Oxpentifylline and cetiedil citrate improve deformability of dehydrated sickle cells

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SUMMARY Erythrocytes from 14 patients with homozygous sickle cell anaemia were treated with the calcium ionophore A23187 to induce loss of cellular potassium and water. The dehydrated cells showed a decrease in filterability (loss of deformability) through pores of 5 μm diameter. Oxpentifylline and cetiedil citrate, which preserve erythrocyte cation and water content, had a significant ($p < 0.01$) protective effect against loss of deformability at a concentration of 1 $\mu\text{mol/l}$. Oxpentifylline showed no adverse effect on the rheology, morphology, or haemolysis of sickle cells at concentrations up to 500 $\mu\text{mol/l}$. Drugs that act on the erythrocyte membrane to maintain cell hydration are of potential rheological benefit in sickle cell anaemia.

The dense erythrocytes in patients with sickle cell anaemia are depleted of potassium¹ and the associated loss of cell water contributes to their high mean cell haemoglobin concentration (MCHC).²⁻⁴ Increase in MCHC greatly increases the intracellular polymerisation of haemoglobin S (HbS)⁵ so that polymer may form at arterial oxygen tension.⁶

It is not known whether sickle cells lose potassium and water by direct leakage through a cell membrane distorted by extended spicules of polymer,⁷ or by opening the Gardos⁸ channel in response to transient increases in cytoplasmic calcium before this cation becomes compartmentalised in membrane vesicles.⁹ Alternatively, young sickle cells that swell in response to a fall in pH at sites of ischaemia may lose potassium and water via the recently described volume sensitive KCl cotransport system.^{10,11} Whatever the cause of their dehydration, the deformability of sickle cells is adversely affected by the increase in MCHC^{12,13} and is substantially improved by rehydration.^{12,14}

Oxpentifylline is a dimethyl xanthine derivative that binds reversibly to erythrocyte membranes and has been shown to increase walking distance in patients with atherosclerotic intermittent claudication.¹⁵ We have recently shown that the drug prevents loss of potassium and therefore water from normal (haemoglobin AA) erythrocytes that had been loaded with calcium using the ionophore A23187.¹⁶ The same experimental model has now been used to study the action of oxpentifylline on sickle cells from

14 patients with homozygous (SS) sickle cell anaemia. Oxpentifylline was compared, at equimolar concentrations, with the iminoester cetiedil citrate which also changes the cation content of sickle cells by increasing passive influx of sodium or preventing loss of potassium induced by calcium gain.¹⁷⁻²⁰ Intravenous infusion of cetiedil has been shown to shorten the duration of vaso-occlusive crisis.²¹

Material and methods

The 14 SS patients were in the asymptomatic steady state. Heparinised venous blood was washed through Imugard IG 500 cotton wool (Terumo Corporation, Tokyo, Japan), using 20 mmol/l HEPES buffered saline (HBS) of osmolality 290 mmol/Kg H₂O and pH 7.4, to give a suspension of leucocyte free washed erythrocytes.²² Passage through cotton wool does not selectively remove irreversibly sickled cells²³ or other dense sickle cells,²⁴ and HBS has been shown not to change the MCHC of sickle cells.²⁵

Filtration of oxygenated sickle cells through polycarbonate membranes with pores of 5 μm diameter and 10-11 μm length (Nuclepore Corporation, Pleasanton, California, USA) was measured at 37°C using a Hemorheometre (IMH, 95470 St Witz, France) to give an index of filtration (IF) corrected for erythrocyte count.¹⁶ This index is the ratio of the flow resistance of erythrocytes suspended in HBS at a cell count of $0.3 \times 10^{12}/\text{l}$ to that of HBS alone, and is expressed as relative resistance rather than absolute units; an increase in IF indicates loss of erythrocyte

Improving the deformability of dehydrated sickle cells

Fig 1 Index of filtration (IF) of sickle cells from 14 patients after incubation of cells for 60 minutes in control buffer and in buffer with calcium ionophore A23187. Increase in IF indicates loss of filterability.

Fig 2 Index of filtration of sickle cells after incubation for 60 minutes in buffer containing A23187 ionophore and either oxpentifylline or cetiedil citrate. Results (mean and 95% confidence interval for 14 patients) expressed as percentage change from IF values for sickle cells incubated with A23187 (fig 1) but without either drug.

deformability. A pore diameter of 5 µm rather than 3 µm was used as this enhances sensitivity to cytoplasmic viscosity and therefore to cytoplasmic hydration.^{26 27} The polycarbonate membranes were cleaned by ultrasonication in 1% w/v sodium dodecyl sulphate and reused.²⁸

A stock solution of the calcium ionophore A23187 (Calbiochem brand, Behring Diagnostics, La Jolla, California, USA) in absolute ethanol (1.9 mmol/l) was stored at -40°C and diluted in ice cold HBS immediately before use. Oxygenated sickle cells ($0.3 \times 10^{12}/l$) were incubated for 60 minutes at 37°C in control buffer (HBS, CaCl₂ 100 µmol/l, MgCl₂ 2 mmol/l, glucose 10 mmol/l) and in control buffer plus A23187. As there was considerable individual variation in response to ionophore a concentration of A23187 (range 0.5-1.3 µmol/l) which increased the IF value by at least two-fold, was selected for each patient. Oxpentifylline or cetiedil citrate, at final drug concentrations of 0, 1, 10, 100 and 500 µmol, was added to the cell suspensions at 37°C, 15 minutes before the addition of ionophore. Oxpentifylline was dissolved in HBS immediately before use. A stock solution of cetiedil citrate in absolute ethanol (125 mmol/l) was stored at -40°C.

Morphology of incubated erythrocytes was as-

sessed by interference microscopy on 300 oxygenated cells fixed with 1.25% w/v glutaraldehyde in HBS, using the Bessis classification²⁹ for stomatocytes and echinocytes. Cells whose length was twice their width or those which had angular contours were counted as irreversibly sickled cells (ISC).³⁰ The extent of haemolysis of the erythrocyte suspensions was assessed from the supernatant haemoglobin concentration, measured spectrophotometrically using tetramethyl benzidine. Significance (two tail) was determined by Wilcoxon's non-parametric signed rank test for paired data.

Results

Calcium loading of sickle cells using A23187 ionophore caused an increase in IF (loss of filterability) after 60 minutes compared with cells in control buffer (fig 1). As there was considerable variation between patients in the extent to which A23187 increased IF, the effects of oxpentifylline and cetiedil citrate were expressed as percentage change from the IF value after incubation with A23187 alone (fig 2). All four concentrations of oxpentifylline significantly improved IF (10 µmol/l, $p < 0.05$; other concentrations, $p < 0.01$) and caused no change in erythrocyte mor-

Table Effect of oxpentifylline and cetiedil on erythrocyte morphology and haemolysis after incubation with A23187 ionophore for 60 minutes (mean and SEM, $n = 14$)

	Stomatocytes (%)	Echinocytes (%)	Irreversibly sickled cells (%)	Supernatant haemoglobin (mg/l)
Control buffer	4.9 (1.9)	8.1 (1.4)	9.7 (2.1)	97 (7)
A23187	3.5 (1.2)	19.1† (4.9)	8.3 (2.2)	101 (4)
Oxpentifylline:				
1 µmol/l	3.7 (1.0)	17.4 (3.0)	9.3 (2.5)	107 (8)
10 µmol/l	4.2 (1.1)	16.3 (4.1)	8.6 (2.0)	114 (7)
100 µmol/l	5.3 (1.7)	11.4 (2.9)	9.3 (2.0)	99 (3)
500 µmol/l	4.5 (2.9)	13.0 (2.8)	8.4 (2.0)	94 (5)
Cetiedil:				
1 µmol/l	5.9 (2.1)	13.1 (2.9)	8.9 (2.0)	97 (5)
10 µmol/l	8.4* (2.0)	8.1* (1.8)	8.2 (1.8)	110 (6)
100 µmol/l	70.1* (4.9)	2.1* (0.8)	5.0 (0.9)	1537* (684)
500 µmol/l	99.1* (0.3)	0* (0.3)	0.8* (0.3)	11316* (104)

† $p < 0.01$ compared with buffer value.

* $p < 0.01$ compared with A23187 value.

phology, percentage of irreversibly sickled cells, or haemolysis (table). Cetiedil concentrations of 1, 10, and 100 µmol/l resulted in a significant improvement ($p < 0.01$) in filterability of erythrocytes treated with A23187 after 60 minutes' incubation (fig 2). A concentration of 10 µmol/l, however, caused a significant increase in stomatocytes, while 100 µmol/l resulted in 70% stomatocytes and a 15-fold increase in haemolysis compared with A23187 alone (table). The highest concentration of cetiedil (500 µmol/l) gave no improvement in filterability compared with A23187 alone, probably because all the cells (99%) had become stomatocytic.

Discussion

Attempts to rehydrate sickle cells by reducing plasma sodium and osmolality are impractical^{31,32} but a change in cation flux across the sickle cell membrane to increase the water content of the cell is a more promising approach. Our study shows that at a concentration of 1 µmol/l cetiedil and oxpentifylline produce a significant beneficial effect on the deformability of ionophore dehydrated but oxygenated sickle cells, as measured using a 5 µm pore filtration technique. This rheological method, which accords with the guidelines of the International Committee for Standardisation in Haematology,³³ was designed to simulate erythrocyte flow in the microvasculature and can detect the rheological effect

of small amounts of intracellular polymer in sickle erythrocytes at arterial oxygen tension.³⁴

Cetiedil is a vasodilator³⁵ that enhances passive entry of sodium into erythrocytes^{17,18} and decreases potassium loss via the Gardos pathway after calcium loading.^{19,20} Both mechanisms could increase cell water, reduce MCHC and cytoplasmic viscosity, and thus improve erythrocyte deformability. Intravenous cetiedil (0.4 mg/kg body weight) has been shown to shorten the duration of vaso-occlusive crisis,²¹ and a single infusion at this dose gives a peak plasma concentration of 0.25 µmol/l.³⁶ A previous in vitro filtration study of deoxygenated sickle cells showed a beneficial effect of cetiedil at 50–200 µmol/l, with no effect on oxygenated cells.³⁷ The higher sensitivity of our filtration technique detected an effect on oxygenated SS cells treated with ionophore at 1 µmol/l, the lowest concentration studied. At 100 µmol/l and above, the drug caused substantial cell swelling (stomatocytosis) and haemolysis, as previously reported.^{17,18}

Oxpentifylline has recently been shown to reduce loss of potassium and therefore water from normal erythrocytes that had been loaded with calcium using the ionophore A23187 or when dehydrated by other ionophores (valinomycin and nystatin).¹⁶ In that study the dehydration was associated with an increase in MCHC, which was partially prevented by oxpentifylline. In the present study oxpentifylline significantly improved the filterability of dehydrated sickle cells at a concentration (1 µmol/l) close to that (0.43 µmol/l) achieved in plasma after ingestion of one 400 mg tablet.³⁸ This contrasts with a concentration of 5 mmol/l required to improve the filterability of normal (AA) cells dehydrated by exposure to this calcium ionophore for 60 minutes.¹⁶

It is difficult to show the action of drugs that change cation flux across membranes when there is no induced change in flux. Previous workers have stressed sickle cells by deoxygenation,³⁷ recurring cycles of deoxygenation-reoxygenation,³⁹ or depletion of adenosine triphosphate and calcium loading¹⁹ to show the in vitro effect of antisickling drugs. Our in vitro model was designed to investigate the protective rheological effect of oxpentifylline and cetiedil when added to sickle cells 15 minutes before they underwent loss of cation and water induced by a calcium ionophore. In the absence of this induced flux neither drug improved the filterability of sickle cells; cetiedil (100 and 500 µmol/l), in fact, impaired filterability further owing to the formation of stomatocytes. Thus the main therapeutic potential of these drugs lies in the prevention of cation and water loss from sickle cells when they are exposed in vivo to stresses such as deoxygenation, calcium loading, low pH or hyperosmolality.

The rheologically beneficial effect of oxpentifylline on sickle cells was, unlike that of cetiedil, independent of change in cell shape, and neither stomatocytosis nor haemolysis were seen at concentrations up to and including 500 $\mu\text{mol/l}$. This accords with the clinical experience of low toxicity of the drug in the long term treatment of atherosclerotic vascular disease. Although one cannot extrapolate from an *in vitro* effect on cells treated with an ionophore at 1 $\mu\text{mol/l}$ to a rheological effect *in vivo*, there have been three longitudinal case reports of individual patients who showed an improvement in the filterability of their sickle cells during treatment with oral oxpentifylline.^{40–42} Clinical benefit obtained with oxpentifylline has been reported in four sickle cell patients with recurrent priapism but not in five patients with painful crises.⁴³ Controlled trials of oral prophylaxis are now indicated, but careful rheological as well as clinical monitoring will be required as partial rehydration of dense sickle cells could improve their otherwise short survival time and thereby cause transient rheological deterioration.

Further studies of the cation sparing actions of oxpentifylline and cetiedil on the erythrocyte membrane are required, but the demonstration of a beneficial effect on the deformability of stressed sickle cells at a drug concentration of 1 $\mu\text{mol/l}$ indicates the potential of sickle cell "hydrotherapy" for improving blood rheology in this disease.

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