# Treatment with Oral Clotrimazole Blocks Ca²⁺-activated K⁺ Transport and Reverses Erythrocyte Dehydration in Transgenic SAD Mice

## A Model for Therapy of Sickle Cell Disease

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## Abstract

Prevention of red cell K⁺ and water loss is a therapeutic strategy for sickle cell disease. We have investigated in vitro and in vivo the effects of clotrimazole (CLT) and miconazole (MIC) on transgenic mice red cells expressing hemoglobin SAD. CLT blocked the Gardos channel ($ID_{50}$ $75 \pm 22$ nM; $n = 3$) and the A23187-induced dehydration of Hbb$^s$/Hbb$^{thal}$ SAD 1 mouse erythrocytes in vitro. Oral treatment with CLT (160 mg/kg per d) and MIC (100 mg/kg per d) inhibited the Gardos channel in both SAD 1 and control (Hbb$^s$/Hbb$^{thal}$) mice. In the SAD 1 mice only, cell K⁺ content increased, and mean corpuscular hemoglobin concentration and cell density decreased. After 7 d of treatment, the hematocrit of SAD 1, CLT-treated animals also increased. All changes were fully reversible. Long-term treatments of SAD 1 mice with oral CLT (80 mg/kg per d for 28 d) lead to sustained increases in cell K⁺ content and hematocrit and sustained decreases in mean corpuscular hemoglobin concentration and cell density, with no changes in animals treated with vehicle alone. Thus, CLT and MIC can reverse dehydration and K⁺ loss of SAD 1 mouse erythrocytes in vitro and in vivo, further supporting the potential utility of these drugs in the treatment of sickle cell anemia. (*J. Clin. Invest.* 1994. 93:1670–1676.) Key words: volume regulation • antisickling agents • K transport • anemia • hemoglobin SAD

## Introduction

The reduced red cell water content observed in homozygous hemoglobin (Hb) S (SS)[1] disease has important implications for the pathogenesis of the disease (1). In sickle cell anemia, Hb S polymerization is a high order exponential function of hemoglobin concentration (2). This polymerization is associated with reduction in cell ion and water content (cell dehydration), increased red cell density, and further acceleration of Hb S polymerization (2). The prevention of sickle cell dehydration is one of the possible therapeutic strategies for decreasing Hb S polymerization and sickling of SS erythrocytes (2).

Studies on red cell membrane cation permeability have characterized two cation transport systems prominently involved in sickle cell dehydration: K/Cl cotransport (1, 3) and Ca²⁺-activated K⁺ transport mediated by the Gardos channel (4). K/Cl cotransport is activated by cell swelling and acidification (1, 3). The activity of this system is elevated above normal not only in SS erythrocytes but also in cells of patients with homozygous Hb C disease (1, 3). This increased activity compared with cells containing normal hemoglobin is probably related to the $\beta^6$ Glu to Val (Hb S) or Lys (Hb C) mutation (5). The second system, the Ca²⁺-dependent K⁺ channel, induces K⁺ loss and erythrocyte dehydration when cytosolic free Ca²⁺ is artificially increased (4, 6) or upon deoxygenation of sickle cells (6, 7). The relative contribution of these two transport systems to the dehydration and K⁺ loss of sickle cells is not known. However, Bookchin et al. (7) have proposed an integrated model suggesting that the deoxygenation-induced influx of Ca²⁺ activates the Gardos channel, with K⁺ loss and cell dehydration. A consequence of the activation of the Gardos pathway is slight cytoplasmic acidification which in turn activates the K/Cl cotransport and produces additional K⁺ and water loss (7, 8).

Recently, we have shown that clotrimazole (CLT) and other imidazole derivatives are specific inhibitors acting on the Ca²⁺-dependent K⁺ channel in SS red cells (9). Furthermore, CLT prevented the dehydration of sickle cells induced by cyclic oxygenation-deoxygenation (9). A necessary requirement before carrying out studies in humans is to demonstrate the expected effects of CLT administration in an animal model for sickle cell disease. Several transgenic mouse models of sickle cell disease are currently in development (10–12). Recently, Trudel et al. (10) have developed a transgenic mouse bearing an integrated concatemer of alpha$^{Human}$ and $\beta^{SAD}$ ($\beta^{S, Antilles, D Punjab}$) genes in the hemizygous state. The presence of all three mutations in the $\beta$ chain of Hb SAD induces a substantial increase in the tendency of this "super Hb S" to polymerize compared with that of Hb S. The red cells of mice in the SAD 1 transgenic line are characterized by the presence of Hb SAD (19% of total Hb) and by erythrocyte sickling in vitro and in vivo (10, 12). The Hbb$^s$/Hbb$^{thal}$ SAD 1 ($\beta$ thal/SAD 1) mice are heterozygous for a murine $\beta$ thalassemia and were obtained by crossing SAD 1 mice with homozygous $\beta$ thalassemia mice (Hbb$^{d/3thal}$). These $\beta$ thal/SAD 1 mice express 26% of Hb SAD (10) and display increased mortality during early development and anemia during late fetal development (10). Survivors to adulthood are characterized by reduced life span, increased erythrocyte turnover with high reticulocytosis, and erythropoietic splenomegaly without anemia (10, 12). Since this animal model is characterized by in vivo

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1. *Abbreviations used in this paper:* CLT, clotrimazole; $D_{50}$, median density; Hct, hematocrit; MCHC, mean corpuscular hemoglobin concentration; MIC, miconazole; SS, homozygous sickle cell disease.

occurrence of cell sickling, it may be useful to study the progression of the disease and the mechanism of vasoocclusion and to evaluate various therapies. The transgenic $\beta$ thal/SAD 1 mouse demonstrates chronic organ and tissue damage consistent with sickle cell disease, such as microvascular occlusions and organized thrombi strongly resembling the pathologic picture of human sickle cell disease, priapism, and acute crises under hypoxia (10, 12).

Unlike human erythrocytes, mouse erythrocytes have a cell Na and K composition similar to that of human erythrocytes (13, 14) but do not seem to express K/Cl cotransport, even after induction of reticulocytosis (15). Consequently, the murine sickle erythrocyte represents a model in which the role of the Gardos channel in $K^+$ loss and red cell dehydration can be evaluated without the confounding presence of K/Cl cotransport. However, we have not directly established the absence of K/Cl cotransport in SAD mouse red cells.

In this report, we investigated the in vitro effect of CLT on red cells of normal and transgenic mice and the in vivo effects on mouse erythrocytes during short- (24 h, 48 h, 7 d) and long-term (28 d) oral treatment with CLT or miconazole (MIC). The aim of our study was to test in vivo the activity of these imidazole compounds as specific inhibitors of the murine Gardos channel and their ability to increase erythrocyte water and $K^+$ content and decrease cell density and mean corpuscular hemoglobin concentration (MCHC) in a murine model of human sickle cell disease.

## Methods

*Drugs and chemicals.* CLT, 7-deoxycholic acid, MIC, NaCl, RbCl, bumetanide, ouabain, Tris, Mops, EGTA, choline chloride, MgCl₂, and Acationox® were purchased from Sigma Chemical Co. (St. Louis, MO). A23187 was purchased from Calbiochem-Novabiochem Corp. (La Jolla, CA). All other chemicals were from Fisher Scientific Co. (Fair Lawn, NJ). MIC was used as Daktarin® oral gel (Janssen Pharmaceutical, Piscataway, NJ). All solutions were prepared using double-distilled water.

*Experimental animals and design of the in vivo trials.* CLT was suspended in a solution containing deoxycholate (5 mg/ml) and cellulose (0.6%) to a final concentration of 20 mg/ml. MIC was used as Daktarin® oral gel.

Normal mice (C57 B16), transgenic Hbb$^s$/Hbb$^s$ SAD 1 mice (SAD 1), and Hbb$^s$/Hbb$^{thal}$ SAD 1 mice ($\beta$ thal/SAD 1) were used in the in vitro experiments. In vivo experiments compared $\beta$ thal/SAD 1 transgenic mice and nontransgenic litter mates heterozygous for $\beta$ thalassemia (Hbb$^s$/Hbb$^{thal}$), used as controls (10). The mouse population was selected from animals 3–6 mo of age. Females (23–27 g) were selected for the long-term treatment protocol and males (28–32 g) were

used for the short-term protocol. No changes in weight were observed during the treatments.

For the short-term treatment we administered CLT (160 mg/kg per d) or MIC (100 mg/kg per d) by gavage twice a day in $\beta$ thal/SAD 1 transgenic mice ($n = 6$) and in $\beta$ thal non-SAD littermates ($n = 6$). The mice were tested before and after 24 h, 48 h, and 7 d of treatment.

For the long-term treatment, $\beta$ thal/SAD 1 transgenic mice ($n = 12$) and $\beta$ thal non-SAD mice ($n = 12$) were divided into two groups, each with six animals: one was treated with CLT (80 mg/kg per d) by gavage twice per day, and the other with the suspension medium without CLT. We studied the four groups of mice before and after 7, 14, and 28 d of treatment. At each time 200 μl of blood was drawn and used for influx measurements, phthalate density distribution curves of the erythrocytes, and determination of hematological parameters. One untreated $\beta$ thal/SAD 1 mouse and one untreated $\beta$ thal non-SAD mouse were lost during the first week because of traumatic complications of the gavage.

*Measurements of Rb$^+$ influx in mouse red cells.* Blood was drawn from mice by retroorbital venipuncture under anesthesia with heparinized microhematocrit (Hct) tubes. Whole blood was incubated for 30 min at room temperature in presence of 1 mM ouabain, 10 mM bumetanide, and 20 mM Tris-Mops, pH 7.4, (final concentrations in plasma, obtained with appropriate dilution of concentrated stock solutions). For in vitro experiments, the desired amount of inhibitor was also added from 10-mM stock solutions in ethanol. The ionophore A23187 was added to the mouse blood to a final concentration of 80 μM, followed by an additional 6-min incubation under stirring at 22°C. At zero time, RbCl was added to the cell suspension to a final concentration of 10 mM in plasma and incubated at 37°C (16, 17). Aliquots were removed after 0, 2, 3, and 5 min, transferred to 2 ml medium containing 150 mM NaCl and 15 mM EGTA, pH 7.40 at 4°C, washed three times at 4°C with the same solution, and lysed in 1.5 ml of 0.02% Acationox®. The lysate was then centrifuged for 10 min at 3,000 g. Rb$^+$ content was measured in the supernatant by atomic absorption spectrophotometry.

*Measurements of Ca$^{2+}$-dependent erythrocyte dehydration in the presence of A23187.* Whole blood buffered with Tris-Mops, pH 7.4 at 37°C to a final plasma concentration of 10 mM, was incubated for 20 min at 37°C with 10 μM CLT. A23187 was added at zero time to a final concentration of 80 μM under stirring. At specified times, cells were diluted with an equal amount of medium containing 150 mM NaCl, 15 mM EGTA, to chelate extracellular Ca$^{2+}$, and 0.1% of BSA to complex A23187 (9). The red cells were washed four times with the same medium at 37°C and immediately used for the phthalate density curves (9, 18).

*Hematological data and cation content.* Hb, Hct, MCHC, and reticulocytes were determined by standard manual methods. After washing the cells three times with PBS (330 mosM), phthalate density distribution curves and median density (D$_{50}$) were measured at 25°C, using 2-μl tubes (Drummond Scientific Co., Broomall, PA) (18). The remaining cells were washed four times with choline wash solution (170 mM choline chloride, 1 mM MgCl₂, and 10 mM Tris-Mops, pH 7.4 at

*Table I. Cation Content and Ca$^{2+}$-activated Rb$^+$ Influx into Mouse Red Cells*

| | Cell cation content | | Ca$^{2+}$-activated Rb$^+$ influx | |
| --- | --- | --- | --- | --- |
| | Na$^+$ | K$^+$ | − CLT | + CLT (100 nM) |
| | *mmol/kg Hb* | | *mmol/liter per cell per min* | |
| C57 B16 | 48.0±3.6 ($n = 5$) | 421±2.1 ($n = 5$) | 12.1±1.1 ($n = 5$) | 5.8±1.8 ($n = 5$)* |
| Hbb$^s$/Hbb$^{thal}$ | 47.4±2.4 ($n = 4$) | 490±4 ($n = 4$) | 10.2±0.1 ($n = 5$) | 5.4±1.7 ($n = 5$)* |
| Hbb$^s$/Hbb$^s$SAD 1 | 46.2±1.2 ($n = 4$) | 370±21 ($n = 4$)‡ | 10.1±0.8 ($n = 5$) | 5.1±1.8 ($n = 5$)* |
| Hbb$^s$/Hbb$^{thal}$SAD 1 | 48.4±3.6 ($n = 5$) | 288±20 ($n = 5$)‡ | 10.6±0.8 ($n = 5$) | 5.0±0.9 ($n = 5$)* |

‡ $P < 0.005$ when compared with the control mice. * $P < 0.005$ when compared with Rb$^+$ influx without CLT.
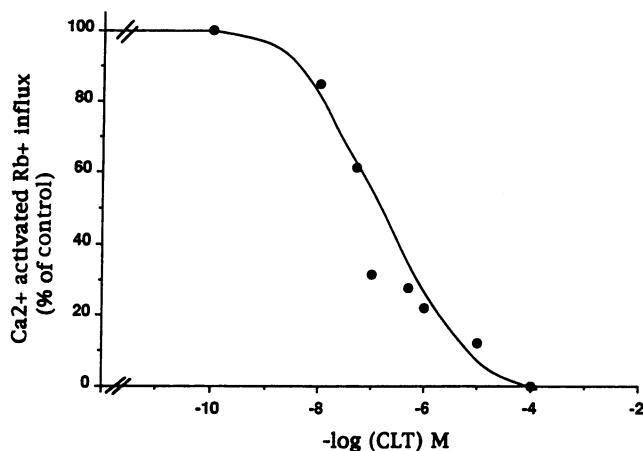
Figure 1. Inhibition of $Ca^{2+}$-activated $Rb^+$ influx in transgenic (Hbb$^s$/Hbb$^{thal}$ SAD 1) mouse red cells by increasing concentrations of CLT. Data points represent mean values of three separate experiments. Curves were computer-fit using a logistic dose–response model. SD for all the data points was between 5 and 15% of the mean values.

4°C, 330 mosM) for measurements of intracellular $Na^+$ and $K^+$ by atomic absorption spectrometry (1).

## Results

*In vitro effect of CLT and MIC in mouse red cells.* The erythrocytes of the mouse strains transgenic for Hb SAD (Hbb$^s$/Hbb$^s$ SAD 1, Hbb$^s$/Hbb$^{thal}$ SAD 1) have similar $Na^+$ content and lower $K^+$ content when compared with normal (C57 B16) and non–SAD 1 (Hbb$^s$/Hbb$^{thal}$) mice red cells (Table I).

$Ca^{2+}$-activated $Rb^+$ influx in whole blood was inhibited 47.8±5.4% ($n = 20$) and 40.6±1.2% ($n = 5$) by 100 nM CLT and 100 nM MIC, respectively (Table I). This inhibitory effect is similar to that observed in human SS cells with a similar concentration of CLT. No differences in $Ca^{2+}$-activated $Rb^+$ influx and in the inhibitory effect of CLT were observed be-

tween normal and transgenic mice (Table I). As shown in Fig. 1, increasing concentrations of CLT lead to progressive inhibition of $Ca^{2+}$-activated $Rb^+$ influx in whole mouse blood, with an $ID_{50}$ of 75±22 nM ($n = 3$) which is similar to the values observed in human red cells (9).

$\beta$ thal/SAD 1 mice red cells exposed to A23187 and $Ca^{2+}$ were markedly dehydrated, as shown by the rightward shift of the phthalate density distribution (Fig. 2). The presence of 10 $\mu$M of CLT prevented this dehydration almost completely (Fig. 2), whereas 10 mM MIC inhibited dehydration by only 30% (data not shown).

*In vivo effect of short-term oral treatment with CLT and MIC on mouse erythrocytes.* $\beta$ thal/SAD 1 and $\beta$ thal non–SAD 1 mice were subjected to a 7-d treatment course with CLT (160 mg/kg per d) and MIC (100 mg/kg per d). $Ca^{2+}$-activated $Rb^+$ influx, red cell cation content, and hematological parameters were tested at zero time and after 1, 2, and 7 d of treatment.

In $\beta$ thal/SAD 1 mice, after 24-h treatment with CLT, we observed a 72% inhibition of $Ca^{2+}$-activated $Rb^+$ influx (Fig. 3 A) and a corresponding increase in the intracellular $K^+$ content (Fig. 3 B) without change in $Na^+$ content (data not shown). Hct and Hb were not modified (Fig. 3, C and D). A decrease in MCHC (Fig. 3 E) was associated with a leftward shift in the density curves: $D_{50}$ changed from 1.105±0.002 to 1.099±0.001 ($n = 5$, $P < 0.005$). These changes are consistent with an increased water content of the erythrocytes. Similar changes in $Rb^+$ influx, $K^+$ content, and cell density were observed after 48 h of treatment, accompanied by a further decrease in MCHC (Fig. 3 E). Again Hct and Hb levels were unmodified (Fig. 3, C and D).

After 7 d of CLT, Hct was significantly increased compared with baseline (from 36.4±1.1 to 38.6±0.5, $n = 5$, $P < 0.005$) (Fig. 3 C). The extent of inhibition of $Ca^{2+}$-activated $Rb^+$ influx, MCHC, and Hb levels remained unchanged from the values at 1 or 2 d of treatment (Fig. 3).

The $\beta$ thal non–SAD 1 (Hbb$^s$/Hbb$^{thal}$) mice presented a similar inhibition of the $Ca^{2+}$-activated $Rb^+$ influx (Fig. 3 A), with no change in cation content (Fig. 3 B), hematological
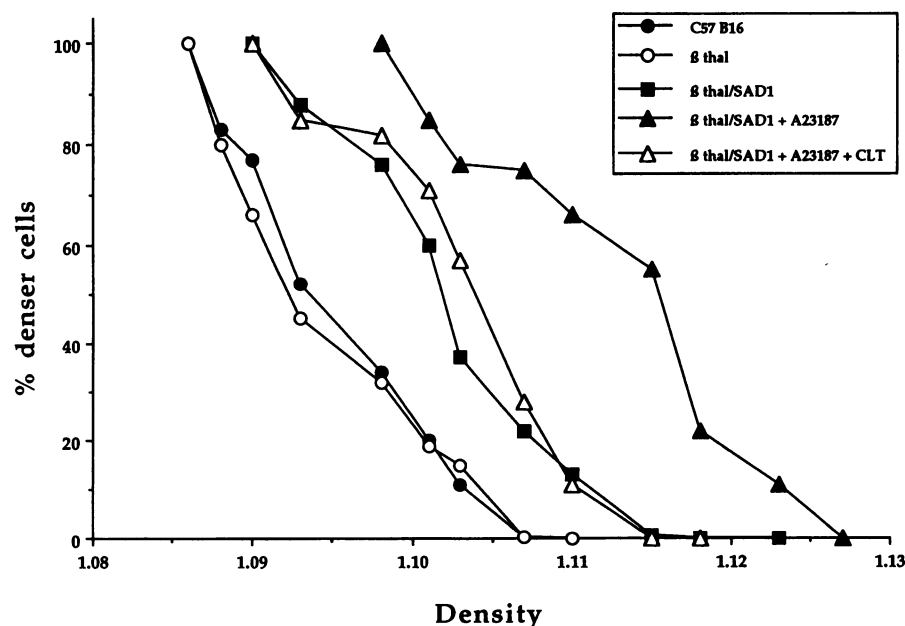


Figure 2. Phthalate density profiles portraying the effect of 10 mM CLT on the dehydration of transgenic (Hbb$^s$/Hbb$^{thal}$ SAD 1) mouse red cells via A23187-induced activation of the $Ca^{2+}$-dependent $K^+$ channel. Data are from one experiment, representative of three separate experiments.
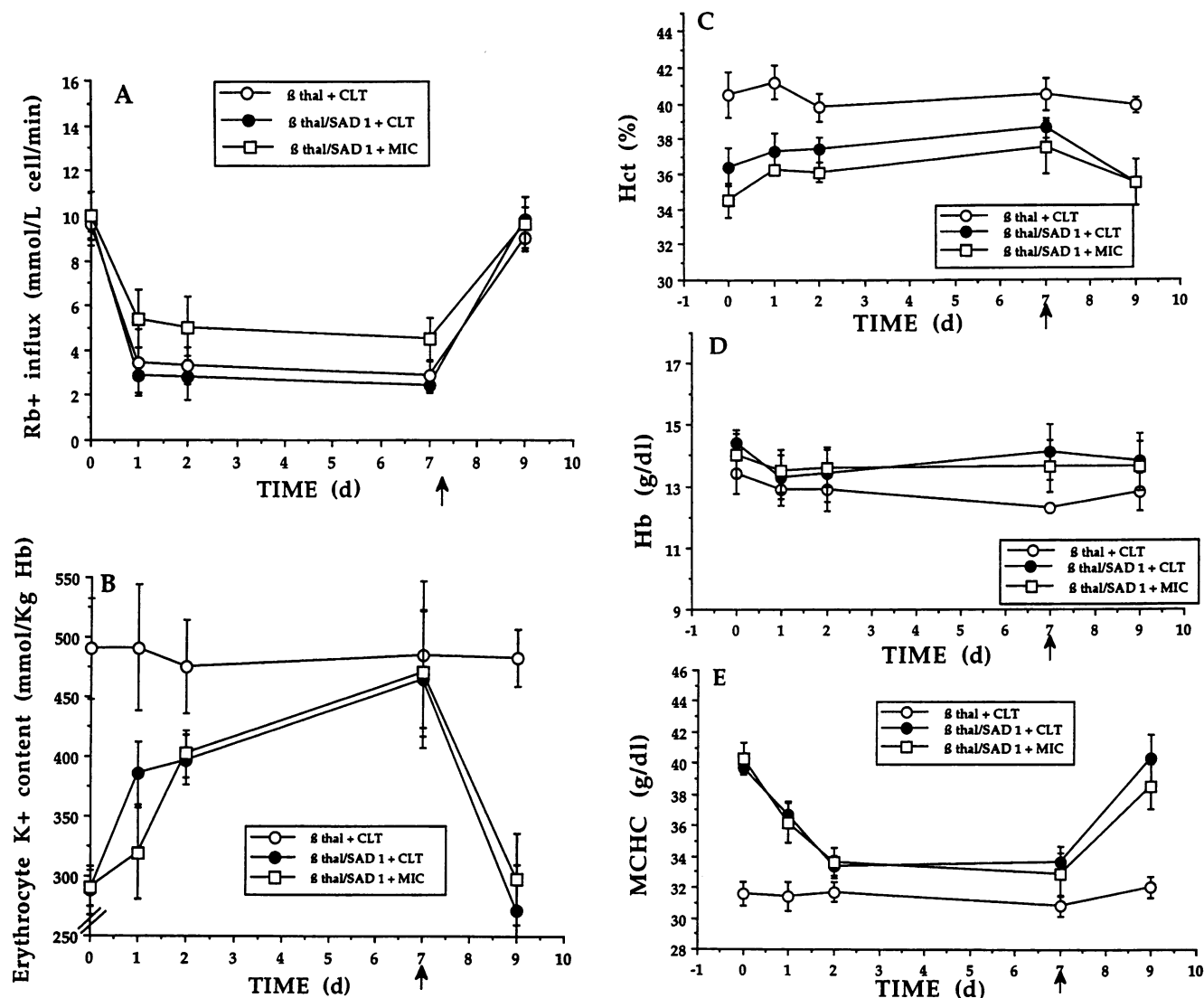
*Figure 3.* In vivo effects of oral CLT (160 mg/kg per d) and MIC (100 mg/kg per d) in transgenic (Hbb$^s$/Hbb$^{thal}$ SAD 1) and control (Hbb$^s$/Hbb$^{thal}$) mouse red cells, during the short-term treatment (24 h, 48 h, and 7 d) and 2 d after stopping treatment (*arrow*). (*A*) Effect on Ca$^{2+}$-activated Rb$^+$ influx. (*B*) Effect on erythrocyte K$^+$ content. (*C*) Effect on Hct. (*D*) Effect on blood Hb concentration. (*E*) Effect on MCHC. The data are expressed as means±SD (*n* = 6).

parameters (Fig. 3, *C–E*), or phthalate density distributions (D$_{50}$ from 1.093±0.001 baseline to 1.092±0.001, *n* = 4, NS) over 7 d of treatment.

The β thal/SAD 1 mice also underwent a brief treatment with oral MIC. The degree of inhibition of the Ca$^{2+}$-activated Rb$^+$ influx was lower than that produced by CLT (Fig. 3 *A*). After 1 and 2 d of administration of oral MIC, Hct and Hb remained constant (Fig. 3, *C* and *D*), MCHC decreased (Fig. 3 *E*), and cell density decreased (D$_{50}$ from 1.104±0.001 to 1.100±0.002, *n* = 6, *P* < 0.005). As observed for CLT-treated mice, the Hct significantly increased after 7 d compared with the baseline value (*P* < 0.05) (Fig. 3 *C*). This was associated with a decrease in MCHC (Fig. 3 *E*) and a larger shift in the phthalate density curves (D$_{50}$ 1.098±0.001, *P* < 0.002) when compared with baseline.

To evaluate the reversibility of these effects of CLT and MIC, treatment was stopped after 7 d. 48 h after cessation of treatment, Rb$^+$ influx, K$^+$ content, Hct, and MCHC were all restored to baseline values (Fig. 3). Phthalate density distribu-

tions were also shifted back to the starting density values (D$_{50}$ 1.105±0.0009, *n* = 5). In the non–SAD 1 (Hbb$^s$/Hbb$^{thal}$) mice, Ca$^{2+}$-activated Rb$^+$ influx returned to normal levels, while the other parameters remained normal (Fig. 3). During these brief treatments with oral CLT and MIC, we observed a positive correlation (*P* < 0.005) between the degree of inhibition of the Gardos channel and the extent of shift in the density curves towards lower values, suggesting that the level of Gardos channel activity directly influenced cell density. No other apparent clinical effects of signs of toxicity were observed in the treated animals. No significant changes in body weight were associated with CLT or MIC treatment.

*In vivo effect of long-term oral treatment with CLT on mice red cells.* Hbb$^s$/Hbb$^{thal}$ SAD 1 mice (β thal/SAD 1) and Hbb$^s$/Hbb$^{thal}$ (non–SAD 1) mice were subjected to a 4-wk trial with oral CLT (80 mg/kg per d). After 7, 14, and 28 d of treatment, Rb$^+$ influx, cation content, density curves, and hematological parameters were evaluated (Tables II–IV). After 7 d, in β thal/SAD 1 and in non–SAD 1 mice treated with CLT,

Table II. Effects of Long-Term Oral Treatment with CLT on Hematological Parameters in $Hbb^s/Hbb^{thal}$ SAD 1 Mice

| | Hct | | Hb | | MCHC | |
|---|---|---|---|---|---|---|
| Time | Hbb$^s$/Hbb$^{thal}$ SAD 1 (CLT) | Hbb$^s$/Hbb$^{thal}$ SAD 1 (Untreated) | Hbb$^s$/Hbb$^{thal}$ SAD 1 (CLT) | Hbb$^s$/Hbb$^{thal}$ SAD 1 (Untreated) | Hbb$^s$/Hbb$^{thal}$ SAD 1 (CLT) | Hbb$^s$/Hbb$^{thal}$ SAD 1 (Untreated) |
| d | % | | g/dl | | g/dl | |
| 0 | 37.1±1.9 (6) | 35.7±0.1 (6) | 14.9±0.9 (6) | 16.6±0.3 (6) | 40.2±1.5 (6) | 43.5±3.9 (6) |
| 7 | 39.3±2.2 (6)* | 36.2±0.5 (5) | 15.3±1.3 (6) | 15.6±0.7 (5) | 36.5±1.3 (6)‡ | 45.0±1.5 (5) |
| 14 | 41.1±1.4 (6)* | 35.8±1.4 (5) | 14.8±0.8 (6) | 15.6±0.7 (5) | 35.1±1.7 (6)‡ | 44.6±1.7 (5) |
| 28 | 40.7±1.5 (6)* | 34.7±2.1 (5) | 14.5±0.5 (6) | 15.3±0.8 (5) | 35.2±1.3 (6)‡ | 44.1±2.1 (5) |

Data are presented as means±SD ($n$ of the determinations). * $P$ = 0.005 when compared with the baseline (time 0). ‡ $P$ < 0.001 when compared with the baseline (time 0).

strong inhibition of the $Ca^{2+}$-activated $Rb^+$ influx was evident, while treatment with suspension medium alone produced no effect (Fig. 4 $A$). In $\beta$ thal/SAD 1 mice, CLT treatment produced a modest increase in cell $K^+$ content (Fig. 4 $B$) with unchanged $Na^+$ (data not shown), an increase in Hct, an unmodified Hb level, and a decrease in MCHC (Table II) which correlated with a shift in the density curves towards normal values (Table IV). The non-SAD 1 mice treated with CLT showed changes in none of these parameters (Fig. 4 $B$, Tables III and IV). In the $\beta$ thal/SAD 1 and non-SAD 1 mice treated with suspension medium alone, cation content (Fig. 4 $B$), hematological data, and density curves were not modified (Tables II–IV).

In the $\beta$ thal/SAD 1 and non-SAD 1 mice, 14 d of treatment with CLT produced no additional change in the inhibition of the Gardos channel (Fig. 4 $A$). $\beta$ thal/SAD 1 mice showed a progressive increase in cell $K^+$ content towards the values of non-SAD 1 mice (Fig. 4 $B$), a further increase in Hct, and a decrease in MCHC (Table II) with density curves still shifted to lower values (Table IV), while the blood Hb concentration was unchanged (Table II). All parameters were unchanged in mice treated with suspension medium alone (Fig. 4, Tables II–IV).

In $\beta$ thal/SAD 1 mice treated with CLT, changes in $K^+$ content (Fig. 4), hematological data (Table II), and density curves (Table IV) at 28 d of therapy were similar to those observed at 14 d of treatment. The inhibition of $Ca^{2+}$-activated $Rb^+$ influx in $\beta$ thal/SAD 1 and non-SAD 1 mice seemed to reach a plateau after 14 d (Fig. 4 $A$). In the animals treated with suspension medium without CLT, all the parameters remained unmodified (Fig. 4, Tables II–IV).

## Discussion

The results presented here document that CLT and MIC are potent inhibitors of the Gardos channel and of the dehydration of transgenic mouse red cells in vitro as well as in vivo. For CLT and MIC, the $ID_{50}$ values and the inhibition of the $Ca^{2+}$-dependent $K^+$ channel (Table I) obtained in transgenic mouse erythrocytes were similar to those described in human SS cells (9), suggesting a similar mechanism of inhibition in mice and human red cells. In addition, as observed for human erythrocytes (9), CLT markedly inhibited, in vitro, the dehydration of $\beta$ thal/SAD 1 red cells induced upon activation of the Gardos channel by A23187 (Fig. 2).

24-h oral treatment with CLT and MIC produced substantial inhibition of the $Ca^{2+}$-activated $K^+$ channel as well as a decreased MCHC (Fig. 3) and a decrease in $D_{50}$. 48-h treatment produced maximum degrees of $Rb^+$ flux inhibition, accompanied by an increase in $K^+$ content and by a decrease in MCHC (Fig. 3) and density. Only after 7 d of treatment did $K^+$ content reach normal mouse values, with stabilization of MCHC and density and a significant increase in the Hct (Fig. 3).

These results suggest that 24 h of CLT treatment, and to a lesser degree MIC treatment, improved hydration of and blocked $K^+$ loss from transgenic mouse red cells. Moreover, the inhibition of $K^+$ and water loss were reversible (Fig. 3), consistent with a major contribution of the Gardos channel to red cell dehydration in the SAD 1 mouse. Even though measurements of CLT blood levels were not carried out in this study, the effect of CLT on the erythrocytes seemed to depend on the presence of the compound in blood. 2 d without treat-

Table III. Effects of Long-Term Oral Treatment with CLT on Hematological Parameters in $Hbb^s/Hbb^{thal}$ non-SAD 1 Mice

| | Hct | | Hb | | MCHC | |
|---|---|---|---|---|---|---|
| Time | Hbb$^s$/Hbb$^{thal}$ (CLT) | Hbb$^s$/Hbb$^{thal}$ (Untreated) | Hbb$^s$/Hbb$^{thal}$ (CLT) | Hbb$^s$/Hbb$^{thal}$ (Untreated) | Hbb$^s$/Hbb$^{thal}$ (CLT) | Hbb$^s$/Hbb$^{thal}$ (Untreated) |
| d | % | | g/dl | | g/dl | |
| 0 | 43.6±1.3 (6) | 41.1±2.1 (6) | 16.5±0.9 (6) | 15.0±1.3 (6) | 34.2±1.7 (6) | 34.8±3.3 (6) |
| 7 | 44.6±1.3 (6) | 43.2±1.2 (5) | 16.8±0.2 (6) | 15.7±1.2 (5) | 34.2±1.4 (6) | 35.1±1.6 (5) |
| 14 | 44.0±1.2 (6) | 41.7±1.2 (5) | 15.7±1.0 (6) | 14.7±0.9 (5) | 34.2±1.1 (6) | 35.3±0.3 (5) |
| 28 | 45.1±2.1 (6) | 44.4±2.0 (5) | 15.7±0.9 (6) | 14.9±0.9 (5) | 34.9±2.1 (6) | 34.8±1.9 (5) |

Data are presented as means±SD ($n$ of the determinations).

*Table IV. Effect of Long-Term Oral Treatment with CLT on $D_{50}$ of $Hbb^s/Hbb^{thal}$ SAD 1 and $Hbb^s/Hbb^{thal}$ Non–SAD 1 Mice Red Cells*

| | $Hbb^s/Hbb^{thal}$ SAD 1 | | $Hbb^s/Hbb^{thal}$ non–SAD 1 | |
| Time | CLT | Untreated | CLT | Untreated |
| --- | --- | --- | --- | --- |
| *d* | | | | |
| 0 | 1.103±0.001 (6) | 1.103±0.001 (6) | 1.092±0.003 (6) | 1.091±0.001 (6) |
| 7 | 1.096±0.002 (6)* | 1.104±0.001 (5) | 1.091±0.002 (6) | 1.090±0.001 (5) |
| 14 | 1.095±0.002 (6)* | 1.104±0.002 (5) | 1.092±0.001 (6) | 1.091±0.001 (5) |
| 28 | 1.095±0.001 (6)* | 1.103±0.001 (5) | 1.092±0.002 (6) | 1.091±0.002 (5) |

Data are presented as means±SD (*n* of the determinations). * $P < 0.005$ when compared with the baseline (time 0).

ment sufficed to allow reappearance of the pathological characteristics of the red cells such as a low $K^+$ content and a high MCHC (Fig. 3), suggesting that CLT reversibly inhibits the $Ca^{2+}$-activated $K^+$ channel in vivo as well as in vitro (9). An HPLC assay for CLT is now available which will allow the study of the correlation between blood levels and cellular effects (Sakamoto, M., C. Brugnara, and N. Rifai, unpublished data).

The long-term oral trial, carried out with a lower dose of CLT (80 mg/kg per d), also produced prompt inhibition of the
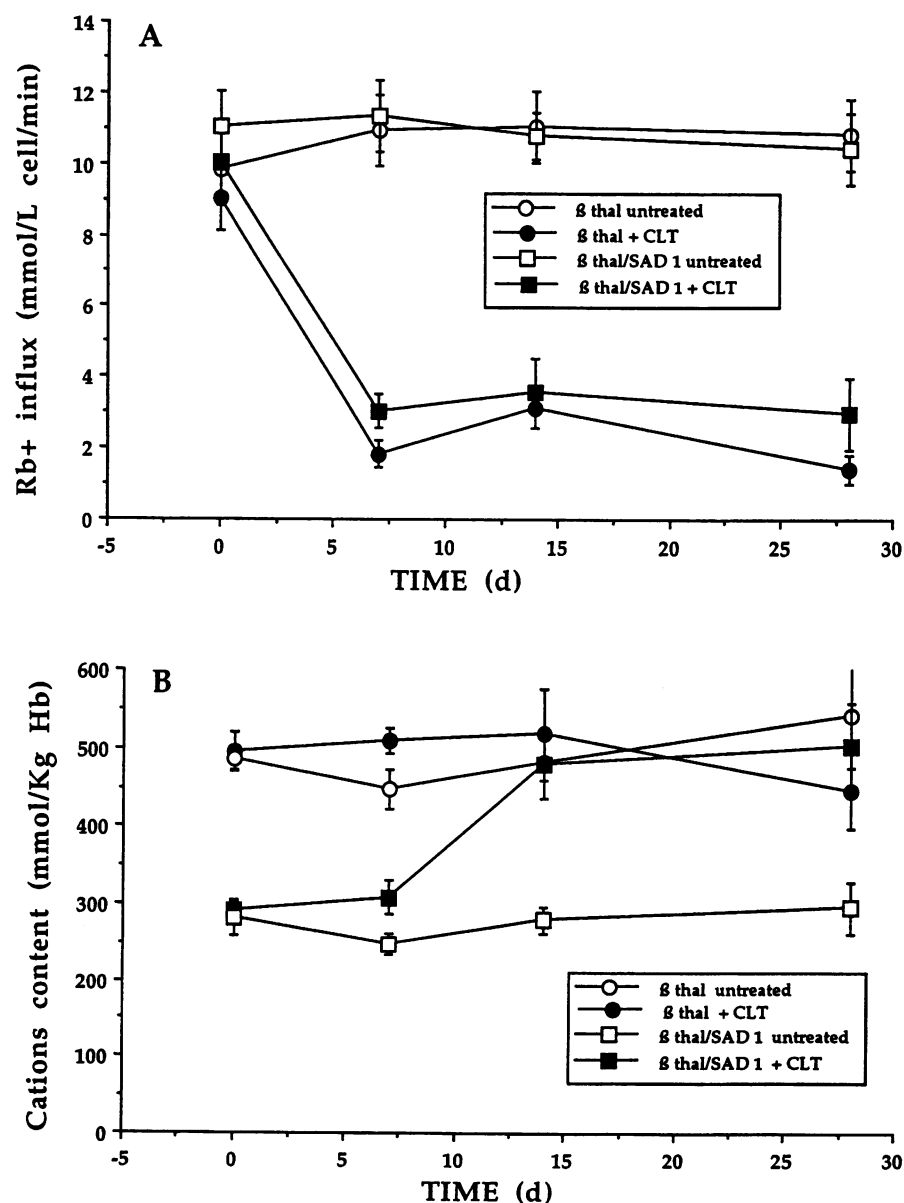


Figure 4. In vivo effect of long-term treatment (28 d) with oral CLT (80 mg/kg per d) and with the suspension medium without CLT in transgenic ($Hbb^s/Hbb^{thal}$ SAD 1) and control ($Hbb^s/Hbb^{thal}$) mouse red cells. (*A*) Effect on $Ca^{2+}$-activated $Rb^+$ influx. (*B*) Effect on intracellular $K^+$ content. The data are expressed as means±SD (*n* = 6).

Gardos channel (Fig. 4 *A*) as well as a decrease in MCHC and an increase in Hct (Table II). However, longer treatment was required to increase K$^+$ content (Fig. 4 *B*). In both short- and long-term treatment, a positive correlation between the extent of inhibition of the Gardos channel and the shift in the density curves was observed. Accordingly, the measurement of the inhibition of the transport could predict the shift in the density distribution and the efficacy of the treatment. The in vivo inhibition of the Gardos channel observed during short- and long-term treatment showed the important role of this pathway in the mechanism of dehydration of $\beta$ thal/SAD 1 erythrocytes. Furthermore, the erythrocyte K$^+$ and water loss was corrected by treatment of the mice with CLT. These results suggest that through the inhibition of the Ca$^{2+}$-dependent K$^+$ channel, CLT might decrease the dehydration of transgenic mouse erythrocytes which may in turn decrease Hb SAD polymerization (19). CLT and MIC did not induce noticeable side effects in mice; weight of the animals as well as behavior and activity did not change.

The therapeutic potential of rehydration of SS red cells was first demonstrated by a clinical study in which the induction of a hyponatremic hyposmotic state in patients reduced the frequency and duration of painful crisis (20), although this regimen posed a significant problem for patient compliance (21). We have attempted rehydration of SS cells by blockade of K$^+$ efflux pathways. Thus, in sickle cell disease, inhibition of the Gardos channel with CLT should decrease the K$^+$ loss and prevent secondary activation of K/Cl cotransport, thereby improving cell hydration. As a rapidly acting and reversible drug, CLT might be useful in sickle cell crisis, in preventing dense cell formation, and in reversing and attenuating the course of vasoocclusive episodes.

In conclusion, these results demonstrate that CLT and MIC are potent and specific inhibitors of the mouse red cell Gardos channel in vitro. We further demonstrate that oral CLT can reverse the dehydration and the K$^+$ loss of transgenic SAD mouse red cells in vivo. These therapeutic agents will be tested in human subjects for the ability to prevent or reverse cell dehydration acutely during sickle crisis and chronically in patients with severe sickle cell disease.

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