Cetiedil: Its Potential Usefulness in Sickle Cell Disease

By Lennette J. Benjamin, Garyfallia Kokkini, and Charles M. Peterson

Cetiedil used clinically in cases of chronic cardiovascular disease in Europe was found to inhibit sickling and improve the filterability of sickle erythrocytes in vitro under hypoxic conditions. The effects were optimal between 50 and 200 μM and were comparable to the effects seen following incubation with 5–10 mM cyanate. Incubation with cetiedil did not inhibit gelation or affect oxygen affinity. Neither isoelectric focusing nor column chromatographic globin chain separation procedures could identify any modified hemoglobin following incubation with radiolabeled compound. Fifteen percent of the radiolabel was associated with erythrocyte membranes. The antisickling properties of the compound therefore appear to be related to membrane effects or to an undefined interaction with hemoglobin. Incubation with cetiedil did not significantly alter the in vivo half-life of reinfused 51chromium-labeled erythrocytes in dogs (16 versus 18 days for controls) or humans with sickle cell disease (9.4 ± 2.8 versus 8.9 ± 4.0 days for the control period), thereby suggesting that the compound is not toxic to the erythrocyte. Combinations of cetiedil and cyanate led to an effect on morphology and filtration that was greater than that seen following incubation of erythrocytes with either compound alone. The fact that the effects of cetiedil, which may exert its action through membrane modification, are additive with those of cyanate, a known modifier of the hemoglobin molecule, suggests the possibility of a polypharmacologic approach to sickle cell disease. Such an approach might allow the rational design of optimal therapeutic regimens and the clinical reevaluation of known modifiers of hemoglobin at subtoxic doses in combination with agents that have their effect on the erythrocyte membrane.

Despite the fact that the molecular biology of sickle cell disease is well characterized, there are at present no specific treatment modalities to prevent or ameliorate the devastating sequelae of the disease. Previous attempts at treatment development have been directed toward inhibition of gelation (polymerization) through the modification of the hemoglobin molecule either covalently, as exemplified by sodium cyanate, or noncovalently, as exemplified by alkyluracils. In addition, the possibility of inhibiting sickling independent of gelation has been considered. The use of zinc and procaine hydrochloride are examples of this approach. The actions of these latter compounds have been ascribed to membrane effects.

Cetiedil has been used in Europe for symptomatic relief of intermittent claudication and pain of the lower extremities caused by arteriosclerosis obliterans, diabetogenic arteriosclerosis, and Raynaud disease. The compound has been demonstrated to have a relaxant action on vascular smooth muscle, to inhibit phosphodiesterase with a resultant increase in cyclic adenosin-3′,5′-monophosphate (cyclic AMP), to block the effect of bradykinin and to inhibit platelet aggregation. In addition, its administration to humans resulted in a decrease in both plasma fibrinogen concentration and in blood viscosity. Viscosity decreased under all slip conditions, particularly at low slip rates that reflect conditions of the microvasculature.

An analgesic with vasodilator properties and the ability to decrease blood viscosity and platelet aggregation would be of interest in treating or preventing the painful crises associated with sickle cell disease. Preliminary uncontrolled studies from the Ivory Coast have reported clinical improvement in patients with sickle cell disease treated with cetiedil during crises. The present study was undertaken to determine the effect of cetiedil on sickle cell morphology and filterability, the distribution of the drug within the red cell, and the mechanism of any antisickling effects observed. We also sought to assess the potential usefulness of cetiedil in combination with drugs known to inhibit sickling via the modification of hemoglobin. In addition, the survival of red cells incubated with cetiedil was also evaluated in dogs and in patients with sickle cell disease.

MATERIALS AND METHODS

Fresh blood samples were obtained from sickle cell patients or normal volunteers at Rockefeller University Hospital. Heparin or EDTA was used as an anticoagulant. All studies were approved by the appropriate hospital committees and informed consent was obtained in all cases. Red cell suspensions were prepared in phosphate-buffered saline (PBS) or PBS-bovine albumin (PBS-BA). The pH was maintained at 7.4 throughout the experiments. Whole blood was incubated with the compound or the appropriate buffer for 1 hr.

Red cells were lysed with three volumes of distilled water in order to isolate hemoglobin. The cellular debris was extracted with 1/4 volume of toluene that was aspirated following centrifugation at 25,000 g for 40 min (Sorvall RC-5). Hemoglobin solutions were then dialyzed for 2 hr against PBS. Cetiedil as the citrate salt was obtained from Cooper Laboratories, Cedar Knolls, N.J. The radiolabeled compound [1,2-14C]-2-(hexahydro-1H-azepin-1-yl)ethyl α-

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cyclohexyl-α-(3-thienyl)acetate (14C-cetiedil) was obtained from Laboratories Innothera, (Areueil Cedex, France). Sodium cyanate was recrystallized as described previously.11

Morphology Studies
After incubation, whole blood was diluted 1:30 with PBS-0.2% NaHCO3 and added to 125-ml Erlenmeyer flasks arranged in series with openings for gas inlet and outlet and a 3-way stopcock that allowed sampling into a 5-mI syringe. The samples were subjected to 100% oxygen for 15 min. Cells were collected at time 0, after which deoxygenation was performed using a gas mixture containing 4% O2, 5% CO2, balance N2. At 10-, 30-, and 60-min intervals, aliquots of the cell suspension were aspirated and transferred to tubes containing deoxygenated phosphate buffer-4% formalin for fixing. The samples were then reoxygenated for 15 min to determine the number of irreversibly sickled cells (IRSC); aliquots were collected as above. The sickling of erythrocytes from individuals homozygous for HbS was determined by phase-contrast microscopy. Two observers counted 300 cells in triplicate under blinded conditions.

Filtration Studies
Whole blood was centrifuged and the plasma removed. The red cells were then washed three times with PBS-BA, diluted with PBS-BA-0.2% NaHCO3 to 1% cell suspension, and divided into 2 aliquots of 30 ml each in 125-mI Erlenmeyer flasks. The suspensions were deoxygenated as above.

Filtration studies utilized polycarbonate sieve membranes of 25 mm diameter with a pore size of 5 μm (Millipore Corp.) placed in a stirred cell over a stainless steel screen (Millipore Corp.) The membranes were saturated with buffer, and the filtration chamber was flushed with N2. The samples were then transferred to the filtration unit, which was maintained at 37°C using an “air blanket,” and filtration was performed by applying a positive pressure of 5 psi N2 gas to the chamber. The filtration time was measured with a stopwatch from the moment the pressure was turned on until the buffer in which the cells were suspended was expressed from the filtration chamber. The filtrate was collected in 5-ml plastic tubes. A 10-μl aliquot of the filtrate was withdrawn into 0.99 ml Drabkin’s solution for determination of its hemoglobin content. The remainder of the filtrate was centrifuged, after which 10 μl of the supernatant was added to Drabkin’s solution as above. Likewise, the hemoglobin concentration of the prefiltration suspension and supernatant was determined.

Optical density (OD) was determined with a Zeiss spectrophotometer (Model PM6), and the percent cells filtered was calculated by the following formula:

\[
\text{% cells filtered} = \frac{\text{OD total filtrate} - \text{OD supernatant}}{\text{OD total initial sample} - \text{OD supernatant}} \times 100
\]

Gelation and Oxygen Affinity
Minimum gelling concentrations were determined by the method of Singer and Singer13 as modified by Bookchin and Nagel.12 SS hemolysates were concentrated by pressure dialysis and ultrafiltration and dialyzed against 0.15 M potassium phosphate buffer at pH 7.35. The hemoglobin concentration of the dialysand was determined by the method of Drabkin,14 after which it was adjusted with potassium phosphate buffer to 22.5 g/dl. The reaction mixtures (total volume of 0.5 ml) were deoxygenated in 10-ml Erlenmeyer flasks on a rotary shaker under a stream of moist N2 for 45 min. The concentration of hemoglobin was then allowed to increase slowly by exposure to dry nitrogen. To insure homogeneity, periodic chilling was performed after the mixture became viscous. The end-point of gelation was defined as the point at which no movement of hemoglobin occurred upon sharp tilting and tapping of the flask. All gelled samples were verified to liquefy upon cooling in an ice bath.

Oxygen affinity studies employed cells from normal individuals (HbA) and were performed using a Hem-O-Scan Apparatus (Aminco).15

Red Cell Distribution Studies and Evaluation of Hemoglobin Binding
After separation from plasma, red cells were washed three times with cold (4°C) PBS, pH 7.4. A 40% cell suspension was incubated with 180 μM 14C-cetiedil for 2 hr at 37°C. All subsequent procedures were performed at 4°C. The cells were washed 3 times with PBS, resuspended in the same buffer and the radioactivity associated with the washed erythrocytes determined. After centrifugation, the red cells were lysed with 2–3 volumes of distilled water and placed at 0°C for 3 hr. The membranes were separated from the hemolysate by centrifugation at 45,000 g for 30 min after which the hemoglobin solution was aspirated and saved. The membranes were washed 3 times with 20 mosmole Tris-HCl buffer, pH 7.4, and the washes were added to the hemoglobin solution. The radioactivity associated with the hemoglobin solution and the globin fraction isolated by 10% trichloroacetic acid (TCA) precipitation was determined as described previously.14 The membranes were dissolved in 0.1 M NaOH, and their radioactivity was also determined. Radioactivity measurements were carried out with a Packard Tricarb liquid scintillation counter using Aquasol-2 (New England Nuclear) as scintillation fluid. Appropriate amounts of unlabelled red cells or hemoglobin were added to the 14C-cetiedil standards in order to equalize the counting error due to color quenching in the sample and standard.

In another experiment, red cells and Hb solutions were incubated with and without radiolabeled cetiedil for 1 hr at 37°C prior to separation of globin chains. Globin was prepared using cold acetic-acetone precipitation.17 Forty milligrams of globin were dissolved in starting buffer (8M urea, 0.05 M β-mercaptoethanol, 0.005 M Na2HPO4, pH 6.7) and dialyzed against the same buffer for 2 hr. The globin solution was applied to a column (1 x 15 cm) of carboxymethylcellulose (Whatman CM 52) at pH 6.7 and developed with a sodium phosphate gradient as described by Clegg et al.18,19

Solutions of hemoglobin (10 mg/ml) were subjected to isoelectric focusing on thin-layer polyacrylamide gels using a pH gradient from 6 to 8.20

Red Cell Survival Studies
These studies were performed with chromium-51 according to the method of Gray and Sterling.21 Radioactivity was determined in a Packard AutoGamma Scintillation Spectrometer (Model 578).

An initial control study was performed on whole blood incubated with buffer alone. Following the disappearance of radioactivity, a second study was performed in the same subject, but this time the whole blood was incubated for 1 hr with 166 μM cetiedil in buffer adjusted to the same osmolality as the control.

RESULTS
Effect of Cetiedil on Sickling and Sickling-Related Phenomena
Cetiedil inhibited sickling, as shown in Fig. 1. In these experiments, inhibitory effects at all concentrations were observed at 10, 30, and 60 min of increasing hypoxia. Inhibition of sickling was maximal at 100 μM
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Fig. 1. Dose–response of cetiedil treatment in vitro on the morphological sickling of hemoglobin SS erythrocytes. Following 1-hr incubation with and without the drug at 37°C, whole blood was diluted 1:30 with PBS-0.2% NaHCO₃ (pH 7.4), oxygenated for 15 min, and then incubated for 1 hr at 37°C under an atmosphere containing 4% O₂, 5% CO₂, balance N₂. Samples for morphology determination were collected into tubes containing buffered formalin at 0, 10, 30, and 60 min.

(p < 0.001). Similarly, the number of irreversibly sickled cells was decreased significantly with 100 and 200 μM cetiedil-treated cells, 10 and 12%, respectively, versus 20% for the control, whereas specimens incubated with 50 and 500 μM cetiedil were not significantly different from control.

Initial filtration experiments suggested that incubation with cetiedil improved the filterability of SS cells and had no adverse effect on AA cells under deoxygenated conditions.

Table 1 shows the change in filterability of SS erythrocytes under hypoxic conditions following incubation with varying concentrations of cetiedil. There was no effect on oxygenated cells. The maximum effect on the filterability of deoxygenated cells (analogous to the effect seen on morphology) occurred at 100 μM cetiedil, as reflected by the ratio of time over percentage of cells filtered. Table 2 shows an experiment using cells from another patient and compares changes in filterability following incubation with 10 mM sodium cyanate and 200 μM cetiedil. Both drugs improved filterability to a comparable degree.

Having shown an effect on sickling, the effect of cetiedil on other sickling-related phenomena was assessed. Cetiedil treatment of SS hemolysate resulted in no change in the minimum gelling concentration when compared with control, nor did incubation of AA erythrocytes with cetiedil for 1 hr result in a change in oxygen affinity.

Red Cell Distribution Studies and Evaluation of Hemoglobin Binding

Of the total radioactivity of the washed red cells (281,790 cpm), 15% was found to be associated with the membranes and 65% with the nonmembrane component. Precipitation of globin with TCA showed that 23% of the nonmembrane radioactivity was associated with globin.

In order to determine whether cetiedil interacted with hemoglobin, globin was separated into α and β chains by column chromatography. The results of these experiments revealed no ¹⁴C labeling of either α or β chains as depicted in Fig. 2. These findings were supported by isoelectric focusing studies that demonstrated no additional bands following incubation of SS hemolysate with 1.5 mM cetiedil at 37°C for 1 hr.

Red Cell Survival Studies

To determine whether cetiedil was toxic to the red cell, chromium-51 (⁵¹Cr) survival of cetiedil-treated cells was compared with the survival of cells treated

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**Table 1. Effect of Cetiedil on the Filterability of Oxygenated and Deoxygenated SS Erythrocytes**

<table>
<thead>
<tr>
<th>Cetiedil (μM)</th>
<th>Oxygenated</th>
<th>Deoxygenated</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>93 ± 4.03</td>
<td>45 30 ± 10</td>
</tr>
<tr>
<td>20</td>
<td>100</td>
<td>58 1.7 29 ± 7</td>
</tr>
<tr>
<td>50</td>
<td>99 ± 2.9</td>
<td>58 1.7 63 ± 12</td>
</tr>
<tr>
<td>100</td>
<td>99 ± 4.0</td>
<td>45 2.2 85 ± 12</td>
</tr>
<tr>
<td>200</td>
<td>100</td>
<td>54 1.9 53 ± 17</td>
</tr>
<tr>
<td>500</td>
<td>93 ± 4.0</td>
<td>56 1.7 114 0</td>
</tr>
<tr>
<td>1000</td>
<td>94 ± 4.0</td>
<td>57 1.7 107 0</td>
</tr>
</tbody>
</table>

*Fl (filtration index) = Percent cells filtered / Filtration time (sec).

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**Table 2. Comparison of Effects of 200 μM Cetiedil and 10 mM Sodium Cyanate on the Filterability of Oxygenated and Deoxygenated Sickle Cells**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Oxygenated</th>
<th>Deoxygenated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer</td>
<td>91 ± 4.0</td>
<td>50 1.8 10 ± 7.2</td>
</tr>
<tr>
<td>Cyanate</td>
<td>94 ± 8.5</td>
<td>50 1.9 44 ± 5.3</td>
</tr>
<tr>
<td>Cetiedil</td>
<td>100</td>
<td>44 2.3 40 ± 1.5</td>
</tr>
</tbody>
</table>

*Fl (filtration index) = Percent cells filtered / Filtration time (sec).
Fig. 2. Distribution of radioactivity between hemoglobin chains from \(^{14}\text{C}\)-cetiedil-treated erythrocytes. Whole blood was incubated with \(^{14}\text{C}\)-cetiedil (specific activity 16 mCi/mM) for 1 hr followed by acid-acetone precipitation of globin. After determination of the radioactivity associated with the precipitated globin, the globin chains were separated on a carboxymethylcellulose column. The radiolabeled proteins eluted in the first 40 ml prior to fraction 0. The fractions were corrected for background counts.

with buffer alone. The red cell survival (T'/2) for canine erythrocytes incubated with 166 \(\mu M\) cetiedil for 1 hr was found to be 16 days in 2 animals compared to 18 days for erythrocytes incubated in buffer alone.

The survival of cetiedil-treated cells was then compared with that of buffer-treated autologous cells from four patients with sickle cell disease (Fig. 3). In three of the patients there was a slight increase in the T'/2 following incubation with the compound. In the fourth patient there was a slight decrease in red cell survival following incubation with cetiedil. The latter patient experienced several crises during the phase of the study when drug-treated cells were being evaluated. The mean control T'/2 for the group was 8.9 ± 4.0 days, while that following incubation with 166 \(\mu M\) cetiedil was 9.4 ± 2.8 days, indicating that the compound is not toxic to the sickle erythrocyte.

Assessment of Drug Combinations In Vitro

Figure 4 shows that 125 \(\mu M\) cetiedil in combination with 5 \(mM\) cyanate causes a reduction in sickling morphology that is greater than that due to 5 \(mM\) cyanate alone but somewhat less than that due to 10 \(mM\) cyanate alone \((p < 0.001)\). In an experiment on erythrocytes from a different patient, the same concentration of the two drugs in combination led to a reduction in sickling \((p < 0.001)\) and an enhancement of filterability greater than that with either 125 \(\mu M\) cetiedil or 5 \(mM\) cyanate alone and comparable to that seen following incubation with 10 \(mM\) cyanate alone (Table 3). Furthermore, there was significant reduc-

Fig. 3. \(^{51}\text{Cr}\) survival curves for cetiedil-treated and untreated erythrocytes in four patients with sickle cell disease. The erythrocytes were incubated with and without cetiedil in vitro, and the excess drug was washed away before reinfusion of the cell suspension into the patient. T'/2 is noted by letter X. Arrows \((\uparrow)\) represent onset of crises in patient GH.

Fig. 4. Effect of combining cetiedil and cyanate treatment in vitro on the morphological sickling of hemoglobin SS erythrocytes. Following a 1-hr incubation at 37°C with and without the drugs, whole blood was diluted 1:30 with PBS-0.2% NaHCO\(_3\) (pH 7.4), oxygenated for 15 min, and then incubated for 1 hr at 37°C under an atmosphere containing 4% O\(_2\), 5% CO\(_2\), balance N\(_2\). Samples for morphology determination were collected into tubes containing buffered formalin at 0, 10, 30, and 60 min.
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Previous studies in nonsickle cell patients have reported that cetiedil, when administered either orally or parenterally, reaches concentrations that are comparable to those that have shown antisickling effects in our in vitro studies. The drug is also well tolerated by normal volunteers and patients with cardiovascular disease. In addition to the studies described here, the drug has several properties that might be of value to patients with sickle cell disease. Its effects on platelets, fibrinogen, blood viscosity in the microvasculature, and pain also show theoretical promise for these patients.

Further studies on the mechanism of action, dose-response, and pharmacokinetic studies need to be performed since individuals with sickle cell disease are known to have differences in renal physiology and hepatic drug metabolism. Should the compound be tolerated as well by patients with sickle cell disease as it appears to be by other patients with chronic cardiovascular disease, then assessment of efficacy in ameliorating and preventing the painful sequelae of in vivo sickling would be warranted.

At present there is no specific therapy for patients with sickle cell disease. The numerous drug studies that have been done make it now seem unlikely that any one compound will be available in the near future that will be useful in providing the necessary efficacy while avoiding toxicity. A minimally toxic compound such as cetiedil, which has an effect on sickling additive with drugs known to have their primary effect on hemoglobin S polymerization might allow the clinical reevaluation of these latter agents at subtoxic doses. Furthermore, these observations suggest the consideration of serial or parallel combinations of drugs that act at different points in the pathophysiology of sickling and its sequelae.

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The mode of action of the compound is not apparent. Cetiedil does not inhibit gelation of hemoglobin S or parenterally, reaches concentrations that are comparable to those that have shown antisickling effects in our in vitro studies. The drug is also well tolerated by normal volunteers and patients with cardiovascular disease. In addition to the studies described here, the drug has several properties that might be of value to patients with sickle cell disease. Its effects on platelets, fibrinogen, blood viscosity in the microvasculature, and pain also show theoretical promise for these patients.

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