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A Stable and Highly Potent Hexamethonium Derivative Which Modulates Muscarinic Receptors Allosterically in Guinea-pig Hearts

G. BEJEUHR, G. BLASCHKE*, U. HOLZGRABE, K. MOHR†, U. SÜRIG AND G. TERFLOTH*

*Institute of Pharmacy, University of Bonn, Kreuzbergweg 26, 53115 Bonn, *Institute of Pharmacy, University of Münster, Hittorfstr. 58–62, 48149 Münster, and †Department of Pharmacology and Toxicology, Institute of Pharmacy, University of Bonn, An der Immenburg 4, 53121 Bonn, Germany*

Abstract—W84 (*N,N,N′,N′*-tetramethyl-*N,N′*-bis-(3-phthalimidopropyl)-*N,N′*-hexane-1,6-diyl-bis-ammonium dibromide) is a potent stabilizer of antagonist binding to muscarinic receptors; however, W84 hydrolyses in aqueous buffered medium. The synthesis of the stable derivative CHIN3/6 is presented containing a 2-phenyl-quinazolinone instead of the labile phthalimide substituent. This compound retarded [^{3}H]*N*-methylscopolamine-dissociation in guinea-pig cardiac membranes with slightly higher potency than W84, the EC50 values amounting to $7{\cdot}5 \times 10^{-7}$ and 13×10^{-7} M, respectively. Molecular modelling revealed differences in the electron density of the substituents and in their molecular shape. It is suggested that the derivatives use partially different sites of attachment when occupying the allosteric binding site of the receptor protein.

The hexamethonium derivative W84 (Scheme 1) is an antagonist at muscarinic acetylcholine receptors distinguished from classical antagonists such as atropine by particular properties; in isolated cardiac preparations the antagonistic effect does not increase in parallel with the concentration but approaches a maximum. The antimuscarinic effect of a combination of W84 with a conventional antagonist is stronger than predicted from the action of the individual compounds (Lüllmann et al 1969). In cardiac membranes, W84 retards the dissociation of radiolabelled antagonists such as [^{3}H]*N*-methylscopolamine concentration-dependently, rendering antagonist binding almost irreversible at high concentrations (Jepsen et al 1988; Mohr et al 1992). These actions are compatible with an allosteric effect at muscarinic cholinoceptors.

Several compounds from various pharmacological groups have been found to stabilize antagonist-binding to muscarinic receptors (Lee & El-Fakahany 1991). However, W84 reveals a particular high potency in this respect. It retards the dissociation of [^{3}H]*N*-methylscopolamine ([^{3}H]NMS) by a factor of two (EC50) at 1·3 μM (Lüß & Mohr 1992). Similar effects have been reported for the structurally closely related heptamethonium derivative (Mitchelson 1975; Choo & Mitchelson 1989).

Unexpectedly from the chemical point of view, the phthalimide group of W84 hydrolyses in aqueous buffered medium (3 mM $MgHPO_4$, 50 mM Tris, pH = 7·4, $t_{\frac{1}{2}} = 5{\cdot}5$ h (Jepsen et al 1988)). To obtain chemically stable allosteric modulators with an activity comparable with that of W84, various hexamethonium derivatives have been synthesized. Screening experiments to assess the ability to stabilize [^{3}H]NMS-binding to cardiac muscarinic receptors pointed to the quinazolinone-substituted hexamethonium derivative, CHIN3/6 (Scheme 1) as a promising candidate.

Correspondence: K. Mohr, Department of Pharmacology and Toxicology, Institute of Pharmacy, University of Bonn, An der Immenburg 4, D-53121 Bonn, Germany.

Chemistry

The quinazolinone skeleton was built in analogy to a pathway described by Büyüktimkin et al (1988) for similar compounds. Anthranilamide (Scheme 1, *1*) was monoalkylated by a twofold excess of dibromopropane. Subsequent refluxing in toluene in the presence of benzaldehyde and piperidine closed the quinazolinone ring (*3*) and the hexamethonium middle chain was generated by conversion of two molecules of the heterocycle with bis(dimethylamino)-hexane in nitromethane.

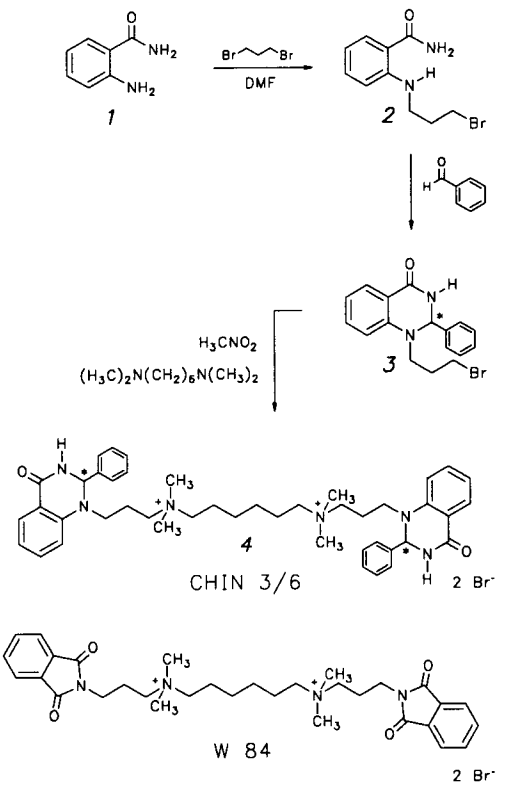
Because a centre of chirality is created by the ring closure, a racemate is obtained. Without previous resolution the final reaction leads to three isomers of *4* (two enantiomers and a meso-isomer). From preliminary investigations it is known that the stereochemistry of the heterocycles at the end of the middle chain strongly influences the biological effects (Bejeuhr et al 1992). Therefore, a chromatographic resolution of the racemate of the quinazolinone (*3*) was attempted on a column with chiral polyphenylethylacrylamide under low pressure conditions.

Materials and Methods

Melting points were determined with a Dr Tottoli melting point apparatus (Büchi, Flawil, Switzerland) and were uncorrected. ^{1}H and ^{13}C NMR spectra were recorded on a Varian XL 300 (^{1}H 299·956 MHz, ^{13}C 75 MHz) and Jeol PMX 60si (60 MHz) instruments. Infrared spectra, recorded as KBr discs, were obtained using a Perkin-Elmer 298 spectrometer. UV/vis spectra were obtained on a Hewlett Packard 8450 A. For column chromatography, Silica Gel 60 (70–23 mesh, Merck 7734) was used. Polyphenylethylacrylamide for HPLC and preparative chromatography was synthesized according to Terfloth (1992). Dry solvents were used throughout.

Synthesis

N-*Bromopropylanthranilamide* (2). 2-Anthranilamide (1·36 g, 0·01 mol) and dibromopropane (6·0 g, 0·03 mol) were



SCHEME 1.

heated under reflux for 12 h in dimethylformamide (DMF, 100 mL). About 80 mL DMF was evaporated in-vacuo. The remaining solution was poured onto ice (200 mL) and allowed to stand overnight. The separated solid was collected and extracted four times with CH_2Cl_2. The combined extracts were dried over Na_2SO_4 and evaporated to give *2* (1·02 g, 40%), mp 110°C, recrystallized from CH_2Cl_2/petroleum ether. IR: 3350, 3160, 1660, 1620 cm^{-1}; ^1H NMR ($CDCl_3$, δ, ppm): 2·16 (m, 2H, 7 Hz, —CH_2—), 3·36 (t, 2H, 7 Hz, N—CH_2), 3·51 (t, 2H, 7 Hz, CH_2—Br), 5·80 (s, br, 3H, NH), 6·42–7·50 (m, 4H, phenyl).

1-(3-Bromopropyl)-2,3-dihydro-2-phenyl-1H-quinazolinone (3). *2* (1·28 g, 5 mmol) and benzaldehyde (0·6 g, 5·5 mmol) were heated in toluene (50 mL) on a water separator for 16 h. Toluene was evaporated in-vacuo and the oil was purified by column chromatography (silica gel, mobile phase: CH_2Cl_2/MeOH = 8:2). *3* was obtained (1·2 g, 70%), mp 136°C, recrystallized from acetone/ether. IR: 3300 (shoulder), 3190, 1655, 1615, 755, 740, 705 cm^{-1}; ^1H NMR ($CDCl_3$, δ, ppm): 1·90–2·46 (m, 2H, —CH_2—), 3·00–3·85 (m, 4H, CH_2—Br, N—CH_2), 5·73 (d, 1H, 3 Hz, CH), 6·70–8·00 (m, 9H, phenyl).

N,N,N′,N′-Tetramethyl-N,N′-bis[3-(4-oxo-2-phenyl-3,4-dihydro-2H-quinazolin-1-yl)-propyl]-N,N′-hexane-1,6-diyl-bis-ammonium dibromide (4). *3* (200 mg, 0·58 mmol) and bis(dimethylamino)hexane (50 mg, 0·28 mmol) were heated under reflux in nitromethane for 16 h. Removal of the solvent gave an oil, crystallized from EtOH (130 mg, 55%), mp 250°C. $C_{44}H_{58}Br_2N_6O_2$ (862·8); Calc. C 61·3 H 6·78 N 9·74, Found C 61·0 H 6·71 N 9·98. IR: 3400, 3200, 1700 (shoulder), 1660, 1600, 755, 740, 695 cm^{-1}; ^1H NMR (DMSO-d_6, δ, ppm): 1·16 (m, 4H, —CH_2—), 1·58 (m, 4H, —CH_2), 1·94 (m, 4H, N—CH_2—CH_2—CH_2—$\overset{+}{N}$), 2·05 (m, 4H, N—CH_2—), 3·01 (s, 12H, $\overset{+}{N}$—CH_3), 3·19 (m, 4H, $\overset{+}{N}$—CH_2—), 3·61 (m, 4H, $\overset{+}{N}$—CH_2—), 5·81 (d, 2H, 4.0 Hz, C_2H), 6·78 (t, 2H, 7·67 Hz, C_6H), 6·92 (d, 2H, 8·35 Hz, C_8H), 7·28–7·34 (m, 10H, phenyl), 7·41 (td, 2H, 8·35 Hz, 7·67 Hz, 1·62 Hz, C_7H), 7·70 (dd, 2H, 7·67 Hz, 1·62 Hz, C_5H), 8·78 (d, 2H, 4·0 Hz, NH). ^{13}C NMR (DMSO-d_6, δ, ppm,): 20·41 (—CH_2—), 21·47 (—CH_2—), 25·12 (—CH_2—), 45·06 (N—CH_2—), 50·15 ($\overset{+}{N}$—CH_3), 60·40 ($\overset{+}{N}$—CH_2—), 62·91 ($\overset{+}{N}$—CH_2—), 70·41 (C2), 112·40 (phenyl), 116·56 (i—C, phenyl), 117·13 (phenyl), 125·90 (phenyl), 127·66, 127·91 (C6/7), 128·24 (C8), 133·56 (C5), 141·12 (C—4a), 145·73 (C8a), 162·06 (C4).

Preparative resolution of 3

The polymer phenylethylacrylamide with particle size 50–100 μm was boiled under reflux in the mobile phase, *t*-butylmethylether/THF (8:2). After cooling the slurry was filled into a glass column (30 cm × 2·5 cm) with Teflon adaptors. The column was packed using a low pressure pump (Duramat Membranpumpe) under a solvent pressure of 1–2 atm. The racemate of *3* (200 mg) was injected. The flow of the mobile phase was 1 mL min^{-1}. Resolution was monitored by HPLC using phenylethylacrylamide bound to silica gel (particle size 4 μm, column 23 cm × 4 mm) with a mobile phase of hexane/dioxane (6:4) containing 1% acetonitrile; no decomposition of *3* was observed under these analytical conditions in control experiments.

Pharmacological testing

The method has been described in detail by Jepsen et al (1988) and Mohr et al (1992). A suspension of myocardial membranes was prepared from guinea-pig hearts. Binding of [^{3}H]NMS (0·5 nM) was measured in 1·5 mL 3 mM $MgHPO_4$, 50 mM Tris, pH 7·3, 37°C. Measurements were made in triplicate. The incubation was terminated by filtration of 1 mL samples of the incubation medium through glass-fibre filters (Schleicher & Schüll, Dassel, Germany) under suction with a vacuum pump. Filter-bound radioactivity was determined by liquid-scintillation counting. Non-specific binding of [^{3}H]NMS was determined in the presence of 10^{-4} M atropine and amounted to less than 5% of the total. Binding of NMS under control conditions was characterized by an equilibrium dissociation constant (K_D) of about 1 nM and a maximum number of binding sites (B_{max}) of about 80 pM in the assay medium.

To determine the effect of CHIN3/6 on the binding of [^{3}H]NMS, the compound was incubated in the indicated concentration range together with the radioligand and the membranes for a period of 120 min before separating the membranes by vacuum filtration. To measure the time course of [^{3}H]NMS dissociation, the radioligand was incubated with the cardiac membranes in a volume of 21 mL for 60 min during which the binding equilibrium was attained. Dissociation was revealed by adding 10^{-4} M atropine to the assay mixture. Samples (1 mL) were drawn at the indicated intervals. To evaluate the effect of CHIN3/6 on the time course of dissociation, the radioligand was applied together with atropine.

The binding data were subjected to nonlinear regression analysis applying the InPlot software (GraphPad, San Diego, USA).

Molecular modelling

Molecular graphics studies were carried out on an IBM 3084/3081 computer and a PS 390/micro VAX system (Evans & Sutherland/Digital Equipment Corporation) using SYBYL software (Tripos Associates, St Louis, MO, USA). Molecules were built up from standard geometries, conformations were optimized by means of force field (MMX, PCMODEL, Serena Software, Bloomington, IN, USA) and quantum chemical methods (AM1, MOPAC 5·1, Program 581, QCPE Bloomington, IN, USA).

Results

Chemistry

On the preparative scale (100–300 mg) only partial enantiomeric resolution of the quinazolinone (*3*) was observed: in different experiments the (+)-enantiomer was enriched to 63% and the (−)-isomer to 90% (Fig. 1). In addition to the unsatisfying resolution, the substance obtained from the column chromatography did not crystallize from the eluent or other solvents added after removing the eluent in-vacuo. From some fractions of the preparative chromatography the anthranilamide *2* could be isolated. To determine whether THF is involved in this decomposition reaction, time-dependent ^{1}H NMR measurements of the quinazolinone *3* in 10% THF in $CDCl_3$ were carried out. By monitoring the signal of the proton at C2 of *3*, a half-life of about 21 days was determined. Thus, THF seems to catalyse the back reaction which was not observed in $CDCl_3$ or other aqueous media. To overcome this problem, THF was replaced with other solvents in the mobile phase, but combinations of $CHCl_3$/methanol, $CHCl_3$/hexane, $CHCl_3$/dioxane, and n-hexane/dioxane each in various proportions resulted only in worse enantiomeric resolution and dioxane combinations led to decomposition. Attempts to resolve *4* on cyclodextrins also failed (Branch et al personal communication).

From these results the question was raised whether *4* is stable in the aqueous Tris-buffered solution used for pharmacological tests. Because ^{1}H NMR and UV/vis spectra change significantly in the case of decomposition, time-dependent spectra were recorded in buffer; there was no sign of reaction in a period of 27 days in contrast to the THF solution. Additionally, ^{1}H and ^{13}C NMR spectra showed only one set of signals indicating that it is likely that either the *meso*-isomer or the racemate of *4* was obtained from the linking reaction, although this reaction should not be stereoselective.

Pharmacology

To check whether CHIN3/6 affected muscarinic cholinoceptors, its effect on the binding of [^{3}H]NMS was measured. As shown in Fig. 2, binding of the radioligand was inhibited by CHIN3/6. However, in the applied concentration range, CHIN3/6 did not fully inhibit receptor occupation by [^{3}H]NMS. The concentration of CHIN3/6 at which [^{3}H]NMS binding was reduced to 50% of the control value was 3×10^{-7} M.

The time course of [^{3}H]NMS dissociation under control

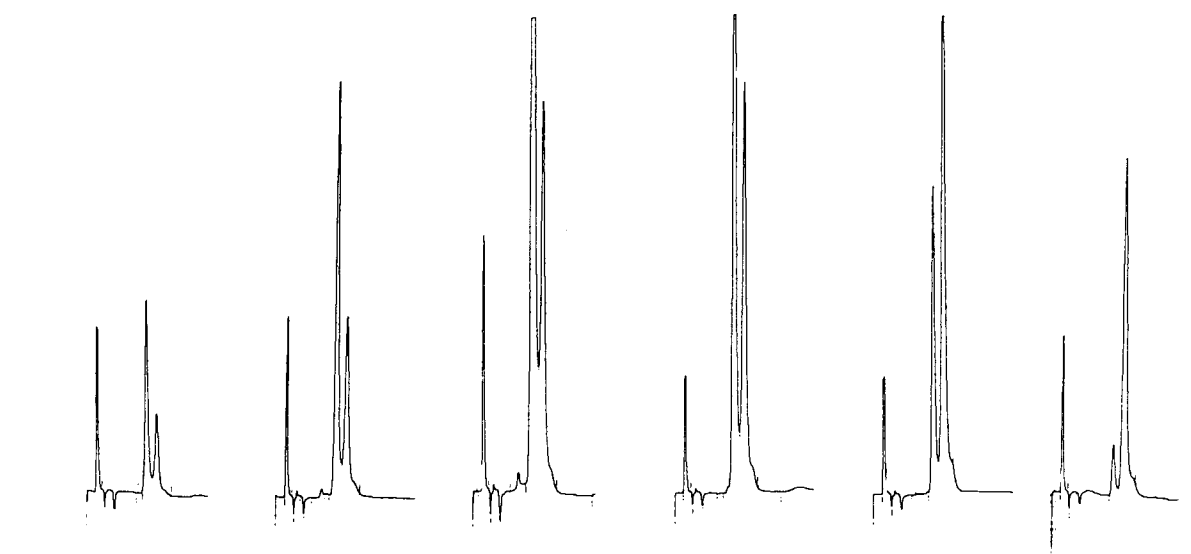


FIG. 1. HPLC of fractions obtained from preparative chiral chromatography of *3*.

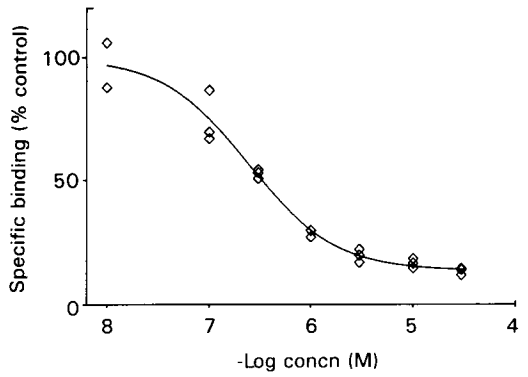


FIG. 2. Effect of CHIN3/6 on the specific binding of [^{3}H]NMS to muscarinic receptors in guinea-pig myocardial membranes. Ordinate: specific binding of [^{3}H]NMS (0·5 nM) as a percentage of the control value measured in the absence of CHIN3/6. Abscissa: concentration of CHIN3/6.

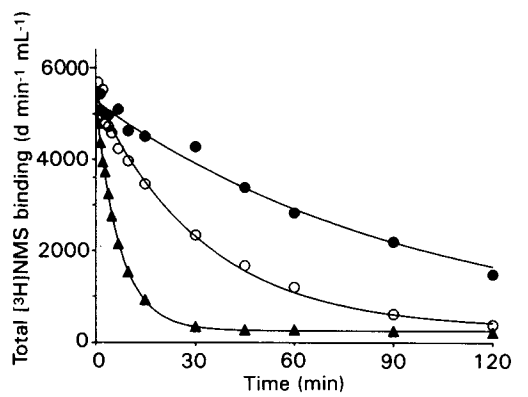


FIG. 3. Effect of CHIN3/6 on the dissociation of [^{3}H]NMS from cardiac membranes in representative experiments. Ordinate: [^{3}H]NMS binding after addition of 10^{-4} M atropine for revealing radioligand dissociation. Abscissa: time after addition of atropine. ▲ Control, $t_{\frac{1}{2}} = 5{\cdot}1$ min; ○ 3 μM, $t_{\frac{1}{2}} = 22$ min; ● 10 μM, $t_{\frac{1}{2}} = 63$ min.

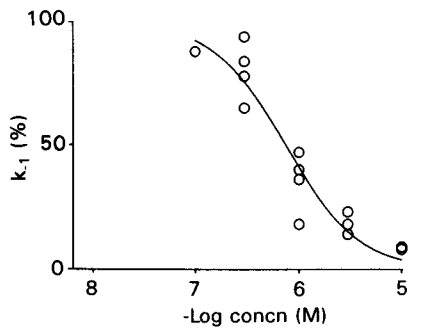


FIG. 4. Concentration-effect curve for the stabilizing action of CHIN3/6 on complexes of [^{3}H]NMS with cardiac cholinoceptors. Ordinate: apparent rate constant of [^{3}H]NMS-dissociation k_{-1} in the presence of CHIN3/6 as a percentage of the value for the control. Abscissa: concentration of CHIN3/6. Points represent results obtained from complete dissociation curves as shown in Fig. 3.

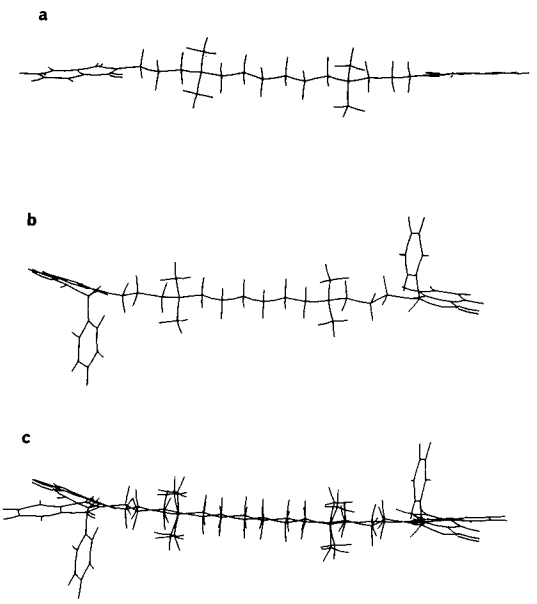


FIG. 5. a. Conformation of W84. b. Conformation of *meso*-CHIN3/6. c. W84 and CHIN3/6 fitted in energetically preferred conformations.

conditions followed a monoexponential function and was characterized by a half-life of about 5 min. In the presence of CHIN3/6 the dissociation of [^{3}H]NMS was retarded (Fig. 3). Nonlinear regression analysis was applied to obtain the half-life of dissociation. From these data, apparent rate constants of dissociation could be calculated. With increasing concentrations of CHIN3/6, the rate of [^{3}H]NMS dissociation declined. To depict the concentration dependency of this effect, the rate constant of [^{3}H]NMS-dissociation measured in the presence of CHIN3/6 was expressed as a percentage of the control value and was plotted against the concentration of CHIN3/6 (Fig. 4). CHIN3/6 reduced the rate of [^{3}H]NMS dissociation with an EC50 value of $7{\cdot}5 \times 10^{-7}$ M.

Molecular modelling

Fig. 5b shows the energetically preferred, elongated conformation of CHIN3/6 resulting from the semi-empirical calculations. The phenyl ring at C2 protrudes out of the plane of the heterocycle at the end of the hexamethonium chain. Additionally calculated isoenergy contours of the *N*-alkylsubstituted quinazolinone and AM1 charges of the whole molecule (not shown) exhibited a relatively large negative area spread over the amido group and the above-mentioned phenyl ring. The highest negative charge is located at the electronegative oxygen of the carbonyl group.

Discussion

CHIN3/6 affected the dissociation of complexes of [^{3}H]NMS with cardiac muscarinic receptors which is indicative of an allosteric action. The stabilizing effect on antagonist-binding was characterized by an EC50 value of $7{\cdot}5 \times 10^{-7}$ M. Under the applied conditions, W84 revealed an EC50 of 13×10^{-7} M (Lüß & Mohr 1992). Thus, CHIN3/6 surpassed W84 in potency as an allosteric stabilizer of [^{3}H]NMS binding. When incubated with [^{3}H]NMS, CHIN3/6 reduced the binding of the radioligand. This effect demonstrated that CHIN3/6 is also capable of inhibiting the association of [^{3}H]NMS. Since

[^{3}H]NMS binding was reduced to half its control value at 3×10^{-7} M, the effect of CHIN3/6 on the association of the radioligand is somewhat stronger than its effect on radioligand dissociation. The stabilizing action of CHIN3/6 on [^{3}H]NMS-receptor complexes tends to promote radioligand binding and thus counteracts the inhibitive effect of CHIN3/6 on [^{3}H]NMS binding. This interplay between the opposing actions of CHIN3/6 offers an explanation for the observation that even high concentrations of CHIN3/6 did not fully suppress specific [^{3}H]NMS binding. At present, it cannot be decided whether the effects on [^{3}H]NMS association and dissociation are mediated via occupation by CHIN3/6 of a common binding site or of distinct sites on the receptor protein.

Previous semi-empirical calculations of W84 (Bejeuhr et al 1992) revealed an elongated conformation (Fig. 5a) of the whole molecule and suggested the carbonyl groups of the phthalimide formed hydrogen bonds with the putative allosteric binding site. Superposition of CHIN3/6 and W84 (Fig. 5c) illustrates the occupation of nearly the same area by the heterocyclic substituents at the ends of the bisquaternary chain; however, the phenyl ring of CHIN3/6 protrudes almost at right angles to the plane of the heterocycles. Additionally, the distribution of the charges is different in the heterocycle groups; whereas the region of high electron density of W84 is orientated to the middle chain, this negative region points to the opposite side of the molecule in CHIN3/6. Nevertheless, it is possible to superimpose similar regions of electron density of the molecules, although this fit is accompanied by a strong distortion, especially of the middle chain.

The above considerations are independent of the individual geometry of the 2-phenylquinazolinone rings of the CHIN3/6 isomers because the phthalimide ring of W84 is symmetrical.

In conclusion, as a stabilizer of antagonist binding to cardiac cholinoceptors, CHIN3/6 is a slightly more potent compound than the lead compound W84. However, it is not possible to obtain a satisfying fit between CHIN3/6 and W84 concerning the regions of electron densities and the phenyl ring. These results suggest that the compounds do not use exactly the same sites of attachment in the receptor protein. CHIN3/6 may represent a valuable tool for further pharmacological research in the field of allosteric modulators of antagonist binding to muscarinic cholinoceptors.

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