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Research Article

Inhibition of Ca^{2+}-dependent K^+ Transport and Cell Dehydration in Sickle Erythrocytes by Clotrimazole and Other Imidazole Derivatives

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Abstract

We have investigated the interaction of clotrimazole (CLT) and related compounds with the erythroid Ca^{2+}-activated K^+ channel, a mediator of sickle cell dehydration. We measured K^+ transport, membrane potential, and cell volume upon activation of this pathway in sickle erythrocytes. CLT blocked almost completely Ca^{2+}-activated K^+ transport in homozygous hemoglobin S cells, with IC_{50} values of 29±15 nM in isotonic 20 mM salt solution and 51±15 nM in normal saline (n = 3). The inhibition of K^+ transport by CLT was caused by a specific interaction with the Ca^{2+}-activated K^+ channel of human red cells, since it displaced bound ^{125}I-Charybdotoxin, a specific ligand of the Gardos channel, with an IC_{50} (12±4 nM in isotonic 20 mM) similar to the IC_{50} values for flux inhibition. When homozygous hemoglobin S cells were dehydrated by incubation in the presence of 100 μM $CaCl_2$ and the ionophore A23187, or by exposure to cycles of oxygenation and deoxygenation, CLT effectively inhibited cell dehydration and K^+ loss. The IC_{50} of CLT for inhibition of Ca^{2+}-activated K^+ transport in sickle cells is significantly lower than plasma concentrations of CLT achievable after nontoxic oral doses. We therefore propose that oral administration of CLT may prevent red cell dehydration in patients with sickle cell anemia. (*J. Clin. Invest.* 1993. 92:520–526.) Key words: erythrocyte • sickle cell anemia • membrane transport • clotrimazole • Ca^{2+}-activated channel

Introduction

The reduced red cell water content observed in sickle cell anemia has important implications for the pathogenesis of the disabling clinical complications of hemoglobin S (Hb S) polymerization and sickling (1). The erythrocyte fractions of highest density in homozygous hemoglobin S (SS)[1] disease are characterized by severe dehydration with mean corpuscular hemoglobin concentrations (MCHC) as high as 40–50 g/dl (2, 3). The high order exponential dependence of the delay time for Hb S polymerization on the concentration of Hb S suggests that extremely small increases in intracellular Hb S concentration will disproportionately reduce the delay time. The resultant acceleration of Hb S polymerization is thought to increase cell sickling before exit of erythrocytes from the confines of the capillary (1).

Prevention of MCHC elevation is one of a limited number of strategies available to decrease Hb S polymerization and cell sickling in SS patients. Several methods of prevention have undergone clinical study, including attempts to increase Hb F production relative to Hb S, and attempts to prevent cell dehydration (1). Prevention of cell dehydration can in theory be approached by osmotic promotion of swelling or by pharmacologic prevention of the cell shrinkage normally provoked by the acidic, hypoxic environment of the capillary bed. The first attempt to reduce MCHC osmotically by therapeutic induction of hyponatremia did, indeed, produce reductions in both cell sickling and clinical symptoms (4). However, prolonged maintenance of the requisite (occasionally symptomatic) degree of hyponatremia required compliance with a very challenging dietary regimen.

Cell shrinkage is mediated by loss of intracellular KCl with accompanying water. Studies of the membrane transport properties of SS erythrocytes have indicated that two transport pathways play a dominant role in K^+ loss from and dehydration of sickle cells. One pathway is the volume and pH-regulated K-Cl cotransport system (3, 5). Though K-Cl cotransport activity is lower in the densest SS cells than in corresponding light and middle-density fractions, activity in these densest fractions is still higher than in AA control cells (normal subjects or red cells containing Hb A) (3). However, there exist no pharmacological inhibitors of this system of sufficiently high affinity and specificity for clinical trials.

The other transport system that has been shown to mediate K^+ loss and dehydration of SS cells when the cytoplasmic free [Ca^{2+}] is increased is the Ca^{2+}-activated K^+ channel first described by Gardos (6, 7). Studies in SS cells have provided evidence for a role of this pathway, also known as the "Gardos channel," in SS cell dehydration (8). The Gardos channel is

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1. *Abbreviations used in this paper:* AA, normal subjects or red cells containing hemoglobin A; CCCP, carbonyl cyanide *m*-chlorophenyl hydrazone; ChTX, charybdotoxin; CLT, clotrimazole; DIDS, diisothiocyano-disulfonyl stilbene; HC, hemoglobin concentration; Hct, hematocrit; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; SS, subjects with homozygous sickle cell anemia or red cells homozygous for hemoglobin S.

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inhibited by quinine, carbocyanine dyes (9), nifedipine (10), and nitrendipine (11) and with greater specificity by charybdotoxin (ChTX), a 37-amino acid peptide derived from the venom of *Leiurus sequestratus* (12–14). None of these drugs has been brought to clinical trial in sickle cell anemia, resulting either from low inhibitory potency (of the former) or from insufficient pharmacological specificity (of the latter) with expected attendant side effects.

Recent work on cytochrome P450-related electron transport has led to a report that imidazole antimycotic drugs are potent inhibitors of the Ca^{2+}-activated K^+ channel in normal human red cells (15). We have investigated the effects of imidazole and triazole antimycotics and of nitroimidazoles in SS erythrocytes, with the intention of evaluating their ability to inhibit K^+ transport and cell dehydration mediated by the Gardos channel. Our long-term objective is to identify compounds that could be clinically useful in SS patients to prevent cell dehydration and to diminish irreversible sickling and microvascular obstruction. Our results suggest that clotrimazole (CLT) or related drugs could be used to prevent dehydration of SS cells in vivo.

Methods

Patient selection

Patients homozygous for Hb S disease were selected for this study. Blood was collected after obtaining informed consent from patients followed in the Hematology Division Clinic at Brigham and Women's Hospital and in the Boston Comprehensive Sickle Cell Center at Boston City Hospital. None of the patients had been transfused in the preceding 120 d.

Drugs and chemicals

Synthetic ChTX was purchased from Peptides International (Louisville, KY). ^{125}I-ChTX and ^{86}Rb were purchased from New England Nuclear (Boston, MA). All preparations of ^{125}I-ChTX were used within 2 mo after radioiodination. A23187 was purchased from Calbiochem-Behring (La Jolla, CA). Sucrose was purchased from Serva Biochemicals (Paramus, NJ). Bovine serum albumin fraction V was purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN). Bumetanide was a gift from Leo Pharmaceutical Products (Ballerup, Denmark). Methazolamide (Neptazane®) was a gift from Lederle Laboratories (Pearl River, NY). Fluconazole was provided by Pfizer Inc. (Groton, CT). Carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP), di-isothiocyano-disulfonyl stilbene (DIDS), EGTA, Mops, CLT, and all other drugs and chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) and Fisher Scientific Co. (Fair Lawn, NJ).

Measurement of ^{86}Rb influx in human red cells

10^7 red cells/ml were incubated at room temperature in the presence of 0.1 mM ouabain, 10 μM bumetanide, and the desired amount of inhibitor in two types of isotonic media. The "low ionic strength" medium contained 18 mM NaCl, 2 mM KCl, 230 mM sucrose, and 10 mM Tris-HCl, pH 8.0. These experimental conditions maximized ChTX binding to human red cells (16). The "physiological ionic strength" medium contained 140 mM NaCl, 2 mM KCl, and 10 mM Tris-HCl, pH 8.0. At the end of the incubation triplicate samples of cell suspension were spun at 3,000 *g* for 10 min, the supernatant was removed and a smaller volume of medium containing A23187 (60 μmol/liter cells), 50 μM $CaCl_2$, and ^{86}Rb (1 μCi/ml) was added to a final hematocrit (Hct) of 4–5%. This cell suspension was agitated at room temperature, aliquots were sampled at 1-, 3-, and 5-min time points, then spun in a microfuge through 0.8 ml of chilled flux medium containing 5 mM EGTA layered over a 0.4 ml cushion of butyl-phthalate oil. The resultant supernatant and the upper layer of oil were carefully aspirated, the remaining tube contents were frozen in a dry ice/acetone mixture, and the tube tips containing the cell pellet were cut off and counted. The initial rate of ^{86}Rb transport, a measure of K^+ transport through the Ca^{2+}-activated pathway, was calculated from the linear least square derived slope. Inhibitory constants were calculated by computer-assisted nonlinear curve fitting (Ultrafit 2.0; Biosoft).

Measurement of ^{125}I-ChTX binding to red cells

White cells were removed by passing 0.8 ml of packed red cells through a 5-ml syringe containing a mixture of equal parts of alfa-cellulose and microcrystalline cellulose as originally described by Beutler and West (17). Red cells were washed three times in binding medium containing 18 mM NaCl, 2 mM KCl, 10 mM Tris-Cl, pH 8.0, 230 mM sucrose, and 0.25% bovine serum albumin. A suspension was made in the same medium at 15% Hct. Cells were added to 3.5 ml of binding medium containing ^{125}I-ChTX to a final concentration of 1×10^7 cells/ml, in the absence or presence of the specified drugs. Tubes containing cell suspension were gently rotated for 90 min at room temperature. At the end of the incubation, aliquots of 1 ml were pelleted by microfuge and washed three times at 4°C with a solution containing 200 mM NaCl, 10 mM Tris-Cl, pH 8.0. The washed red cell pellet was lysed in 1 ml of 0.01% Acationox®, and counted in a gamma counter. Aliquots of cell-free binding medium were counted before addition of cells and at the end of the assay.

Measurement of membrane potential in red cells

Measurements of the proton distribution ratio in unbuffered media in the presence of the proton ionophore CCCP were used to estimate red cell membrane potential (18).

Measurement of Ca^{2+}-dependent erythrocyte dehydration

Experiments with A23187. Cells were incubated for 60 min at 37°C in a medium containing 140 mM NaCl, 4 mM KCl, 100 μM $CaCl_2$, 1 mM K-phosphate buffer, 10 mM Tris-Mops, pH 7.40, at 1–2% Hct with the specified inhibitors. A23187 was subsequently added to a final concentration of 60 μmol/liter cells under stirring. At the specified times, the cell suspension was diluted with an equal amount of medium containing 5 mM EGTA and 0.1% BSA, to chelate extracellular Ca^{2+} and remove A23187. The red cells were then washed four times with the same medium at 37°C. An aliquot of the washed cells was used to measure the distributions of cell volume and hemoglobin concentration with a blood analyzer (H*2 Technicon; Miles Diagnostic Inc., Tarrytown, NY; reference 19). The remaining cells were washed four more times with choline washing solution (144 mM choline Cl, 1 mM $MgCl_2$, 10 mM Tris-Mops, pH 7.40 at 4°C) for measurements of internal Na and K contents by atomic absorption spectrometry (3).

Exposure to oxygenation-deoxygenation cycles. Cells were incubated at 37°C and 10% Hct in a medium containing 110 mM NaCl, 25 mM Na bicarbonate, 5 mM KCl, 1 mM Na-phosphate buffer, pH 7.40, 1 mM $MgCl_2$, 5 mM glucose, and 0.01% bovine serum albumin. Media also contained either 2 mM Ca Cl_2 (±10 μM CLT) or 1 mM EGTA. Cells were exposed to repeated cycles of 1 min at 15% O_2, 5% CO_2, and 4 min at 5% CO_2 (gases were balanced with N_2 and hydrated in gas washers containing assay medium). After 1 or 3 h, cells were harvested for cation content, cell volume, and hemoglobin concentration measurements.

Measurements of ^{35}S-sulfate influx into AA erythrocytes

^{35}S-sulfate uptake into AA red cells was measured as described by Schofield et al. (20).

Results

Imidazole antimycotics inhibit Ca^{2+}-activated ^{86}Rb influx in SS erythrocytes. The inhibitory effect of CLT on the Ca^{2+}-activated ^{86}Rb influx of SS cells from three patients was assessed in low ionic strength media (18 mM NaCl, 2 mM KCl, 230 mM

sucrose) and in normal saline (140 mM NaCl, 2 mM KCl) in the presence of 50 μM $CaCl_2$ and 60 μmol A23187/liter cell. As shown in Fig. 1 *A*, CLT markedly inhibited Ca^{2+}-activated ^{86}Rb influx. For red cells from each patient, the inhibition was greater when measured at low ionic strength. Mean values of IC_{50}±SD were 29±15 nM (n = 3) at low ionic strength and 51±15 nM (n = 3) for normal saline.

Other antimycotic drugs were tested for their ability to inhibit Ca^{2+}-activated ^{86}Rb influx in sickle erythrocytes. As shown in Table I, the rank order of inhibitory potency determined in normal saline was clotrimazole (IC_{50} = 51±15 nM) = miconazole (IC_{50} = 110 nM) > econazole (IC_{50} = 270 nM), with no inhibition by fluconazole. The degree of transport inhibition produced by 10 μM clotrimazole (87%) was significantly greater than with 10 μM miconazole (63%). ^{86}Rb influx was partially inhibited by metronidazole (IC_{50} = 440 nM), a member of the nitroimidazole class, but only marginally by 10 μM ornidazole and tinidazole, two other members of this class.

To address the specificity of action of the imidazole antimycotics, we assessed the effect of these drugs on ^{35}S-sulfate influx into normal human red cells (sulfate/chloride exchange, reference 20). This assay is an index of function of the major anion transport system of red cells, the AE1 (band 3) chloride/bicarbonate exchanger (21). We found no inhibition of AE1 function by any of the above-mentioned compounds at 10 μM concentrations (not shown). Gardos channel–mediated cell shrinkage requires net movement of Cl^- or another anion to accompany K loss from the cell. AE1 is thought to mediate the dominant Cl^- conductance of the red cell membrane, as well as the much greater, electrically silent membrane permeability to Cl^-. Thus, the lack of inhibition of sulfate uptake by CLT makes primary inhibition of electrogenic anion permeability an unlikely explanation for the inhibitory effect of CLT on Ca^{2+}-activated ^{86}Rb influx. Furthermore, there was no effect of 10 μM or of 1 μM CLT on the activities in normal human red cells of the Na-K pump, Na-K-Cl cotransport, or K-Cl cotransport (not shown).

When the Gardos channel is activated, the increased membrane permeability for K^+ shifts the membrane potential away from the equilibrium potential for Cl^- (E_{Cl}) toward the equilibrium potential for K^+ (E_K). This effect is magnified when the membrane Cl^- permeability is inhibited by DIDS (18). The effect of CLT on the membrane potential of SS cells in which the Gardos channel is activated is shown in Fig. 1 *B*. CLT markedly reduced K^+ permeability and shifted E_m from E_K toward E_{Cl}. The inhibitory potency of CLT in this assay was higher in low ionic strength conditions than in normal saline (Fig. 1 *B*, IC_{50} values of 33±6 nM and 208±13 nM, respectively).

Imidazole antimycotics displace bound ^{125}I-ChTX from SS erythrocytes. ^{125}I-ChTX is a specific ligand of the Ca^{2+}-activated K^+ channel of human red cells. In normal red cells, 60–70% of the total binding can be displaced by an excess (50 nM) of cold ChTX (16). Levels of specific binding of ^{125}I-ChTX are more heterogeneous in SS patients, with markedly increased specific binding in the least dense cells (14). To examine the interaction between CLT and the Ca^{2+}-activated K^+ channel of SS cells, we measured displacement of ^{125}I-ChTX (50 pM) bound to SS cells by increasing concentrations of CLT. As shown in Fig. 1 *C*, CLT displaced ^{125}I-ChTX in a dose-dependent manner. The IC_{50} for displacement (12±4 nM, average of triplicate experiments with red cells from two individuals) was

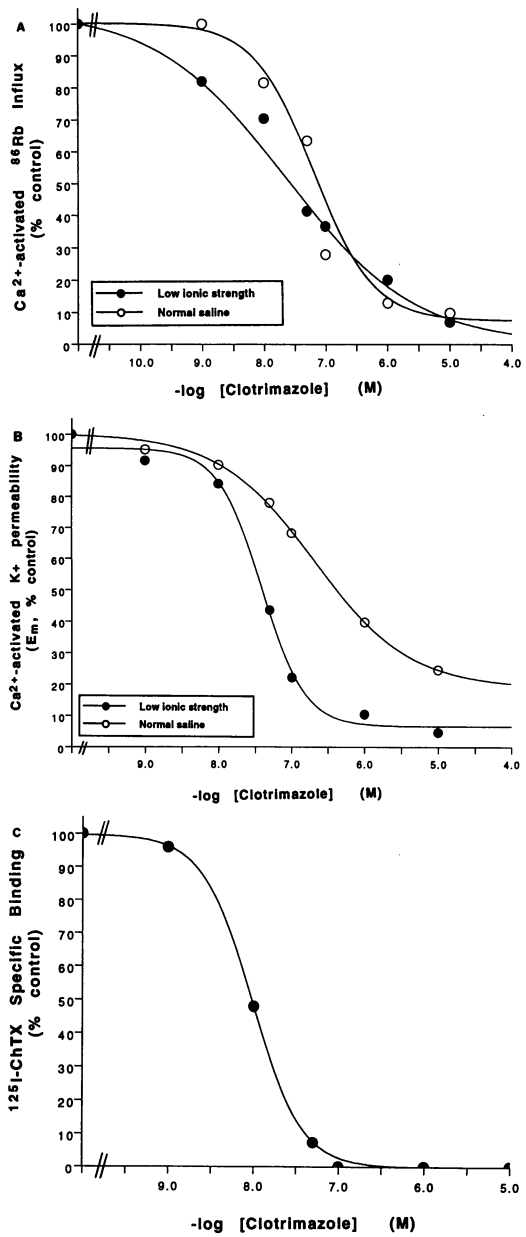

Figure 1. (*A*) The effect of clotrimazole on Ca^{2+}-activated ^{86}Rb influx in sickle erythrocytes. 86Rb influx was measured in SS cells incubated in low or normal ionic strength conditions. Data represent mean values±SD of triplicate experiments performed with red cells from three individuals. Flux media contained 18 mm NaCl, 2 mM KCl, 230 mM sucrose, 10 mM Tris-HCl, pH 7.40 (closed circles) or 140 mM NaCl, 2 mM KCl, 10 mM Tris-HCl, pH 7.40 (open circles). All media contained 50 μM $CaCl_2$ and 60 μmol/liter cell A23187. (*B*) The effect of clotrimazole on the membrane potential (E_m) of sickle erythrocytes. Suspended at 5% Hct in unbuffered low ionic strength (*closed circles*) or normal saline media (*open circles*) in the presence of A23187 and 50 μM $CaCl_2$. All media contained 100 μM ouabain, 10 μM bumetanide, 10 μM DIDS, 10 μM methazolamide, and 50 μM CCCP. Data are mean values of triplicate experiments performed with red cells from two individuals. Control values in the absence of A23187 refer to normal saline conditions. (*C*) Displacement of ^{125}I-ChTX binding from sickle erythrocytes by clotrimazole. SS cells were incubated at 1 × 10^7 cells/ml with 50 pM ^{125}I-ChTX, in the presence of increasing concentrations of clotrimazole in low ionic strength medium (18 mm NaCl, 2 mM KCl, 230 mM sucrose, 10 mM Tris-HCl, pH 8.0). Data represent the average of triplicate determinations in two SS patients.

Table I. Inhibition of Ca^{2+}-activated ^{86}Rb Influx and Displacement of Specific ^{125}I-ChTX Binding in Sickle Erythrocytes

Drug	Inhibition of ^{86}Rb influx				Displacement of specifically bound ^{125}I-ChTX	
	Low ionic strength		Normal saline			
	IC_{50}	Maximum inhibition (%)	IC_{50}	Maximum inhibition (%)	IC_{50}	Percent displacement by 10^{-5} M
	nM		*nM*		*nM*	
Imidazoles						
Clotrimazole	29±15	93	51±15	87	12±4	98
Miconazole	100	49	100	63	165	77
Econazole	575	31	270	47	205	50
Triazoles						
Fluconazole	—	0	—	0	—	26
Nitroimidazoles						
Metronidazole	850	28	440	36	580	66
Ornidazole	—	11	—	21	—	—
Tinidazole	—	21	—	3	—	—
Charybdotoxin	0.085±0.025	95.5	1.2±0.5	52	0.033	100

Results for clotrimazole and charybdotoxin are expressed as mean±SD (n = 3) for ^{86}Rb influx, and as the average of two experiments for displacement of bound ^{125}I-ChTX. Data for other drugs are from single experiments. All experiments were performed in triplicate. IC_{50} values were caclulated with nonlinear curve fitting. 100% specific binding represented displacement of 74.5% of total bound ^{125}I-ChTX by 50 nM cold ChTX. Drug concentrations tested ranged from 10^{-9} M to 10^{-5} M.

in the same range as the IC_{50} for inhibition of Ca^{2+}-activated ^{86}Rb influx (29±15 nM). Other imidazole antimycotics that inhibited Ca^{2+}-activated ^{86}Rb influx also displaced specifically bound ^{125}I-ChTX from sickle erythrocytes (Table I). The IC_{50} values for flux inhibition and for ^{125}I-ChTX displacement by the active inhibitors tested showed reasonably good agreement.

Imidazole antimycotics block Ca^{2+}-dependent dehydration of SS erythrocytes. Several protocols of deoxygenation have been used to demonstrate the role of the Ca^{2+}-activated K channel in promoting sickle cell dehydration (7, 8, 13, 22). We first assessed the inhibitory effect of CLT and other antimycotics on the dehydration mediated by the Ca^{2+}-activated K^+ channel under conditions of maximal activation of this pathway by exposure to A23187 for 15 min in the presence of 100 μM $CaCl_2$. After rapid removal of ionophore and chelation of Ca^{2+}, single cell volume and hemoglobin concentration were measured with the (Technicon H*2; Miles Diagnostics Inc.) blood analyzer.

Fig. 2 *A* shows a typical plot of hemoglobin concentration (HC, x axis) versus cell volume (V, y axis) in the dual parameter histogram display in the upper row, and distribution histograms for red cell volume and hemoglobin concentration in the lower row. Arbitrary gate values (hemoglobin concentration < 28 and > 41 g/dl; cell volume < 60 and > 120 fl) are used to define hypo- and hyperchromic cells and micro- and macrocytic cells, respectively. Reticulocytes and cells with low HC and high mean corpuscular volume (MCV) occupy the top of the middle and left zones, discocytes occupy the central quadrant, and dehydrated dense cells are found in the right middle zone. SS cells exposed to A23187 and Ca^{2+} were markedly dehydrated, as shown by the shift of the HC histogram rightward to higher values (Fig. 2 *A, left column*). The presence of 10 μM CLT substantially prevented this dehydration such that the HC and volume distributions of SS cells reverted nearly to those of control conditions (Fig. 2 *A, right column*). Measurements of cell cation content at the end of the incubation supported the hypothesis that the effects of CLT on volume and HC were caused by inhibition of K loss. Mean values from experiments with SS cells from three individuals indicated that cell K was decreased in the presence of Ca^{2+}, and this change was markedly inhibited by 10 μM CLT (Table II). Similar experiments were carried out with miconazole and econazole at 10 μM concentrations, but the inhibitory effects on dehydration were substantially smaller than those observed for CLT (not shown).

To evaluate the effect of CLT on the changes in cation content induced by deoxygenation, SS cells were exposed for periods of 1–3 h to repeated cycles of 4 min deoxygenation and 1 min oxygenation. As shown in Fig. 2 *B*, this deoxygenation protocol produced a Ca^{2+}-dependent cell dehydration of lesser degree than did A23187. This dehydration was inhibited to equal extent by Ca^{2+} chelation with EGTA or by 10 μM CLT. Similar experiments for SS cells from three individuals are summarized in part B of Table II. Table II also documents the ability of CLT to prevent completely the deoxygenation-induced, Ca^{2+}-dependent loss of cellular K^+.

Discussion

We have demonstrated in this report that imidazole derivatives are potent and specific inhibitors of the Ca^{2+}-activated K^+ channel (Gardos channel) of sickle erythrocytes. CLT appears to be the most potent inhibitor of the Gardos channel of sickle cells among the tested members of this class of compounds. CLT displayed IC_{50} values for inhibition of Ca^{2+}-activated K^+ transport of 29±15 nM in low ionic strength medium and 51±15 nM at physiologic ionic strength (Fig. 1 *A*). Similar values were obtained for the inhibition by CLT of the membrane potential changes induced by activation of the Gardos channel in sickle erythrocytes (Fig. 1 *B*). CLT also displaced bound

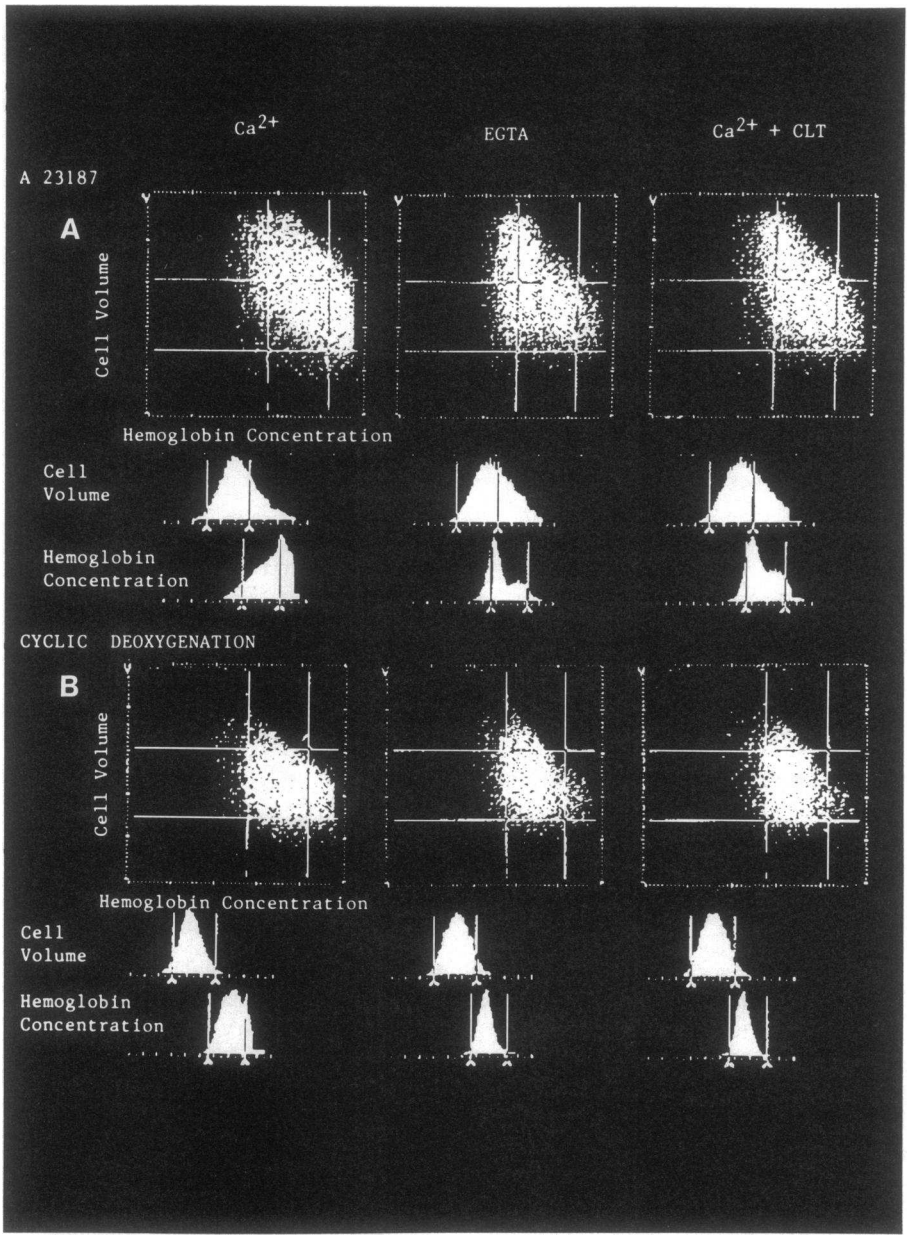

Figure 2. The effects of 10 μM clotrimazole on the dehydration of SS cells via the Ca^{2+}-activated K channel. (*A*) Experiments with A23187: Medium contained 140 mM NaCl, 4 mM K-Cl, 1 mM K-phosphate buffer, pH 7.40, 10 mM glucose, 10 mM Tris-Mops, pH 7.40 at 37°C, and A23187 (60 μmol/liter cells) with additional compounds as indicated. *Left column,* 100 μM $CaCl_2$; *middle column,* 1 mM EGTA; *right column,* 100 μM $CaCl_2$ + 10 μM CLT; values for cell volume and hemoglobin concentration (±SD) for the patient presented here were 108±27 fl and 38.2±6 g/dl in the presence of A23187 and $CaCl_2$, 116±28 fl and 32±5.1 g/dl in control with 1 mM EGTA, and 113±28 fl and 33.7±5.3 g/dl with 10 μM CLT, respectively. (*B*) Experiments with cyclic oxygenation-deoxygenation. Medium contained 110 mM NaCl, 25 mM Na bicarbonate, 5 mM KCl, 1 mM Na-phosphate buffer, pH 7.40, 1 mM $MgCl_2$, 5 mM glucose and 0.01% bovine serum albumin. Cells at 10% Hct were incubated for 3 h at 37°C and exposed to 36 consecutive cycles of oxygenation and deoxygenation (1 min with 15% O_2, 5% CO_2, 80% N_2, and 4 min with 5% CO_2, 95% N_2). *Left column,* 2 mM Ca Cl_2; *middle column,* 1 mM EGTA; *right column,* 2 mM Ca Cl_2 + 10 μM CLT. Values for cell volume and hemoglobin concentration (±SD) for the patient presented here were 86±16 fl and 37.2±4.4 g/dl in the presence of 2 mM $CaCl_2$, 95±18 fl and 33.8±3.3 g/dl with 1 mM EGTA, and 97.5±17 fl and 33.2±3.0 g/dl with 2 mM $CaCl_2$ and 10 μM CLT, respectively.

The distributions of cell volume and hemoglobin concentration were measured with a blood analyzer (H*2 Technicon; Miles Diagnostics Inc.). Plots of hemoglobin concentration (HC, x axis) versus cell volume (V, y axis) in the dual parameter histogram display are shown the upper parts of each panel, and distribution histograms for red cell volume and hemoglobin concentration are presented in the lower parts of each panel. Histogram goal post values for hemoglobin concentration are 28 and 41 g/dl and for cell volume 60 and 120 fl.

^{125}I-ChTX, a specific ligand for the Ca^{2+}-activated K channel of human red cells (16) with an IC_{50} (12±4 nM) similar to that for its inhibition of ^{86}Rb transport (Fig. 1 *C*). In addition, CLT markedly inhibited the dehydration of sickle cells induced by activation of the Gardos channel, whether by A23187 or by cycles of oxygenation-deoxygenation (Fig. 2 and Table II).

Based on the similarities in the IC_{50} values of CLT for inhibition of ^{86}Rb influx influx and for displacement of ^{125}I-ChTX, we propose that CLT inhibits the Gardos channel by direct binding to an inhibitory site on the external surface of the channel which partially overlaps with the ChTX binding site or otherwise promotes ChTX dissociation. The inhibition by CLT of net K^+ efflux was probably not secondary to blockage of the anion permeability, since the AE-1-mediated influx of sulfate was not decreased by CLT. This lack of effect of CLT on anion permeability was also observed in normal red cells by Alvarez et al. (15).

Other imidazole antimycotics, as well as nitroimidazole derivatives, were tested for their inhibitory potencies on the Gardos pathway. Important differences were found among the drugs in IC_{50} for ^{86}Rb influx inhibition and in the maximal inhibition achieved by 10^{-5} M concentrations. The drugs revealed a rank order of inhibitory potency of clotrimazole > miconazole > econazole > metronidazole. Fluconazole was inactive as an inhibitor of K^+ transport. The IC_{50} values obtained in SS cells for miconazole (110 nM) and econazole (270 nM) differed greatly from those measured with a different method in normal AA cells (1.5 and 1.8 μM, respectively; reference 15). Though CLT has been shown also to inhibit cytochrome P450 activity and plasmalemmal calcium influx (15), the data pre-

Table II. Effect of Clotrimazole on Ca^{2+}-dependent Dehydration of SS Erythrocytes

	Cell Na	Cell K	Total cations	MCV	HC
		mmol/kg hemoglobin		*fl*	*g/dl*
A. Exposure to A23187					
100 μM $CaCl_2$	79±6	85±24	164±18	105±4	39.6±1.5
1 mM EGTA	57±8	279±20	336±23	115±2	32.7±0.5
100 μM $CaCl_2$ + 10 μM CLT	91±12	211±30	300±22	110±3	35.0±1.0
B. Exposure to oxygenation/ deoxygenation cycles					
2 mM $CaCl_2$	115±10	180±4	295±7	81±6	37.4±0.3
Ca^{2+}-free, 1 mM EGTA	118±20	229±10	347±23	89±6	33.5±1.2
2 mM $CaCl_2$, 10 μM CLT	134±15	220±7	355±10	88±7	33.8±0.5

For each of the two dehydration conditions, data are the mean±SD of three experiments in SS patients. For experiments reported in (*A*), all the cells were incubated with the indicated compounds in the presence of 60 μmol/liter cell A23187. The increased MCV in all these cells, greatest in the presence of EGTA can be attributed to the swelling that is usually observed in the presence of A23187 when the $MgCl_2$ concentration in the external medium is above 0.15 mM (30).

sented in this paper suggest that these lower affinity actions of CLT probably play little or no role in the ability of CLT to inhibit the Gardos pathway.

Generation of irreversibly sickled cells under conditions of ATP depletion has been shown to depend on the presence of external Ca^{2+}. The possible involvement of the Gardos effect in this phenomenon was first advocated by Glader and Nathan in 1978 (7). However, they also found that deoxygenation in the absence of ATP depletion did not change the total cation content of red cells. Experiments performed in thiocyanate (SCN^-) media have indicated that Ca^{2+}-dependent K^+ loss does take place during deoxygenation and is associated with formation of dense cells (8). The integrated red cell model developed by Lew and Bookchin suggests a major role for the Gardos pathway in cell dehydration (23). Experiments in SS reticulocytes have shown that 30 min deoxygenation in the presence of Ca^{2+} induces significant dehydration that can be blocked by quinine, a low affinity inhibitor of the Gardos pathway (24).

Antimycotics of the imidazole class include clotrimazole, miconazole, ketoconazole, and econazole, whereas terconazole, itraconazole, and fluconazole are members of the triazole class (25). Their antimycotic effects have been attributed to their inhibition of fungal sterol-14-demethylase, a cytochrome P-450 dependent system (25). All these drugs are available as topical preparations, and a number are available for intravenous administration. CLT, ketoconazole and itraconazole, can also be administered orally. Side effects of oral CLT include infrequent nausea, vomiting, diarrhea, and very infrequent neurologic effects. The latter, however, have been attributed to release of endotoxins from lysed fungi (26). Plasma levels of the drug in patients receiving two oral doses of 1 g spaced 4 h apart varied between 1.2 and 4 μg/ml at 9–12 h after the second dose (27). These levels correspond to plasma concentrations of 3.5–11.6 μM. Another study that administered CLT in three daily oral doses of 20 mg/kg body wt reported serum levels between 0.3 and 1 μg/ml, corresponding to 0.9–2.9 μM (28). A single oral dose of 200 mg/d has resulted in CLT plasma concentrations of 0.2–0.35 mg/liter (0.58–1.0 μM; reference 25).

Oral doses of 522 and 1,000 mg of miconazole in normal subjects yielded peak plasma concentrations of 0.37–1.16 μg/ml (0.77–2.42 μM; reference 29). However, use of intravenous miconazole is associated with phlebitis, pruritus, nausea, fever, and chills in 11–28% of patients and, more importantly, with anemia secondary to marrow hypoplasia in 40–50% of patients (29). This hematologic side effect probably will prohibit trials of miconazole in SS disease. Fluconazole, the only triazole antimycotic that has been extensively studied with systemic administration, does not inhibit the Ca^{2+}-activated K^+ channel of SS cells.

Among the compounds tested here, CLT displayed the most potent and the most complete inhibition of the Ca^{2+}-activated K^+ channel of sickle cells. Most importantly, the measured IC_{50} for inhibition of ^{86}Rb influx by CLT is lower than the levels achievable in plasma after oral administration. CLT plasma and serum levels reported in the literature after high but nontoxic doses are 10–50-fold higher than the IC_{50} for inhibition of ^{86}Rb influx, suggesting that nearly complete inhibition of the Gardos pathway might be therapeutically achievable. High dose oral CLT administration also has been associated with relatively mild side effects.

In conclusion, the widely used, inexpensive drug clotrimazole is a potent and specific inhibitor of the Ca^{2+}-activated K^+ channel of sickle erythrocytes. The ratio between clinically achievable plasma concentrations and in vitro inhibitory potency, together with the relatively mild side effects, suggest that it could be used to prevent dehydration of SS cells in vivo. Clotrimazole may also lend itself to combination therapy with other promising therapeutic agents such as hydroxyurea and sodium phenylbutyrate.

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Note added in proof. Though the ability of CLT to displace ^{125}I-ChTX leaves little doubt about its mechanism of action at nanomolar concentrations, the 10-μM concentration used in the experiments of Fig. 2 and Table II allows for a contribution to the observed effects by possible inhibition of P450 epoxygenase activity (30).

References

1. Eaton, W. A., and J. Hofrichter. 1987. Hemoglobin S gelation and sickle cell disease. *Blood.* 70:1245–1266.

2. Clark, M. R., R. C. Unger, and S. B. Shohet. 1978. Monovalent cation composition and ATP and lipid content of irreversibly sickled cells. *Blood.* 51:1169–1178.

3. Brugnara, C., H. F. Bunn, and D. C. Tosteson. 1986. Regulation of erythrocyte cation and water content in sickle cell anemia. *Science (Wash. DC).* 232:388–390.

4. Rosa R. M., B. E. Bierer, R. Thomas, J. S. Stoff, M. Kruskall, S. Robinson, H. F. Bunn, and F. H. Epstein. 1980. Study of induced hyponatremia in the prevention and treatment of sickle cell crisis. *N. Engl. J. Med.* 303:1138–1143.

5. Canessa, M., A. Spalvins, and R. L. Nagel. 1986. Volume-dependent and NEM-stimulated K^+, Cl^- transport is elevated in SS, SC and CC human red cells. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 200:197–202.

6. Gardos, G. 1958. The function of calcium in the potassium permeability of human erythrocytes. *Biochim. Biophys. Acta.* 30:653–654.

7. Glader, B. E., and D. G. Nathan. 1978. Cation permeability alterations during sickling: relationship to cation composition and cellular hydration of irreversibly sickled cells. *Blood.* 51:983–989.

8. Bookchin, R. M., O. E. Ortiz, and V. L. Lew. 1986. Red cell calcium transport and mechanisms of dehydration in sickle cell anemia. *In* Approaches to the Therapy of Sickle Cell Anemia. Y. Beuzard, S. Charache, and F. Galacteros, editors. Les Editions INSERM, Paris. pp. 291–300.

9. Latorre, R., A. Oberhauser, P. Labarca, and O. Alvarez. 1989. Varieties of calcium-activated potassium channels. *Annu. Rev. Physiol.* 51:385–399.

10. Kaji D. 1990. Nifedipine inhibits calcium-activated K transport in human erythrocytes. *Am. J. Physiol.* 259 (*Cell Physiol.* 28):C332–C339.

11. Ellory, J. C., K. Kirk, S. J. Culliford, G. B. Nash, and J. Stuart. 1992. Nitrendipine is a potent inhibitor of the Ca^{2+}-activated K^+ channel of human erythrocytes. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 296:219–221.

12. Wolff, D., X. Cecchi, A. Spalvins, and M. Canessa. 1988. Charybdotoxin blocks with high affinity the Ca-activated K^+ channel of Hb A and Hb S red cells: individual differences in the number of channels. *J. Membr. Biol.* 106:243–252.

13. Ohnishi, S. T., H. Katagi, and C. Katagi. 1989. Inhibition of the in vitro formation of dense cells and of irreversibly sickled cells by charybdotoxin, a specific inhibitor of calcium-activated potassium efflux. *Biochim. Biophys. Acta.* 1010:199–203.

14. Brugnara, C., L. De Franceschi, L. McMahon, and S. L. Alper. 1992. Inhibitors of the Ca^{2+}-activated K^+ channel in sickle erythrocytes. A possible role for clotrimazole as a new therapeutic agent for sickle cell disease. *Blood.* 80:344a.

15. Alvarez, J., M. Montero, and J. Garcia-Sancho J. 1992. High affinity inhibition of Ca^{2+}-dependent K^+ channels by cytochrome P-450 inhibitors. *J. Biol. Chem.* 267:11789–11793.

16. Brugnara, C., L. De Franceschi, and S. L. Alper. 1993. Ca^{2+}-activated K^+ transport in erythrocytes: comparison of binding and transport inhibition by scorpion toxins. *J. Biol. Chem.* 268:8760–8768.

17. Beutler, E., and C. West. 1976. The removal of leukocytes and platelets from whole blood. *J. Lab. Clin. Med.* 88:328–333.

18. Halperin, J. A., C. Brugnara, M. T. Tosteson, T. Van Ha, and D. C. Tosteson. 1989. Voltage activated cation transport in human erythrocytes. *Am. J. Physiol.* 257 (*Cell Physiol.* 26):C986–C996.

19. Mohandas, N., A. Johnson, J. Wyatt, L. Croisille, J. Reeves, D. Tycko, and W. Groner. 1989. Automated quantitation of cell density distribution and hyperdense cell fraction in RBC disorders. *Blood.* 74:442–447.

20. Schofield, A. E., D. M. Reardon, and M. J. A. Tanner. 1992. Defective anion transport activity of the abnormal band 3 in hereditary ovalocytic red blood cells. *Nature (Lond.).* 355:836–838.

21. Milanick, M. A., and R. B. Gunn. 1982. Proton-sulfate cotransport: mechanism of H^+ and sulfate addition to the chloride transporter of human red blood cells. *J. Gen. Physiol.* 79:87–113.

22. Horiuchi, K., S. K. Ballas, and T. Asakura. 1988. The effect of deoxygenation rate on the formation of irreversibly sickled cells. *Blood.* 71:46–51.

23. Lew, V. L., C. J. Freeman, O. E. Ortiz, and R. M. Bookchin. 1991. A mathematical model of the volume, pH and ion content regulation in reticulocytes. *J. Clin. Invest.* 87:100–112.

24. Bookchin, R. M., O. E. Ortiz, and V. L. Lew. 1991. Evidence for a direct reticulocyte origin of dense red cells in sickle cell anemia. *J. Clin. Invest.* 87:113–124.

25. Goodman, A. G., T. W. Rall, A. S. Nies, and P. Taylor. 1990. Goodman and Gilman's. The pharmacological basis of therapeutics. Pergamon Press, New York. p. 1169.

26. Sawyer, P. R., R. N. Brogden, R. M. Pinder, T. M. Speight, and G. S. Avery. 1975. Clotrimazole: a review of its antifungal activity and therapeutic efficacy. *Drugs.* 9:424–447.

27. Seo, M., H. Iida, and Y. Miura. 1977. Basic experiments with clotrimazole administered orally. *Curr. Med. Res. Op.* 5:169–178.

28. Weuta, H. 1974. Clinical studies with oral clotrimazole. *Postgrad. Med. J.* 50(Suppl. 1):45–49.

29. Heel, R. C., R. N. Brogden, G. E. Pakes, T. M. Speight, and G. S. Avery. 1980. Miconazole: a preliminary review of its therapeutic efficacy in systemic fungal infections. *Drugs.* 19:7–30.

30. Wang, W., A. Cassola, and G. Giebisch. 1992. Arachidonic acid inhibits the secretory K+ channel of cortical collecting duct of rat kidney. *Am. J. Physiol.* 262:F554–559.