 ± 0.35	2.7
Triose phosphate isomerase	84.3	0.20 ± 1.35	0
Glyceraldehyde phosphate dehydrogenase	72.6	0.165 ± 0.33	0
Diphosphoglyceromutase	100.0	16.2 ± 2.3	27.3
Diphosphoglycerate phosphatase (no P-glycolate)	ND†	25.8, 43.5‡	81.6
Diphosphoglycerate phosphatase (10 μM P-glycolate)	ND	52.4, 65.2‡	48.3
Phosphoglycerate kinase	79.8	1.71 ± 1.36	0
Monophosphoglyceromutase	100.0	23.7 ± 6.16	22.4
Enolase	100.0	1.72 ± 1.06	0
Pyruvate kinase	100.0	27.9 ± 1.6	19.4
Lactic dehydrogenase	65.6	0.008 ± 0.008	−0.6
Glucose-6-P dehydrogenase	35.8	0.0 ± 0.0	0
6-Phosphoglyceric acid	29.4	0.18 ± 0.18	0
Glutathione peroxidase	72.6	1.2 ± 1.2	0
Glutathione reductase	54.1	−0.68 ± 0.683	0
Adenylate kinase	85.3	0.2 ± 0.2	3.4
Adenosine deaminase	100.0	3.90 ± 0.73	−2.86
Serum glutamic oxaloacetic transaminase w/pyridoxal 5'phosphate	100.0	0.0 ± 0.0	3.6

* Mean ± SE.
† Not determined.
‡ N = 2.

DISCUSSION

The left shift that occurs progressively during red cell storage was first noted by Valtis and Kennedy [9] but did not receive general attention until the discovery by Chanutin and Curnish [1] and Benesch and Benesch [2] that it could be explained by the loss of 2,3-DPG that occurred in acid citrate medium. Suggestive evidence [10] has been presented that the transfusion of blood with red cells with high oxygen affinity may be clinically undesirable, although the oxygen dissociation properties normalize within about 24 hr of reinfusion of red cells [11, 12]. Many efforts have been made to devise means of preventing 2,3-DPG loss from stored red cells. These include the use of artificial storage medium with a high pH [13], the addition of dihydroxyacetone [14], and the use of ascorbate [15], which is now known to exert its putative effect through the oxalate that contaminates commercial ascorbate preparations [16, 17].

In the present investigation, we have been able to confirm that BW A440C greatly improves the preservation of 2,3-DPG in red cells stored in CPDA-1. Some compounds have been found to influence 2,3-DPG levels merely by altering the intracellular pH. Citrate, itself, exerts such an influence through the Donnan membrane effect [18]. Propranolol was thought to exert an effect on red cell 2,3-DPG levels [19], but this was later shown to be due merely to changes in intracellular hydrogen ion concentrations [20]. For this reason we monitored the intracellular pH of the stored cells. Since BW A440C had no effect on pH, we turned our attention to the effect of BW A440C on the activities of red cell enzymes. The higher of the two drug concentrations tested is in the concentration range that increases 2,3-DPG

Table 2. Effect of BW P440C on the storage of rabbit blood

Storage medium	ATP (μmol/g Hb)		2,3-DPG (μmol/g Hb)		Viability (%) Days 21–22
	Day 0	Day 21	Day 0	Day 21	
CPD	7.34	1.76	28.65	13.12	71.2, 68.8, 65.6, 64.4
CPD + BW A440C		0.67		9.65	21.4, 29.2, 20.1, 17.2

levels when added to stored blood. At this concentration of BW A440C, there was complete or nearly complete inhibition of many essential red cell enzymes, including hexokinase, phosphofructokinase, pyruvate kinase, phosphoglycerate kinase, and aldolase. It is unlikely that red cells could survive metabolically for even a day or two if the effective concentration of the drug were sufficiently high to totally inhibit these enzymes. Very likely the effective concentration of BW A440C in the red cell is much lower than the concentration in the plasma, possibly because of limited permeability of the red cell to the compound. Therefore, the more selective inhibition produced by 0.050 mM BW A440C is probably of greater significance. Here the enzymes that were most sensitive were pyruvate kinase (PK) and diphosphoglycerate phosphatase in the presence of 10 μM phosphoglycolate.

Inhibition of either PK or DPGP could well result in an elevation of red cell 2,3-DPG levels. Diphosphoglycerate mutase/diphosphoglycerate phosphatase (DPGM/DPGP) is a bifunctional enzyme that catalyzes both the synthesis of 2,3-DPG from 1,3-DPG and its hydrolysis to 3-phosphoglyceric acid [21, 22]. Since the synthetic (DPGM) activity of the enzyme is scarcely inhibited by BW A440C, whereas the catabolic (DPGP) activity is strongly inhibited, an increase in the steady-state level of 2,3-DPG would be the expected result. The inhibitory effect of BW A440C on DPGP activity was greater in the presence of the potent activator 2-phosphoglycolate than in its absence. This may be due to an effect of BW A440C on the ability of 2-phosphoglycolate to bind to or react with DPGP.

The effect of PK deficiency in elevating 2,3-DPG levels is well known from observations of the genetic deficiency state [23, 24]. The effect of PK deficiency is a consequence of the accumulation of phosphoenol-pyruvate (PEP), the substrate of the PK reaction. This, in turn, causes an increase of the levels of 2-phosphoglyceric acid (2-PGA) and 3-phosphoglyceric acid (3-PGA) which are in equilibrium with PEP through the enolase and monophosphoglycerate mutase reactions. The increase in 3-PGA serves as a potent stimulus for the accumulation of 2,3-DPG since it both inhibits the DPGP reaction and is an essential co-factor for the DPGM reaction.

The usefulness of any additive to blood preservatives depends both on its systemic toxicity and on its effect on the viability of the stored cells. Our results using rabbit red cells suggest that BW A440C may seriously impair red cell viability. The effects of this compound on rabbit red cell enzymes were very similar to the effects on human cells. Nonetheless, one must be cautious in extrapolating these results to humans. Indeed, it has been found that in baboons, a species much more closely related to man, red cell survival was not impaired.*

Of course the systemic toxicity of an acceptable blood additive must be extremely low. At present, insufficient data are available to draw definitive conclusions about the suitability of this agent as a blood additive. Even in the event that BW A440C proves to be unsuitable as an additive, the principles developed in understanding the mechanism of action of such a drug may prove useful in the future design of agents that influence red cell 2,3-DPG levels either *in vivo* or *in vitro*.

* C. R. Valeri, unpublished data, cited with permission.

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