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The Synthesis and Some Pharmacological Actions of the Enantiomers of the K^+-Channel Blocker Cetiedil

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Abstract

Cetiedil ((±)-2-cyclohexyl-2-(3-thienyl)ethanoic acid 2-(hexahydro-1*H*-azepin-1-yl) ethyl ester) possesses anti-sickling and analgesic, antispasmodic, local anaesthetic and vasodilator activities. A total synthesis and circular dichroism spectra of the enantiomers of cetiedil is described, together with a comparison of their effectiveness as blockers of the Ca^{2+}-activated K^+ permeability of rabbit erythrocytes; the contractile response of intestinal smooth muscle to acetylcholine; the Ca^{2+}-dependent contraction of depolarized intestinal muscle; and the cell volume-sensitive K^+ permeability (K_{vol}) of liver cells.

The enantiomers did not differ substantially in their ability to block the Ca^{2+}-activated K^+ permeability of rabbit red cells or in their effectiveness as blockers of the contractile response of depolarized smooth muscle to externally applied Ca^{2+}. There was a clear difference in the muscarinic blocking activity of the enantiomers, as assessed by inhibition of the contractile response of intestinal smooth muscle to acetylcholine; (+)-cetiedil was 7·7 ± 0·2 (s.d.) times more active than the (−) form. The enantiomers also differed in their potency as blockers of the increase in membrane conductance which occurs when liver cells swell. The concentration of (+)-cetiedil needed to reduce the conductance increase by 50% was 2·04 ± 0·54 (s.d.) μM; (−)-cetiedil was 2·6 ± 0·8 (s.d.) times less active (IC50 of 5·2 ± 1·2 μM).

Differences in the biological actions of the enantiomers of cetiedil indicate that a more extensive study could be rewarding in relation to the use of the enantiomers both in therapeutics and in the study of K^+ channels.

Cetiedil ((±)-2-cyclohexyl-2-(3-thienyl)ethanoic acid 2-(hexahydro-1H-azepin-1-yl) ethyl ester) is one of a series of compounds with spasmolytic, local anaesthetic and analgesic activity (Robba & Le Guen 1967). It was introduced to therapeutics as a vasodilator but has since become of interest for treatment of the vaso-occlusive crises that occur in sickle cell disease (Benjamin et al 1986). The demonstration (Berkowitz & Orringer 1981) that cetiedil blocks the Ca^{2+}-activated K^+ permeability ($P_{K(Ca)}$) of erythrocytes led to the suggestion (Berkowitz & Orringer 1982) that this action could underlie the beneficial effect of cetiedil in this condition. It is supposed that the opening of Ca^{2+}-activated K^+ channels in the membrane of the sickle cells causes a net loss of K^+ and water. The resulting cell-shrinkage and dehydration then leads to the polymerization of HbS, the haemoglobin variant that is the primary cause of the disease, with the outcome that the cells become more rigid and therefore less able to pass through capillaries (for a review, see Joiner 1993). Cetiedil, by blocking the Ca^{2+}-activated K^+ permeability, would prevent the initiation of this sequence of events.

Although this is a plausible explanation, it must be kept in mind that cetiedil has other actions that could be involved. For example, it is a tissue-selective vasodilator (Simaan & Aviado 1976; Boissier et al 1978) which inhibits muscarinic responses and Ca^{2+}-dependent contractions of depolarized vascular smooth muscle (Boissier et al 1978). It is also known to block cell volume-sensitive K^+ channels in lymphocytes (Sarkadi et al 1985; De Coursey et al 1987) and hepatocytes (Sandford et al 1992).

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Cetiedil has a chiral centre, and it is noteworthy that all previous work has been conducted with a racemic mixture of its two enantiomers. Although these have been mentioned in a patent (Innothéra 1975), no chemical, physical or biological data have been reported. In this paper, we describe the synthesis of these enantiomers, and some of their pharmacological actions. Although our primary aim was to establish the importance or otherwise of the stereochemistry of the molecule in relation to its $P_{K(Ca)}$-blocking activity in red cells, we also had in mind that a comparison of the activity of the enantiomers as blockers of Ca^{2+}-activated and volume-sensitive K^+ channels, of muscarinic receptors and of Ca^{2+}-dependent contractions of smooth muscle could be of value in relation to the difficult problem of establishing the mechanism of action of cetiedil in sickle cell disease. Further, this comparison could have relevance for the therapeutic applications of cetiedil because the balance between desirable and disadvantageous actions is likely to differ between the enantiomers.

Materials and Methods

Chemistry

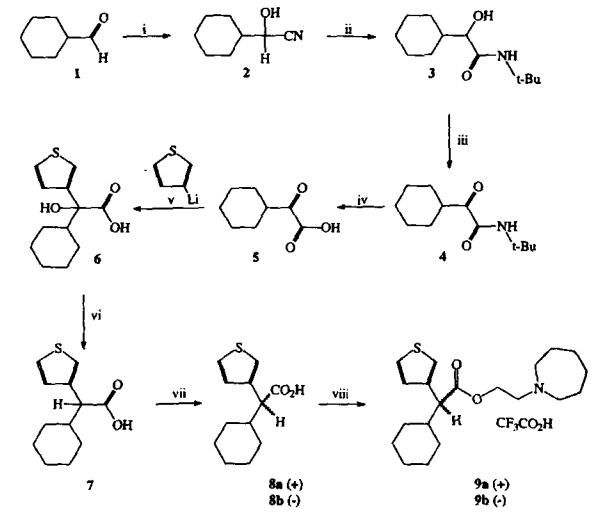
Melting points were determined in open capillary tubes with an Electrothermal melting point apparatus and are uncorrected. Optical rotation of solutions in absolute ethanol was measured on an Optical Activity AA-10 polarimeter. IR spectra were recorded with a Perkin-Elmer 983 spectrometer. ^1H NMR spectra were recorded at 60 MHz on a Jeol PMXSI spectrometer, at 200 MHz on a Varian XL200 spectrometer, or at 400 MHz on a Varian VXR 400 spectrometer; samples were dissolved in $CDCl_3$ and TMS was used as internal standard.

Multiplicities are reported as (s) singlet, (d) doublet, (t) triplet, (q) quartet, (m) multiplet. Assignments of hydroxyl and ammonium protons were checked by deuterium exchange. Mass spectra were recorded with a VG 7070H mass spectrometer interfaced with a Finnegan Incos data system. Circular dichroism (CD) spectral measurements were recorded with a Jasco J600 spectropolarimeter using methanol solutions (1 mg mL^{-1}) in 0·02- and 0·05-cm cylindrical silica cells. Thin-layer chromatography was performed over glass plates coated with Merck silica gel 60 F_{254}; flash chromatography was performed using Merck 7734 silica gel (20–63 μm). Preparative and analytical high-performance liquid chromatography (HPLC) were performed with a Gilson binary grading system with an LB diode-array detector set at 240 nm. Elemental analyses were performed by A. A. T. Stones, UCL microanalytical services, and were within 0·4% of the calculated values. Chemical intermediates were from Aldrich Chemical Company unless otherwise indicated.

Direct resolution of racemic cetiedil by fractional crystallization of salts with chiral acids was investigated first. Di-*p*-toluoyl- or dibenzoyltartaric acids gave oils. Tartaric, mandelic, and D-camphorsulphonic acids gave solids but there was no evidence of optical resolution after several recrystallizations, even after very slow crystallization lasting one week. It is probable that the ion pair between the amino group and the chiral acid formed in the crystal is too far away from the chiral centre (a distance of five single bonds) to afford sufficient difference in the solubilities of the two diastereomeric salts.

The chiral precursor, (±)-2-cyclohexyl-2-(3-thienyl)ethanoic acid (**7**) was, therefore, resolved by fractional crystallization of the (−)-quinine salt and each enantiomer (**8a, b**) was then esterified with 2-(hexahydro-(1*H*-azepin-1-yl)ethanol to afford the corresponding enantiomers of cetiedil (**9a, b**) isolated as oily trifluoroacetates after final purification by HPLC. The synthesis of the chiral precursor **7** is shown in Scheme 1. The key intermediate is cyclohexyl 2-ketoethanoic acid (**5**) (Luyten et al 1989) but in our hands the reported procedure from diethyloxalate (**10**) (Tanaka et al 1978; Scheme 2) was not reproducible and (**5**) was obtained in 16% yield only. We therefore devised Scheme 1, starting from cyclohexane carboxaldehyde cyanohydrin (**2**), which was converted into the hydroxy-*t*-butylamide (**3**) using a Ritter reaction with butanol in sulphuric acid (Anatole and Medète 1971). The hydroxyamide (**3**) was oxidized with CrO_3 to the α-ketocarboxamide (**4**) which, on acid-catalysed hydrolysis, furnished cyclohexyl-2-ketoethanoic acid (**5**) in 60% overall yield. **5** was then reacted with 3-thienyl lithium (Gronowitz 1954) to yield the hydroxy acid (**6**) (Robba & Le Guen 1966) which, on treatment with stannous chloride, was dehydroxylated to furnish the required (±)-2-cyclohexyl-2-(3-thienyl)ethanoic acid (**7**) (Robba & Le Guen 1967).

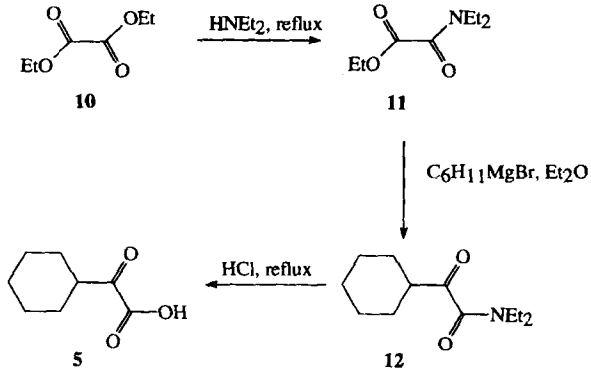
The circular dichroism spectra of the (−)- and (+)-2-cyclohexyl-2-(3-thienyl)ethanoic acids (**8a, b**) are reproduced in Fig. 1. Esterification of the (−) and (+) acids, **8a** and **8b**, respectively, with 2-(hexahydro-1*H*-azepin-1-yl)ethanol in a Dean and Stark apparatus using a slight molar excess of *p*-toluenesulphonic acid yielded the (−)- and (+)-2-cyclohexyl-2-(3-thienyl)ethanoic acid 2-(hexahydro-1*H*-azepin-1-yl)ethyl esters (**9a, b**) (cetiedil); the circular dichroism spectra of these enantiomers (as trifluoroacetates) are also reproduced in Fig. 1; the two sets of curves are seen to be almost superimposable indicating that absolute stereochemistry and enantiomeric purity have been maintained.



SCHEME 1. The synthesis of the chiral precursor **7**. Methods: (i) (a) aq $NaHSO_3$, 4°C, 24 h (b) aq EtOAc, KCN, 5 h; (ii) *t*-BuOH, conc H_2SO_4, < 50°C, 1 h, 75°C, 1 h; (iii) CrO_3, acetic acid, 80°C, 4 h; (iv) conc HCl-acetic acid, reflux, 11 h; (v) 3-thienyl Li, diethyl ether, − 78°C, 5 h; (vi) $SnCl_2$, conc HCl-acetic acid, reflux, 5·5 h; (vii) (a) (−)-quinine, ethanol, fractional crystallization (b) HCl; (viii) (a) *p*-TsOH, toluene, $HOCH_2CH_2N(CH_2)_6$, Dean and Stark reflux (b) prep HPLC.

2-Cyclohexyl-2-hydroxyacetonitrile, 2

To a cooled (ice bath) stirred suspension of cyclohexane carboxaldehyde (5 g; 44·6 mmol) in water (110 mL) was added a solution of sodium metabisulphite (4·64 g; 44·6 mmol) in water (35 mL). The resulting mixture was stirred at room temperature for 3 h and then stored at 4°C overnight. The reaction mixture was left to warm to room temperature; EtOAc (200 mL) was added, then a solution of potassium cyanide (2·9 g) in water (45 mL). After 5 h stirring the layers were separated. The organic layer was washed with water (350 mL), dried (Na_2SO_4), filtered, and concentrated to yield **2** (6·3 g; 100%) as a viscous colourless oil (Grunewald et al 1976).



SCHEME 2. The synthesis of cyclohexyl 2-ketoethanoic acid (**5**) as reported by Tanaka et al (1978).

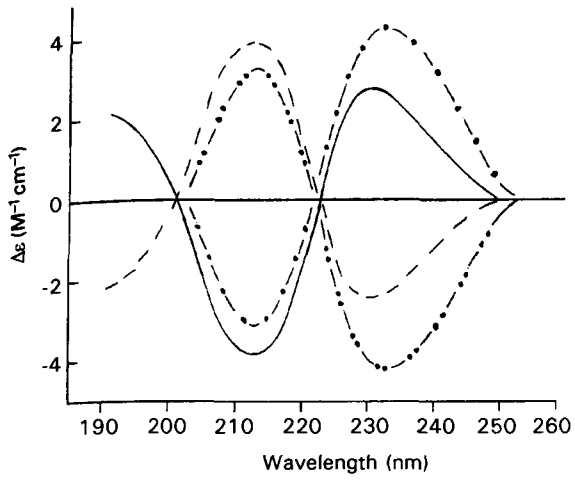


FIG. 1. Circular dichroism (CD) spectra of the (+)(—) and (−)(- - - -) enantiomers of 2-cyclohexyl- 2-(3-thienyl)ethanoic acids **8a** and **8b** and of the (+) (·····) and (−) (— · —) enantiomers of cetiedil trifluoroacetates **9a** and **9b** in methanol at 1 mg mL^{-1} concentrations.

2-Cyclohexyl-2-hydroxyethanoic acid N-t-*butylamide, 3*
H_2SO_4 (conc; 4·2 mL) was added dropwise to 2-cyclohexyl-2-hydroxyacetonitrile (5 g; 36 mmol) in *t*-butanol (36 mL) cooled (ice bath) to keep the temperature below 50°C. After 1 h the mixture was warmed at 75°C on a steam bath for a further 1 h; water (40 mL) was then added and the cooled mixture neutralized (NaOH). The mixture was extracted with EtOAc; the aqueous layer was saturated with NaCl and again extracted. The combined extracts were dried (Na_2SO_4) and concentrated under reduced pressure to yield a viscous oil which crystallized on standing to give 2-cyclohexyl-2-hydroxyethanoic acid *N-t*-butylamide (6·49 g; 86%), mp 119–120°C, (Müller & Zeeh (1965) report mp 121–122°C).

2-Cyclohexyl-2-ketoethanoic acid N-t-*butylamide, 4*
Chromium trioxide (2·35 g; 24 mmol) in glacial acetic acid (10 mL) was added to 2-cyclohexyl-2-hydroxyethanoic acid *N-t*-butylamide (5 g; 23 mmol) dissolved in glacial acetic acid (30 mL). The mixture was left to stand for 0·5 h then heated on a steam bath at 80°C for 4 h. The acetic acid was removed under reduced pressure to yield an oil to which water (25 mL) was added. The mixture was then extracted with diethyl ether and the combined extracts dried (Na_2SO_4) and concentrated to an oil which was subjected to flash chromatography, using diethyl ether as the eluant, to yield 2-cyclohexyl-2-ketoethanoic acid *N-t*-butylamide (4·5 g; 93%) as a semi-solid (Gibby and Gubler 1982).

2-Cyclohexyl-2-ketoethanoic acid, 5
2-Cyclohexyl-2-ketoethanoic acid *N-t*-butylamide (4 g; 19 mmol) was dissolved in a mixture of conc HCl and glacial acetic acid (1 : 1; 90 mL) and heated under reflux for 11 h until the starting material, as identified by TLC, had disappeared. The mixture was then concentrated under reduced pressure to give an oil which was dissolved in diethyl ether, shaken with one equivalent of K_2CO_3, filtered, and washed with diethyl ether. The resulting solid was collected, dissolved in water, acidified (dil HCl) and extracted with diethyl ether. The extract was dried (Na_2SO_4) and evaporated under reduced pressure to give 2-cyclohexyl-2-ketoethanoic acid as an oil which solidified on standing (3·8 g; 76%), mp 50–51°C, (Robba & Le Guen (1966) report mp 53°C; Tanaka et al (1978) report mp 48–49°C). IR ($CHCl_3$): 2520–3510 cm^{-1} (*v* COOH); 1720 cm^{-1} (*v* COOH). ^1H NMR (60 MHz; $CDCl_3$) δ (ppm): 1·4–2·4 (10H, m, cyclohexyl); 3·0–5·5 (1H, m); 8·45 (1H, s, OH).

(±)-2-Cyclohexyl-2-hydroxy-2-(3-thienyl)ethanoic acid, 6
2-Cyclohexyl-2-ketoethanoic acid (10 g; 64 mmol) in 100 mL dry diethyl ether, under N_2 at − 78°C, was added to 3-thienyllithium (89 mmol) prepared, using the method of Gronowitz (1954), from 3-bromothiophene (14 g) and a solution of nBuLi (10 M; 6·8 mL) in dry diethyl ether (100 mL). The mixture was stirred for 5 h at − 78°C and the temperature was then left to increase to − 15°C when ice (100 mL) was carefully added. The ethereal layer was discarded; diethyl ether (200 mL) was then added and the mixture acidified (5M HCl) and stirred. The ether layer was separated, dried (Na_2SO_4), and concentrated under reduced pressure. The resulting residue was crystallized from EtOAc-petroleum ether (bp 60–80°C), 1 : 10, to yield **6** as a white crystalline solid (11 g; 72%) mp 134–137°C (Robba & Le Guen (1966) report mp 162°C). IR ($CHCl_3$) 3440 cm^{-1} (*v* OH); 2400–3600 cm^{-1} (*v* CO_2H); 1720 cm^{-1} (*v* CO_2H). ^1H NMR (60 MHz; $CDCl_3$): δ (ppm): 0·8–2·0 (11H, m, cyclohexyl), 6·4 (1H, bs, OH), 7·0–7·3 (3H, m, thiophene).

(±)-2-Cyclohexyl-2-(3-thienyl)ethanoic acid, 7
The acid **6** (14 g; 58 mmol) was dissolved in a mixture of glacial acetic acid and conc HCl (1 : 1; 200 mL) and stannous chloride dihydrate (47·2 g; 209 mmol) was added. The resulting solution was stirred and heated under reflux for 5·5 h. The volume was then reduced to one third and ice (150 g) was added. The mixture was extracted with EtOAc and the combined extracts were dried (Na_2SO_4) and concentrated under reduced pressure to give a thick dark oil. Flash chromatography of this oil using EtOAc-petroleum ether (bp 60–80°C) (3 : 7) as eluant yielded (±)-2-cyclohexyl-2-(3-thienyl)ethanoic acid as a white crystalline solid (7·6 g; 58%) mp 127°C, (Robba & Le Guen (1967) report mp 128°C). ^1H NMR (400 MHz; $CDCl_3$) δ (ppm): 0·7–2·0 (11H, m, cyclohexyl); 3·4 (1H, d, $J_{vic} = 10$ Hz, CH); 7·0–7·25 (3H, m, thiophene).

(+)- and (−)-2-Cyclohexyl-2-(3-thienyl)ethanoic acids, ***8a, b***
(−)-Quinine (4·8 g; 15 mmol) was added to a solution of (±)-2-cyclohexyl-2-(3-thienyl)ethanoic acid (3 g; 13 mmol) in ethanol (6 mL). The mixture was warmed for 15 min in a solution of ethanol-water (2 : 1; 90 mL) and then left to cool to room temperature and stored in a refrigerator for several days. The crystals that formed were filtered off and recrystallized three more times (until no increase in optical rotation was observed) from ethanol-water (2 : 1) to give (−)-quinine-(+)-2-cyclohexyl-2-(3-thienyl)ethanoate as densely matted needles (1·23 g; 16%) mp 148–149°C. The needles were added to ether (50 mL) and the resulting suspension acidified with 1 M hydrochloric acid. The ether layer was separated, dried (Na_2SO_4) and evaporated under reduced pressure to give (+)-

2-cyclohexyl-2-(3-thienyl)ethanoic acid (0·42 g; 14%) mp 105–106°C, (Innothéra (1975) reports mp 108°C). $[\alpha]_D^{25} + 37°$ (c = 1 mg mL^{-1} in ethanol). ^1H NMR (400 MHz; $CDCl_3$) δ (ppm): 0·7–2·0 (11H, m, cyclohexyl); 3·4 (1H, d, $J_{vic} = 10$ Hz, CH); 7·0–7·25 (3H, m, thiophene). The filtrate from the above mixture was reduced in volume to approximately one third and left in a refrigerator for several days. The crystals which formed were filtered off and recrystallized twice more (until no further increase in optical rotation was noted) from ethanol-water (2:1) to give (−)-quinine-(−)-2-cyclohexyl-2-(3-thienyl)ethanoate as lightly matted needles (0·69 g; 9%) mp 116–117°C. This was treated as described above to yield (−)-2-cyclohexyl-2-(3-thienyl)ethanoic acid as a white crystalline solid (0·28 g; 9%) mp 103–104°C (Innothéra (1975) reports mp 106°C). $[\alpha]_D^{25} - 31°$ (c = 1 mg mL^{-1} in ethanol). The spectral data were identical to those for the (+) enantiomer.

(+)- and (−)-2-(Hexahydro-1H-azepin-1-yl)ethyl 2-cyclohexyl-2-(3-thienyl)ethanoate (cetiedil) trifluoroacetates ***9a, b***

p-Toluenesulphonic acid monohydrate (0·372 g; a slight molar excess) and (−)-2-cyclohexyl-2-(3-thienyl)ethanoic acid (0·2 g; 0·89 mmol) were added to a solution of 2-(hexahydro-1*H*-azepin-1-yl)ethanol (0·255 g; 1·78 mmol; Lancaster Synthesis Ltd) in dry hot toluene and the mixture was heated in a Dean and Stark apparatus for 45 h. The toluene was removed under reduced pressure to afford an oil which was treated with water (5 mL) and ethanol (50 mL), and then K_2CO_3 until basic. The ethereal layer was separated and the basic layer extracted twice more with diethyl ether. The combined extracts were dried (Na_2SO_4) and concentrated to give an oil which was purified by preparative HPLC to give (−)-2-(hexahydro-1*H*-azepin-1-yl)ethyl-2-cyclohexyl-2-(3-thienyl)ethanoate trifluoroacetate as a colourless oil (72·2 mg; 31%; yield based on the chiral acid consumed after allowing for acid recovered from the reaction mixture after acidification with 5 M HCl and extraction into ether). $[\alpha]_D^{25} - 15°$ (c = 1 mg mL^{-1} in ethanol). Anal: $C_{20}H_{31}NO_2S,CF_3CO_2H$ (C,H,N). IR (liq film) 2923 cm^{-1} (ν sat CH); 1728 cm^{-1} (ν C=O ester); 771 cm^{-1} (mono-substituted thiophene). ^1H NMR (400 MHz; $CDCl_3$) δ (ppm): 0·7–2·0 (19H, m, aliphatic); 2·6 (4H, m, $N^+CH_{ax,eq}$); 2·75 (2H, t, $OCH_2CH_2N^+$); 3·4 (1H, d, $J_{vic} = 10$ Hz, CH); 4·2 (2H, m, OCH_2); 7·1–7·3 (3H, m, thiophene). MS m/z 350 (MH^+, 100%).

The (+) enantiomer was prepared and purified as for the (−) enantiomer, to yield (+)-2-(hexahydro-1*H*-azepin-1-yl)ethyl-2-cyclohexyl-2-(3-thienyl)ethanoate trifluoroacetate as a colourless oil. $[\alpha]_D^{25} + 17·5°$ (c = 1 mg mL^{-1} in ethanol). Spectral data were identical to those of the (−) enantiomer. Anal: $C_{20}H_{31}NO_2S,1·4CF_3CO_2H$ (C,H,N).

Biological testing

Effect on erythrocyte $P_{K(Ca)}$. The potency of the enantiomers was assessed from their ability to inhibit the net loss of cell K^+ that follows the application of the Ca^{2+} ionophore A23187 to rabbit erythrocytes. The ionophore increases the cytosolic concentration of Ca^{2+}, with the consequences that the Ca^{2+}-activated K^+ channels in the membrane open and K^+ leaves the cells. The amount of K^+ lost can be measured using a K^+-sensitive electrode placed in the cell suspension, and this provides a convenient, if indirect, measure of changes in the number of open K^+ channels (Burgess et al 1981; Cook & Haylett 1985). Because only small quantities of the cetiedil enantiomers were available, and because cetiedil becomes approximately threefold more active when applied in a solution containing less than the normal concentration of K^+, the experiments were performed with a low-K^+ solution. In detail, blood (2–5 mL) was drawn from the ear vein of adult New Zealand white rabbits and mixed with heparin (500 units mL^{-1} whole blood). After centrifugation (3 min at 1600 g) the supernatant and buffy coat were aspirated and discarded, leaving a pellet of packed cells. This was then re-suspended in a solution (5 vols) containing (mM): NaCl 145, KCl 0·1, EGTA 1, Tris 10, inosine 10, pH adjusted to 7·4 by adding 1 M NaOH. The suspension was centrifuged as before, and the pellet re-suspended twice. After the final centrifugation, the cells were stored as a pellet in this solution at 4°C, for up to 3 days.

The assays were performed using a solution containing (mM) NaCl, 145; KCl, 0·1; $MgSO_4$ 1; $CaCl_2$ 1; inosine 10; Tris 10; pH adjusted to 7·4 by adding 1 M NaOH. Because earlier experiments (Shiner, unpublished results) had shown that the action of racemic cetiedil develops slowly ($t\frac{1}{2} = 4$ min), the cells were incubated with the enantiomers for 1 h before the tests proper. 'Control' cells were incubated under the same conditions (20 μL of packed cells in 2 mL of the low-K^+ solution at 37°C; haematocrit approximately 1%). The cell suspension was then transferred to a water-jacketed bath which contained the K^+-sensitive and reference electrodes, and was maintained at 37°C. When the signal from the K^+ electrode had become steady, A23187 (2 μM) was added, and the resulting loss of K^+ from the cells was recorded for 3 min. Finally, digitonin (100 μM) was introduced to lyse the cells and so enable estimation of their total K content (see Cook & Haylett (1985) for further details). The amount of K^+ lost in response to the 3-min application of A23187 could then be calculated as a fraction of this total. The value observed in the presence of cetiedil was expressed as a percentage of the K^+ loss from the 'control' cells.

Muscarinic blocking action. The muscarinic antagonist activities of the enantiomers were compared using the classical guinea-pig ileum preparation. A 1·5–2 cm length of ileum was mounted in an organ bath held at 37°C, and contractions elicited by acetylcholine were recorded isotonically under a load of 0·5 g. The bathing fluid contained (mM): NaCl, 116; KCl, 4·6; $MgSO_4$, 1·18; NaH_2PO_4, 1·16; $CaCl_2$, 2·5; $NaHCO_3$, 25; glucose 11, and was oxygenated with 95% O_2–5% CO_2. Hexamethonium (10 μM) was included to block nicotinic responses. Two series of experiments were performed. In the first, concentration-response curves were determined by the cumulative application of increasing concentrations of acetylcholine (5–500 nM) at 30-s intervals. Reproducible curves were obtained on repeating the cumulative additions at 15-min intervals. The enantiomers of cetiedil applied 45 min previously caused a parallel displacement of the log concentration-response curves, and the EC50 values for acetylcholine before and in the presence of cetiedil were determined by fitting the Hill equation to the curves. The factor (the concentration ratio) by which the concentration of acetylcholine had to be increased to give the same response as before addition of cetiedil could then be calculated. The second series of experiments employed the method of Arunlakshana & Schild (1959)

in which single rather than cumulative concentrations of acetylcholine were applied, and the concentrations producing approximately 30 and 70% of the maximum response were determined. This was then repeated in the presence of cetiedil, applied 45 min previously. A control preparation which was not exposed to cetiedil was used to correct for changes in the sensitivity to acetylcholine during the experiment. The two sets of experiments gave similar concentration ratios, and these were combined.

Inhibition of the cell-volume-sensitive K^+ conductance of hepatocytes. The blocking action of the cetiedil enantiomers on the K^+ conductance ($g_{K(vol)}$) which is activated when liver cells swell was examined by recording the membrane potential and conductance of single isolated hepatocytes by means of an intracellular electrode. Electrolyte leakage from the electrode causes swelling, resulting in turn in an increase in membrane potential electronegativity and an increase in cell conductance as the $g_{K(vol)}$ channels open. The method has been described in detail by Sandford et al (1992) and only minor changes were made. Briefly, single isolated guinea-pig or rat hepatocytes were penetrated using a micro-electrode (resistance 90–120 MΩ) filled with 1 M KCl (rather than 1 M potassium citrate, as employed by Sandford et al (1992)). The subsequent opening of the K_{vol} channels was signalled by a slow increase in the negativity of the membrane potential, and by an increase in membrane conductance (as assessed by the voltage deflections resulting from the passage of 100 pA current pulses through the micro-electrode). The application of cetiedil in the perfusion fluid reversed these changes; the magnitude of the reduction in conductance was used to compare the potencies of the enantiomers. In these experiments the cells were bathed in a solution containing (mM): NaCl, 134; KCl, 4·7; $MgCl_2$ 1·2; $CaCl_2$; 2·5, glucose, 5·6; Hepes, 10; pH adjusted to 7·4 with 1 M NaOH.

Inhibition of Ca^{2+}-evoked contractions of depolarized smooth muscle. This was assessed by the method of Spedding (1982) in which smooth muscle is exposed to a K^+-rich, low-Ca^{2+} bathing solution. After an initial contraction attributable to depolarization of the cells, the tissue relaxes as the extracellular spaces become depleted of Ca^{2+}. Subsequent application of higher concentrations of Ca^{2+} then initiates contraction as a result of entry of Ca^{2+} through open Ca^{2+} channels. In these experiments, 1-cm lengths of the taenia of the guinea-pig caecum were mounted in a 2-mL organ bath at 37°C. The tissue was connected to an isometric transducer, and was placed under an initial tension of 1 g. The original bathing solution, of the same composition as was used in the experiments with guinea-pig ileum, was then replaced by one containing (mM): KCl, 123; $CaCl_2$, 0·1; $MgSO_4$, 1·2, KH_2PO_4, 1·2; $KHCO_3$ 25; glucose 11 (oxygenated with 95% O_2–5% CO_2). Mepyramine (1 μM) and atropine (1 μM) were also included. Under these conditions, and using a 15-min dose cycle and a 5-min exposure time, the addition of $CaCl_2$ to the bathing fluid, to give final Ca^{2+} concentrations of 0·4 and 3·1 mM, initiated contractions that were approximately 20 and 60%, respectively, of the maximum attainable with higher concentrations. The cetiedil enantiomers were then added to the solution and, after 30–45 min, increasing concentrations of $CaCl_2$ were applied until the resulting contractions matched the originals. The factor by which the concentration of Ca^{2+} had to be increased to overcome the action of cetiedil was then calculated.

Analysis of pharmacological data. A least-squares curve-fitting program (CVFIT, written by Professor D. Colquhoun, University College, London, UK) was used to analyse the relationship between the concentration of cetiedil and its inhibitory action. Either the Hill equation or the Schild equation (when examining the muscarinic blocking action of cetiedil) was fitted to the data in order to obtain estimates (± the approximate standard deviation) of either the IC50 (the concentration causing 50% inhibition) or the potency ratio of the enantiomers.

Results

The availability of the enantiomers in the pure form, albeit in limited quantity, enabled us to complete the biological tests summarized in Figs 2 and 3. Fig. 2a shows that (+)- and (−)-

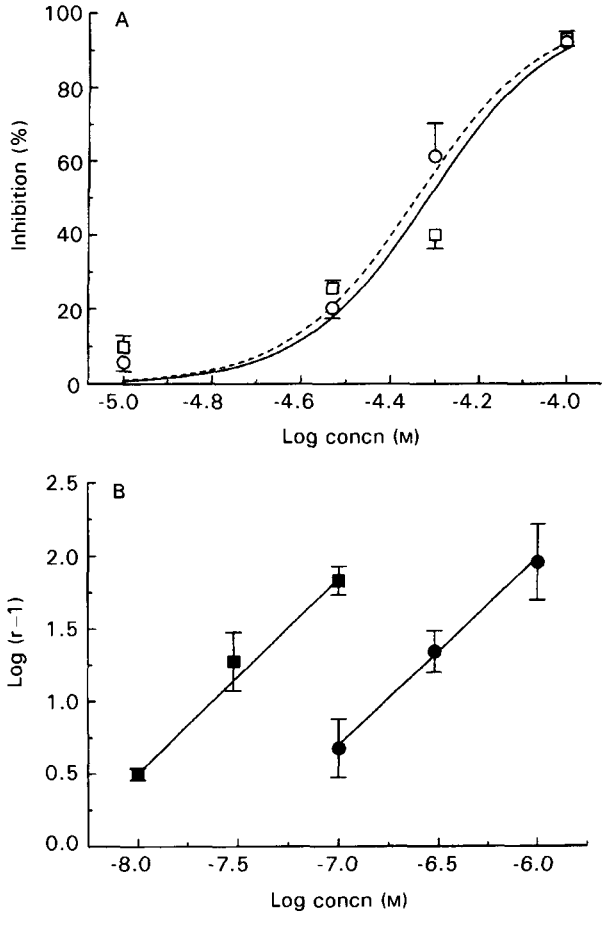


FIG. 2. A. Log molar concentration-effect relationships for the inhibition by (+)-cetiedil (□) and (−)-cetiedil (○) of A23187-induced K^+ loss from rabbit erythrocytes. The values plotted are the means of 3 to 5 observations and the vertical bars indicate the standard errors of the means. The lines have been fitted using the Hill equation with a Hill coefficient of 3·07 and the maximum inhibition constrained to 100%. B. Schild plots for the antagonism by (+)- and (−)-cetiedil (■ and ●, respectively) of the contractile response of guinea-pig ileum to acetylcholine. The values plotted are the means of 3 to 8 observations and the vertical bars indicate the standard deviations of the means.

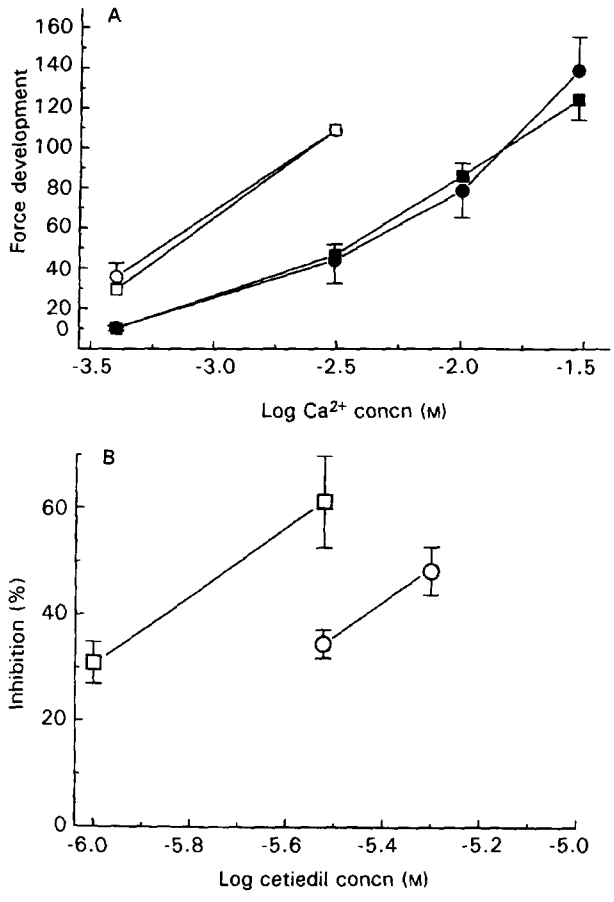


FIG. 3. A. Comparison of the effects of (+)-cetiedil (3 μM, ■; control □) and (−)-cetiedil (3 μM, ●; control ○) on the log molar concentration-response curves for the action of Ca^{2+} in causing contraction of depolarized smooth muscle (guinea-pig taenia caeci). The responses observed at each calcium concentration were normalized by expressing them in terms of the force developed by each preparation when Ca^{2+} was applied at a concentration of 3·1 mM, before cetiedil. The vertical bars indicate the standard errors of the means of four observations. B. Comparison of the percentage inhibition by (+)-cetiedil (□) and (−)-cetiedil (○) of the increase in membrane conductance which follows the swelling of isolated guinea-pig hepatocytes. The values plotted are the means of 3 to 6 observations, and the vertical bars indicate the standard errors of the means.

cetiedil did not differ substantially in their ability to block the Ca^{2+}-activated K^+ permeability of rabbit red cells. The (+) enantiomer caused half-maximum inhibition at 48·3 ± 2·9 (s.d.) μM, compared with 45·5 ± 2·8 mM for the (−) form. The corresponding equimolar potency ratio was 0·93 ± 0·08 (s.d.).

In contrast, there was a clear difference in the muscarinic blocking activity of the enantiomers, as assessed by inhibition of the contractile response of intestinal smooth muscle to acetylcholine. The magnitude of the inhibition was expressed as a concentration ratio, r (the factor by which the concentration of acetylcholine had to be increased in the presence of cetiedil in order to restore a submaximum response). Fig. 2b shows Schild plots, i.e. graphs of log (r − 1) against log [cetiedil]. The slopes of the lines were 1·34 ± 0·10 (s.d.) for (+)-cetiedil, and 1·29 ± 0·32 (s.d.) for (−)-cetiedil. Because these values were not significantly different, an equimolar potency ratio for the enantiomers could be calculated, assuming a common slope. By this criterion, (+)-cetiedil was 7·7 ± 0·2 (s.d.)-fold more active than the (−) form. It should, however, be noted that the slopes of the Schild plots were greater than unity (and significantly so for the (+) enantiomer) so that the predictions of simple competitive antagonism were not fulfilled. Insufficient material was available to enable this to be analysed further. It was not possible, therefore, to calculate the dissociation equilibrium constants for the combination of the cetiedil enantiomers with the muscarinic receptors (predominantly of the M3 subtype) mediating this action of acetylcholine. Extrapolation of the unconstrained regression lines to the abscissa yielded pA_2 values of 8·37 ± 0·03 (s.d.) and 7·54 ± 0·06 (s.d.) for (+)- and (−)-cetiedil, respectively.

Fig. 3a shows that the enantiomers do not differ in their effectiveness as blockers of the contractile response of depolarized smooth muscle to externally applied Ca^{2+}. Thus the application of 3 μM (+)-cetiedil made it necessary to increase the concentration of Ca^{2+} needed for a submaximum contraction by a factor of 5·2 ± 1·1 (s.d.), compared with 5·5 ± 2·3 for (−)-cetiedil. These values do not differ significantly. Interestingly, they are close to the figure (5·6) which can be estimated from the results of similar experiments to study the action of (±)-cetiedil on the depolarized rat aorta preparation (Boissier et al 1978).

The final comparison (Fig. 3b) shows that the enantiomers differ in their potency as blockers of the increase in membrane conductance which occurs when liver cells swell. This change in conductance is largely attributable to an increase in K^+ permeability (Sandford et al 1992). The concentration of (+)-cetiedil needed to reduce the conductance increase by 50% was 2·04 ± 0·54 (s.d.) μM, and (−)-cetiedil was 2·6 ± 0·8 (s.d.) times less active, with an IC50 of 5·2 ± 1·2 μM. These values are consistent with the figure of 2·3 ± 0·3 μM reported by Sandford et al (1992) for similar experiments with (±)-cetiedil.

Discussion

These findings have several implications. The equipotency of the enantiomers as blockers of $P_{K(Ca)}$ in red cells suggests that the shape of the esterified end of the molecule is not important for this action. This is in keeping with preliminary evidence (Benton et al 1994) suggesting that lipophilicity is a more important factor. It must, however, be kept in mind that cetiedil is not a potent blocker of this channel; nitrendipine (Ellory et al 1992) and clotrimazole (Alvarez et al 1992) have recently been shown to be much more effective, with IC50 values of 130 and 50–100 nM, respectively. More active analogues of cetiedil might, therefore, show greater shape-dependence.

The results shown in Figs 2a and b, taken together, suggest that it would be useful to compare the clinical effectiveness of the enantiomers of cetiedil. If cetiedil's effectiveness in the treatment of sickle cell disease (Benjamin et al 1986) is indeed a consequence of its ability to block the Ca^{2+}-activated K^+ channel in erythrocytes, it should be possible to achieve the same degree of block by using the (−) enantiomer at a dose which would produce less muscarinic block than seen with the racemic mixture currently used in therapeutics. Admittedly, it

is conceivable that a certain extent of muscarinic block, or indeed some quite different action, could be required for therapeutic benefit. Comparing the actions of the enantiomers with each other, and with (±)-cetiedil, could enable these possibilities to be distinguished.

The results presented in Fig. 3 add to the evidence that the geometry of the chiral centre influences some of the actions of cetiedil, although not others. Thus, (+)-cetiedil is more than twice as effective as (−)-cetiedil in blocking the specific K^+ channel which opens when hepatocytes swell. In contrast, there is no significant difference between the abilities of the enantiomers to block the Ca^{2+}-induced contraction of depolarized smooth muscle. This response is initiated by the inward movement of Ca^{2+} through voltage-dependent Ca^{2+} channels of the L subtype (see, e.g., Spedding & Paoletti 1992). Whereas the present results (and those of Boissier et al 1978) could be explained by supposing that cetiedil is able to block these channels, further work would be needed to rule out other possible explanations.

To summarize, our finding of differences in the biological actions of the enantiomers of cetiedil indicates that a more extensive study could be rewarding in relation to the use of the enantiomers both in therapeutics and in the study of K^+ channels.

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