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# Uptake of antimalarial bis(benzyl)polyamine analogs by human erythrocytes

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It is known that the natural polyamines putrescine, spermidine and spermine [1, 2], as well as a number of polyamine analogs [2–5], are accumulated in various cells, including human erythrocytes [6], by a carrier-mediated mechanism. Polyamine transport into most cells has also been shown to be energy dependent [7]; however, energy dependence for polyamine uptake into human erythrocytes has not been demonstrated unambiguously [1, 6]. We recently reported on the growth inhibitory effects of a series of bis(benzyl)polyamine analogs on the erythrocytic stages of both human (*Plasmodium falciparum*) and rodent (*Plasmodium berghei*) malaria parasites [8]. Because our new antimalarial agents are polyamine analogs, it was of interest to explore the possibility that these novel antimalarial polyamines were accumulated in erythrocytes by the uptake mechanism used for the natural polyamines.

In the present report we show that antimalarial bis(benzyl)polyamine analogs (e.g. *N,N'*-bis[3-[(phenylmethyl)amino]propyl]-1,8-diaminooctane; MDL 27391) are concentrated by human erythrocytes *in vitro*. The accumulation process shows saturation kinetics and is apparently carrier-mediated. However, the uptake system may be distinct from the transport system responsible for uptake of the natural polyamines and other polyamine analogs described previously. This drug uptake system may, in part, determine the efficacy of the novel polyamines against malaria parasites.

## *Methods*

*Preparation of erythrocytes.* Venous blood from two of the authors (A.J.B. (B+) and J.A.D. (A+) was used in all experiments. The blood was anticoagulated with a citrate:phosphate:dextrose solution and washed three times by centrifugation and resuspension using RPMI 1640 medium containing 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) (pH 7.2). Washed erythrocytes were stored as a 50% suspension in the wash buffer at 4° for no more than 1 week, during which time ATP levels in the erythrocytes, as measured by a bioluminescence assay [9], remained constant.

*Polyamine and polyamine analog uptake by erythrocytes.* Uptake experiments with erythrocytes were carried out as described by Moulinoux *et al.* [6]. Erythrocytes were washed three times in 0.14 M NaCl and resuspended in the same solution prior to uptake measurements. Erythrocyte suspensions ($2 \times 10^9$ in 0.3 ml) were mixed with 0.3 ml of homologous human serum, [$^{14}$C]-spermidine or a [$^{14}$C]-polyamine analog and various competitive inhibitors or other drugs to make a final volume of 0.63 ml for uptake incubations. The pH of the incubations under these conditions was 7.4. Identical results for uptake of polyamines into erythrocytes were obtained if RPMI 1640 medium supplemented with 10% human serum and 25 mM HEPES (pH 7.4) was substituted for the 0.14 M NaCl/serum mixture. Some experiments were carried out using RPMI 1640 medium with 10% human serum and 25 mM (piperazine-*N,N'*-bis(2-ethanesulfonic acid) (PIPES) (pH 6.5).

Uptake incubations were done at 37° with vigorous shaking in a water bath for either 15 or 30 min at pH 7.4 or pH 6.5, respectively, and then terminated by adding 2.5 ml of ice-cold 0.14 M NaCl and sedimenting the erythrocytes by centrifugation for 4 min in the cold. The cells were washed twice more with 2.5 ml of ice-cold 0.14 M NaCl and then lysed with two packed cell volumes (0.4 ml) of distilled water followed by precipitation of protein in the hemolysate with two packed cell columns (0.4 ml) of ice-cold 10% perchloric acid. Samples were then centrifuged at 15,000 *g*, and 0.5-ml aliquots of the supernatant fractions were taken for determination of radioactivity by liquid scintillation counting in 10 ml of Aquasol. Uptake was linear for 30 min at pH 7.4 and at least 60 min at pH 6.5.

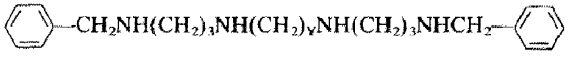
*Polyamine analog analysis by HPLC.* Polyamine analogs were measured in erythrocytes by derivatization with dansyl chloride and separation by HPLC as described previously [10].

*Chemicals.* [Tetramethylene-1,4-$^{14}$C]-Spermidine (80 mCi/mmol) was purchased from New England Nuclear. Other reagents were purchased from Sigma. All polyamine analogs including *N,N'*-bis[3-[(phenylmethyl)amino]-propyl]1,8-octane-$^{14}$C]-diamine (48 mCi/mmol) were synthesized at the Merrell Dow Research Institute.

## *Results*

A series of antimalarial bis(benzyl)polyamine analogs, differing only in the length of the central methylene chain, were accumulated by human erythrocytes during 24 hr of culture at 37° (Table 1). Concentrations of polyamine analogs within the erythrocytes were four (MDL 27693) to twenty-four (MDL 27701) times higher than the concentrations found in the culture medium after 24 hr. A polyamine analog with free terminal amines (MDL 26547) rather than terminal benzyl groups was neither taken up nor concentrated by the erythrocytes. Thus, the terminal

Table 1. Accumulation of polyamines analogs by human erythrocytes and competition for spermidine uptake

| | Y | Compound number | Concentration of polyamines after 24 hr (μM) | | $K_i$ for competition with [$^{14}$C]spermidine (μM) |
|---|---|---|---|---|---|
| | | | Erythrocytes | Medium | |
| Bis(benzyl)polyamines | | | | | |
|  | 6 | MDL 27693 | 14 | 3.3 | 135 ± 19* |
| | 7 | MDL 27695 | 32 | 1.9 | 52 ± 12 |
| | 8 | MDL 27391 | 43 | 2.3 | 160 ± 39 |
| | 9 | MDL 27701 | 66 | 2.8 | 223 ± 18 |
| | 10 | MDL 27700 | 50 | 2.0 | 513 ± 93 |
| Free amines | | | | | |
| $H_2N(CH_2)_3NH(CH_2)_YNH(CH_2)_3NH_2$ | 8 | MDL 26547 | 0 | p.8 | 103 ± 30 |
| Spermine | 4 | — | — | — | 0.25 ± 0.1 |

Incubations of erythrocytes in culture with polyamine analogs and subsequent measurement of polyamine analog concentrations by dansylation and HPLC were done as described in Methods. The initial concentration of polyamine analogs was 5 μM. A mean volume of 86.1 $\mu l/10^9$ erythrocytes was used to calculate the concentration of polyamine analogs in the erythrocytes [11]. Using the latter value, recoveries of 80–100% were obtained for the polyamine analogs from medium plus erythrocytes. A representative experiment is shown for the concentration of polyamine after 24 hr. This experiment was repeated three times with the same relative uptake values being obtained in each experiment. Apparent $K_i$ values were obtained from graphs of $1/v$ vs $1/$[spermidine] at spermidine concentrations of 2, 3, 5 and 10 μM and three different polyamine analog concentrations.

* Mean ± SD, N = 3.

benzyl groups seem to be necessary for uptake of the polyamine analogs by the erythrocytes. Accumulation of polyamine analogs was similar whether or not erythrocytes were parasitized with *P. falciparum*, a human malaria parasite.

It was possible that the accumulation and concentration of the polyamine analogs could have been due to transport by the same carrier system which mediates uptake of the natural polyamines putrescine, spermidine and spermine into cells. To test this, the uptake of [$^{14}$C]-spermidine was measured in the presence and absence of polyamine analogs. A $K_m$ = 1.98 ± 1.1 μM (mean ± SD, N = 12) was determined for spermidine which is similar to values determined previously in erythrocytes [6] and L1210 cells [4].

Apparent $K_i$ values for competitive inhibition of [$^{14}$C]-spermidine uptake by polyamine analogs were calculated using Lineweaver–Burk analysis. All the polyamine analogs were relatively weak competitive inhibitors of spermidine uptake. In contrast, spermine was a potent inhibitor of [$^{14}$C]-spermidine uptake (Table 1).

A radioactive prototype polyamine analog, [$^{14}$C]-MDL 27391, was used to further characterize the uptake of polyamine analogs by erythrocytes. Saturable uptake kinetics for MDL 27391 could not be shown when erythrocytes were incubated at 37° in pH 7.4 medium (Fig. 1A). However, if the incubation temperature was lowered to 23° (Fig. 1A) or the pH of the incubation was lowered to pH 6.5 (Fig. 1B), a saturable component of uptake was resolved. A $K_m$ of 0.7 ± 0.2 μM (N = 5) was obtained for uptake of MDL 27391 by erythrocytes (Fig. 1, A and B). With the saturable component of uptake resolved, it was possible to show competition for uptake of [$^{14}$C]-MDL 27391 by the structural analog MDL 27695 (Fig. 1B). Putrescine, spermidine and spermine at 100-fold excess concentrations failed to compete for the uptake of [$^{14}$C]-MDL 27391. Uptake of this polyamine analog was also unaffected by metabolic poisons including 2-deoxyglucose, iodoacetic acid, 2,4-dinitrophenol, KCN and $NaN_3$.

Human erythrocytes were preloaded by incubation with either 0, 10, 100, or 500 μM unlabeled MDL 27391 for 60 min and then washed free of the drug remaining in the medium. Subsequent to the preloading period, 2 μM [$^{14}$C]-MDL 27391 was added to the cells, and its uptake was followed for 15 min. Preloading with unlabeled drug resulted in increased velocity of uptake of [$^{14}$C]-MDL 27391 (Fig. 2).

The uptake of [$^{14}$C]-MDL 27391 by human erythrocytes was markedly temperature dependent, as shown in Table 2. Uptake into erythrocytes was stimulated approximately 50-fold at pH 7.4 or 10-fold at pH 6.5 when the temperature of the incubations was raised from 0° to 37°.

*Discussion*

The data contained herein suggest that antimalarial bis(benzyl)polyamine analogs are taken up into human erythrocytes by a carrier-mediated uptake system as well as by passive diffusion. This uptake system may be responsible for the accumulation of the bis(benzyl)polyamines within the erythrocyte to concentrations higher than the surrounding medium. The uptake system also may, in part, determine the antimalarial efficacy of the polyamine analogs. The bis(benzyl)polyamines which are accumulated in erythrocytes are potent antimalarial agents *in vitro* [8], whereas free-amine analogs such as MDL 26547 which are not accumulated in the erythrocytes have only very weak antimalarial effects [8]. Furthermore, the antimalarial potency of the series of bis(benzyl)analogs positively correlates with the magnitude of their accumulation in human erythrocytes.

The polyamine analog uptake system was characterized using a radioactively-labeled bis(benzyl)polyamine analog, [$^{14}$C]-MDL 27391, as a prototype substrate. The uptake system meets the criteria established for classical membrane carrier-mediated uptake systems [12], namely, uptake kinetics which are saturable, competitive inhibition of uptake by close structural analogs, transacceleration of uptake by high intracellular concentrations of unlabeled drug, and marked temperature dependence. Uptake was not inhibited by several metabolic poisons, suggesting that the uptake is probably a facilitated rather than an active process.

The resolution of the saturable component of MDL 27391 uptake by erythrocytes was not possible under the conditions used for measuring spermidine uptake because passive diffusion was apparently a large component of the total uptake of the MDL 27391 under those conditions. However, lowering the incubation temperature from 37° to 23° allowed a saturable component of uptake to be resolved, and an apparent $K_m$ was determined. This is similar to the uptake of sugars by Ehrlich ascites tumor cells [13]. Saturation kinetics for uptake of the polyamine analog was also obtained if the pH of the incubations was lowered to

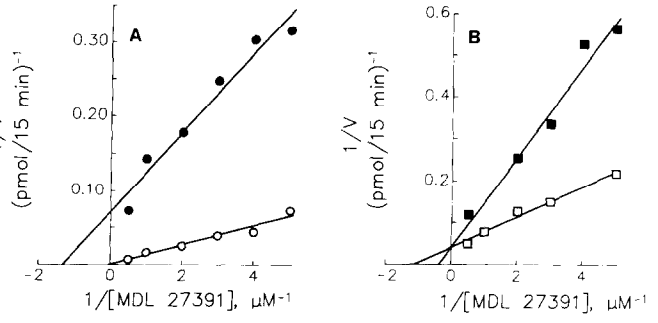



Fig. 1. Kinetics of uptake of [$^{14}$C]-MDL 27391 into human erythrocytes. (A). Human erythrocytes were incubated with [$^{14}$C]-MDL 27391 (0.2 to 2 μM) for 15 min at either 37° (○) or 23° (●) in medium containing 0.14 M NaCl and human serum, and the internalized [$^{14}$C]-MDL 27391 was measured as described in Methods. Double-reciprocal plots ($^1/_v$ vs $^1/_{[MDL\,27391]}$) were drawn to obtain kinetic constants. (B) Human erythrocytes were incubated with [$^{14}$C]-MDL 27391 (0.2 to 2 μM) for 15 min at 37° with no inhibitor (□) or in the presence of 50 μM MDL 27695) (■) in RPMI 1640 medium containing 25 mM PIPES (pH 6.5). Double-reciprocal plots were used as in Panel A to determine kinetic constants.

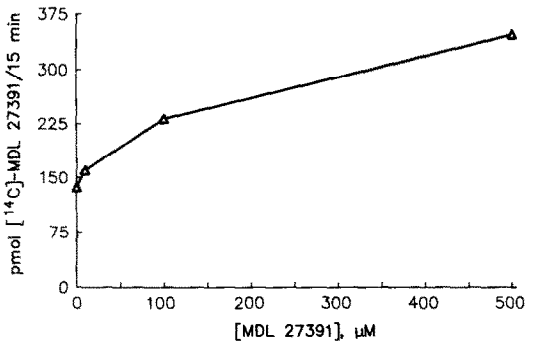



Fig. 2. Transacceleration of [$^{14}$C]-MDL 27391 uptake by preloading with MDL 27391 in human erythrocytes. Human erythrocytes were incubated with 0, 10, 100, or 500 $\mu$M MDL 27391 for 60 min at 37° in medium containing 0.14 M NaCl and human serum (see Methods). The cells were then washed rapidly three times in the same ice-cold medium and incubated for an additional 15 min at 37° at pH 7.4 with 2 $\mu$M [$^{14}$C]-MDL 27391, and uptake of the radioactive compound was measured as described in Methods.

Table 2. Temperature dependence of MDL 27391 uptake into erythrocytes

| | Uptake (pmol/15 min) | |
|---|---|---|
| Temperature (°C) | pH 7.4 | pH 6.5 |
| 0 | 1.7 | 1.4 |
| 12 | 2.5 | 2.0 |
| 24 | 17 | 3.9 |
| 37 | 99 | 11 |

Erythrocytes ($2 \times 10^9$) were incubated with 2 $\mu$M [$^{14}$C]-MDL 27391 at the temperatures indicated for 15 min at pH 7.4 or for 30 min at pH 6.5. The cells were then washed, and intracellular [$^{14}$C]-MDL 27391 was measured as described in Methods. The data are from a single representative experiment.

pH 6.5 by using PIPES-buffered RPMI 1640 medium for incubation of MDL 27391 with the erythrocytes. These data may indicate that the charged species of MDL 27391 (more of which would be present at the lower pH) is transported by a carrier-mediated mechanism, whereas the uncharged molecule is taken up primarily by passive diffusion, similar to what was shown previously for the transport of methylglyoxal bis(guanylhydrazone) into leukemic leukocytes [14]. Alternatively, these data may indicate that diffusion must simply be slowed sufficiently to demonstrate the saturable component since total uptake of MDL 27391 is less at either pH 6.5 or 23° than at pH > 7.0 and at 37°.

Evidence of carrier-mediated uptake is bolstered by the demonstration of transacceleration of MDL 27391 uptake by preloading of the erythrocytes with this analog. Transacceleration represents an uphill transport of a compound independent of metabolic energy [12]. The energy for this uptake against a concentration gradient is provided by the downhill movement of a second substrate molecule on the same membrane carrier.

It has been shown previously that many cells have the ability to accumulate the natural polyamines putrescine, spermidine and spermine as well as structural analogs of the polyamines such as methylglyoxal bis(guanylhydrazone) by a specific transport system [1–7]. Polyamine transport had saturable uptake kinetics, was inhibited competitively by structural analogs, and was inhibited by metabolic poisons, suggesting that the transport was energy dependent. All of the antimalarial polyamine analogs tested in the present work were relatively weak competitive inhibitors of the polyamine transport system as shown by their weak inhibition of [$^{14}$C]spermidine uptake. However, accumulation and concentration of the analogs by erythrocytes were not positively correlated with their efficacy as polyamine transport inhibitors. The compounds which concentrated to the greatest extent were the weakest inhibitors of spermidine uptake. It cannot be ruled out that binding to intraerythrocytic sites is responsible for some of the concentration of the polyamine analogs by the erythrocytes. However, our data more likely suggest an uptake system specific for the polyamine analogs and different from that which mediates uptake of the natural polyamines.

Uptake of antimalarial bis(benzyl)polyamine analogs has been characterized in human erythrocytes. Uptake was found to have both nonsaturable and saturable components, which could be experimentally resolved by variations in either the pH or the temperature of the uptake medium. An apparent $K_m$ of approximately 1 $\mu$M was obtained for a prototype bis(benzyl) analog (*N,N'*-bis[3-[(phenylmethyl)amino]propyl]-1,8-diaminooctane) in erythrocytes. The uptake of the polyamine analog was temperature dependent, was inhibited competitively by a close structural analog, and showed transacceleration upon preloading of the erythrocytes with the analog. However, uptake was not sensitive to several metabolic poisons and the natural polyamines putrescine, spermidine and spermine did not compete for the uptake of the polyamine analogs. We conclude, therefore, that uptake of the analogs may involve a separate carrier mechanism. The possible natural substrates for this carrier system remain to be elucidated.

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# Characteristic β-adrenergic receptors in a rat ascites hepatoma cell line (AH130)

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The β-adrenergic receptors are functionally coupled to adenylate cyclase and subdivided into $\beta_1$- and $\beta_2$-subtypes according to their different affinities for several adrenergic ligands. In the normal mammalian liver, it has been reported that the functional adrenergic receptor is the $\beta_2$-subtype [1, 2]. Uncontrolled tumor cell growth may result from some malignant transformation accompanied by changes in the hormonal membrane receptors and the cAMP production system [3, 4].

Lacombe *et al.* [5] had suggested that there is a switchover of adrenergic receptors from the normal $\beta_2$-type to $\beta_1$-type in Zajdela hepatoma, which was derived from rat liver treated with dimethylaminoazobenzene, judging from the change in the relative potency of various β-agonists in adenylate cyclase activation. We have studied the adrenergic responsiveness of AH130 cells, an ordinary cell line among a series of rat ascites hepatoma cell lines that were induced in the liver by dimethylaminoazobenzene treatment and established as transplantable tumors [6], and indicated that the cells hardly respond to $\beta_2$-adrenergic agonists in adenylate cyclase activation [7]. We have also reported that AH130 cells have many β-adrenergic receptors and α-adrenergic receptors dominated by $\alpha_2$-like receptors [8]. In this paper, we identified the qualitative and quantitative differences of β-adrenergic receptors in AH130 cells from those in normal rat hepatocytes.

## *Materials and methods*

*Cells.* AH130 cells were maintained serially by intraperitoneal passage at weekly intervals in female Donryu rats (5–7 weeks old, Shizuoka Laboratory Animal Center, Hamamatsu, Japan) and used 7 days after the cell inoculation. Normal rat hepatocytes were isolated from female Donryu rats by collagenase digestion *in situ* by the method of Berry and Friend [9].

*Adenylate cyclase assay.* It has been reported for liver and hepatoma cells that adrenergic responsiveness in the plasma membrane was lower than that in the cell homogenate [10, 11]. Therefore, the cell homogenates were used in this assay. A reaction mixture contained 2 mM ATP, 8 mM phosphoenolpyruvate, 20 μg/ml pyruvate kinase, 10 mM theophylline, 5 mM $MgCl_2$, 1 mM EDTA, 10 μM GTP, and various concentrations of a β-adrenergic agent, *l*-isoproterenol (IPN), *l*-epinephrine (Epi), *l*-norepinephrine (NE, Sigma Chemicals Co., St Louis, MO), *dl*-salbutamol (Sankyo Co., Tokyo, Japan), or *dl*-turobuterol (Hokuriku Seiyaku Co., Katsuyama, Japan), in the absence or presence of 100 μM *dl*-phentolamine (Ciba-Geigy Co., Summit, NJ) in 30 mM Tris–HCl buffer (pH 7.4); this was incubated for 5 min at 37°. After the incubation, the reaction was started by the addition of cell homogenate and continued for 10 min at 37°. The cAMP formed was measured by radioimmunoassay using a cAMP assay kit (Yamasa Shoyu, Choshi, Japan). $K_a$ values for β-adrenergic agents were determined as the concentration necessary for half-maximum activation of adenylate cyclase. Relative efficacies were calculated from the maximum activation by each agent compared to IPN.

*Binding assay.* Membranes (50–150 μg protein/tube) of AH130 cells and hepatocytes prepared by Percoll (Pharmacia Fine Chemicals, Uppsala, Sweden) sedimentation [12] were incubated with various concentrations of [$^{125}$I]iodocyanopindolol ([$^{125}$I]ICYP, 2000 Ci/mmol, Amersham, U.K.) in the absence or presence of 10 μM *dl*-propranolol (Sigma) in a total volume of 0.25 ml of 42 mM Tris–HCl buffer (pH 7.5) containing 15 mM $MgCl_2$, for 45 min at 37°. The reaction was then stopped by the addition of 3 ml of Tris buffer and immediate vacuum filtration of the samples on a GF/C filter (Whatman, Maidstone, U.K.). Non-specific binding at the $K_d$ value of ICYP in the presence of propranolol was about 8.1% and 1.6% of the total binding in hepatocytes and AH130 cells, respectively. To measure the potency of *dl*-propranolol, *dl*-metroprolol (Ciba-Geigy), and *dl*-salbutamol in inhibiting ICYP binding, samples were incubated with various concentrations of each agent as described above. The concentration of [$^{125}$I]ICYP was 50 pM for normal rat hepatocytes or 100 pM for AH130 cells, which was close to the $K_d$ of ICYP for each cell membrane (61.2 pM for hepatocytes and 121.6 pM for AH130 cells [8]). The $K_d$ values for [$^{125}$I]ICYP binding were calculated by Scatchard analysis. $K_i$ values for the inhibition of ICYP binding by