Chapter 7

Effect of antimalarials on human erythrocyte membrane acetylcholinesterase

Introduction

Chloroquine (CQ), primaquine (PQ) and quinine (Q) are the three most widely used antimalarial drug (1). Besides being used for management of malaria and prophylaxis, the antimalarials are also recommended for management of rheumatoid arthritis, porphyria cutanea tarda and solar urticaria (1) and in more recent years hydroxychloroquine – an anti-inflamatory agent- has also found application in the treatment of Alzheimer's disease (2,3). Chloroquine is also being used as an anti-inflamatory agent and has shown promise for use in graft verses host disease (GVHD) (4).

One of the major defects in Alzheimer's disease is the lack of adequate amounts of neurotransmitter acetylcholine (ACh) in the brain due to destruction of acetylcholine producing neurons in specific brain regions (5). Most of the drugs used for treatment of Alzheimer's disease are acetylcholinesterase (AChE) inhibitors the strategy being that this will increase the ACh concentration in the brain (6). In the light of this it was of interest to find out whether the antimalarials exert their beneficial effects by acting as AChE inhibitors or there are alternate mechanisms by which they act. Previously it has been reported that chloroquine inhibit eel AChE and horse serum BuChE (7). However, the effects on human AChE or BuChE have not been described thus far.

To evaluate this possibility we examined the effect of the three above-mentioned antimalarials on the AChE activity in human erythrocyte membrane AChE The results are summarized below.

Materials and Method

Chemicals

Acetylthicholine iodide (ACTI), bovine serum albumin (BSA) fraction V and ethopropazine hydrochloride (ETPZ HCl) were purchased from Sigma Chemical Co. U S.A. 5,5' -dithiobis (2-nitrobenzoic acid) (DTNB) was purchased from SRL, India. All other chemicals were of analytical reagent grade and were purchased locally.

Sources of the antimalarials are as given in Chapter 2 of the thesis.

Isolation of erythrocyte membranes

Blood was collected from normal healthy volunteers (both males and female, age 22-45 years) in heparinized vials. RBC and plasma were separated by centrifugation at 2000 rpm for 10 min. The RBCs were washed twice with saline and then lysed with 10 mM potassium phosphate buffer, pH 7.4. The membranes were separated by centrifugation at 10,000 rpm for 30 min. The membranes were then washed twice with 14 mM tris-HCl buffer,pH 7.4, by centrifugation at 10,000 rpm for 30min. The membranes were finally suspended in 14 mM tris-HCl buffer,pH 7.4 at a final concentration of Ca. 1 mg protein/ml (8). All operations were carried out at 0-4 ° C in a Sorvall RC5 Centrifuge.

Assay of AChE activity

The AChE activity was assayed by the method of Ellman et al. with some modification described previously (9,10). The assay medium (final voloume 1.0 ml) contained 100 mM potassium phosphate buffer, pH 8.0, 5 mM ACTI, 320μM DTNB, and about 80-100

µg membrane protein as the source of the enzyme. 10µM ETPZ.HCl was included for inhibition of BuChE. The membranes were pre-incubated in the assay medium for 2 min at 37 °C and the reaction was started by the addition of the substrate and the increase in absorbance at 412 nm was recorded spectrophotometrically at intervals of 5 sec. The activity is expressed as µmoles of ACTI hydrolyzed / min / mg protein.

For the substrate kinetics studies ACTI concentration was varied over the range of 0.025 mM to 5 mM

Studies with inhibitors

For inhibition studies the antimalarial were used in the range of 0.01 µM to 10 mM as indicated in Fig. 1.

The data for substrate kinetics were analyzed by the Lineweaver-Burk, Eadie-Hofstee and Eisenthal and Cornish-Bowden methods for the determination of Km and Vmax (11) The values of Km and Vmax obtained by the three methods were in close agreement and were averaged.

Inhibition kinetics analyses were carried out using the equation (11):

$$Ki = \frac{i}{\frac{Kp(1+i/Ki)}{Ks} - 1} \qquad \text{and } K'i = \frac{i}{\frac{V}{Vp} - 1}$$

Where Kp and Ks are Michaelis constant in the presence and the absence of inhibitor respectively, and Vp and V are the respective velocities in the presence and the absence of inhibitor(11). and the Ki values were calculated.

Hill plot analysis for substrate-dependent increase in the velocity was by using the equation:

$$\log \left[\frac{v}{V-v} \right] = n \log [S] - \log k'$$

Where V is the maximum velocity, v is the velocity in the presence of substrate concentration [S], k' is the constant. The Hill's coefficient (n) reflects the number of substrate molecules bound. The plot of log [v/(V-v)] vs log [S] is expected to be a straight line, the slope of which gives the Hill coefficient(11).

Hill plot analysis for antimalarials concentration-dependent inhibition of the enzyme velocity was carried out by using the equation (11):

$$\log \left[\frac{V-v}{v} \right] = n \log [I] - \log k'$$

Where V is the maximum velocity in the presence of inhibitor, v is the velocity in the presence of inhibitor concentration [I], k' is the inhibitor constant and n (Hill's coefficient) reflects the number of inhibitor molecules bound The plots of log [(V-v)/v] vs log [S] is expected to be a straight line, the slope of which gives the Hill coefficient(11).

All the kinetics data were computer analyzed employing Sigma plot version 5.0 (12,13).

Protein was estimated by the method of Lowry et al. with bovine serum albumin (BSA) used as the standard(14).

Results

In the initial experiments the effect of pre-incubation with antimalarials on erythrocyte membrane AChE activity was examined. The data are shown in Fig. 1. Quinine concentration upto 1mM had marginal inhibitory effect; concentrations beyond 1mM resulted in rapid inhibition. CQ progressively inhibited the enzyme activity with increasing concentration whereas the pattern for PQ inhibition was similar to that obtained for quinine except that the inhibitory effect was apparent at much lower concentration i.e. 10μM and beyond. It was thus clear that the three antimalarials inhibited AChE activity over different concentration ranges. To illustrate this point the concentrations which inhibited the enzyme activity by 33 % (IC33) and 67 % (IC67) were computed from individual inhibition plots. The averaged values are given in Table 1. Thus quinine exerted its inhibitory effect at 3.2 and 7.5mM (33 and 67 % inhibition respectively) These concentrations for chloroquine were 22 and 192 μM. Corresponding concentrations for primaquine were 38 and 247 μM. Thus chloroquine seemed to be most potent inhibitor while quinine had the least inhibitory action. Also, the concentrations required for 67% inhibition were 9 and 7 times higher for chloroquine and primaquine. This value for quinine was 2.4 times higher. The result thus suggested that the concentration-dependent effects were different for the three antimalarials. The inhibition data were further analyzed by Hill plot analysis. The typical Hill plots are shown in Fig 2 The plots were linear for quinine and primaquine whereas that for chloroquine was biphasic. The Hill coefficients are given in Table 2, which suggest that

Fig. 1 Inhibition of AChE by (A) Quinine, (B) Chloroquine and (C) Primaquine. The enzyme activity was determined in the presence of varying concentrations of the antimalarials. The % of remaining activity (compared to the activity of uninhibited enzyme under standard assay conditions) is plotted on ordinate against log of antimalarial concentration (I) used on abscissa. The results are typical of 8 independent observations.

Fig. 1

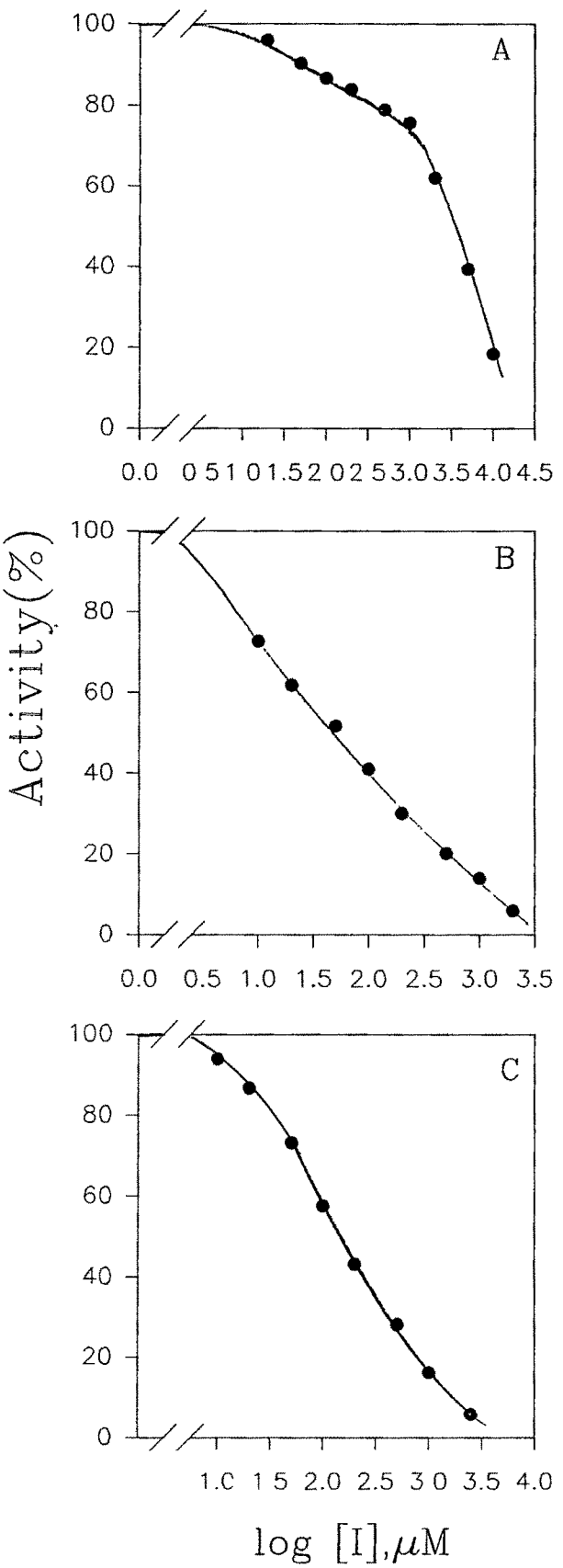

A
B
C
100
80
60
40
20
0
0.0 0 5 1 0 1.5 2 0 2 5 3.0 3.5 4.0 4.5
0.0 0.5 1.0 1.5 2.0 2.5 3.0 3.5
1.0 1 5 2.0 2.5 3 0 3.5 4.0
Activity(%)
log [I],μM

Table I IC33 and IC67 Concentrations of Antimalarials for Human Erythrocyte Membrane Acetylcholinesterase

Antimalarial	IC33	IC67
	μM	
Quinine	3201.6±370.63	7541.6±246.08
Chloroquine	22.3±3.04	192.7±24.42
Primaquine	38.2±2.18	247.2±14.48

IC33 and IC67 represents the concentration of antimalarial (μM) at which 33% and 67% inhibition was obtained. The results are given as mean ± SEM of eight independent observations.

Fig. 2 Typical Hill plots for AChE inhibition in the presence of (A) Quinine, (B) Chloroquine and (C) Primaquine The experimental details and the concentration of antimalarials are as given in the text and in Fig. 1. The transition point at which chloroquine shows de-binding is shown by vertical line.

Fig. 2

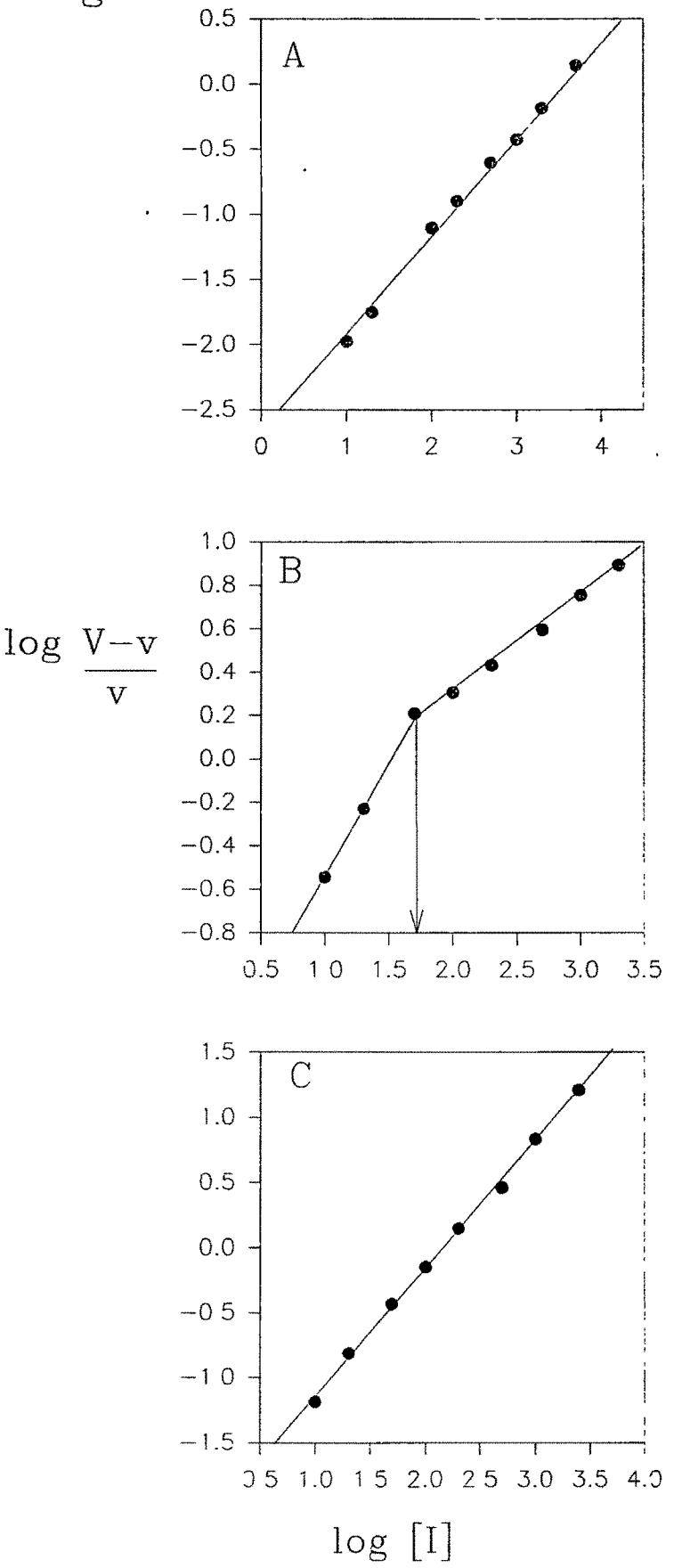

A
B
C
log V−v / v
log [I]
0.5
0.0
−0.5
−1.0
−1.5
−2.0
−2.5
0
1
2
3
4
1.0
0.8
0.6
0.4
0.2
0.0
−0.2
−0.4
−0.6
−0.8
0.5
1.0
1.5
2.0
2.5
3.0
3.5
1.5
1.0
0.5
0.0
−0.5
−1.0
−1.5
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0

Table 2 Antimalarial binding to erythrocyte membrane AChE.

Antimalarial	Hill coefficient		Transitition point conc.(μM)
	n1	n2	
Quinine	0.84±0.04	--	--
Chloroquine	1.17 ±0.09	0.54±0.04	39.17 ± 3.53
Primaquine	0.81±0.03	--	--

The Hill coefficients were calculated from the corresponding Hill plots.

The results are given as mean ± SEM of eight independent observations.

over the entire concentration range tested, 1 molecule of quinine and primaquine bound to the enzyme. in the case of chloroquine also 1 molecule was bound upto 40μM concentration. however, beyond this concentration de-binding occurred

In the next set of experiments the nature of inhibition was evaluated by examining the substrate saturation kinetics of the AChE in the presence of IC33 and IC67 concentrations of the three antimalarials. The typical plots are shown in Fig 3. The data were then analyzed by Lineweaver-Burk plot method to find out the nature of inhibition and the effect of antimalarials on the Km and Vmax.

The typical substrate saturation curve and the corresponding Lineweaver-Burk, Eadie-Hofstee and Eisenthal Cornish-Bowden plots for uninhibited enzyme are shown in Fig 4 Consistent with our previously reported values the enzyme activity resolved in two components and the Km and Vmax values matched with our earlier observation (10).

Fig 5 shows the typical Lineweaver-Burk plots obtained in the presence of three antimalarials As can be noted that even under these conditions the enzyme activity resolved in two components

In order to better illustrate the nature of inhibition the data were re-plotted taking into consideration the points corresponding to component I and component II. These Lineweaver-Burk plots are shown in Fig. 6. The computed values of the Km and Vmax are given in Tables 3-5 Thus increasing concentrations of quinine resulted in progressive decrease in the Vmax of both the components, while the Km increased by 2.5 and 12 fold (Table 3) With chloroquine also a similar trend for Vmax could be noted However,

Fig 3 Substrate saturation plots for AChE in the presence of (A) Quinine, (B) Chloroquine and (C) Primaquine. The experimental details are as given in the text. The results are typical of 8 independent observations for each group

Fig. 3

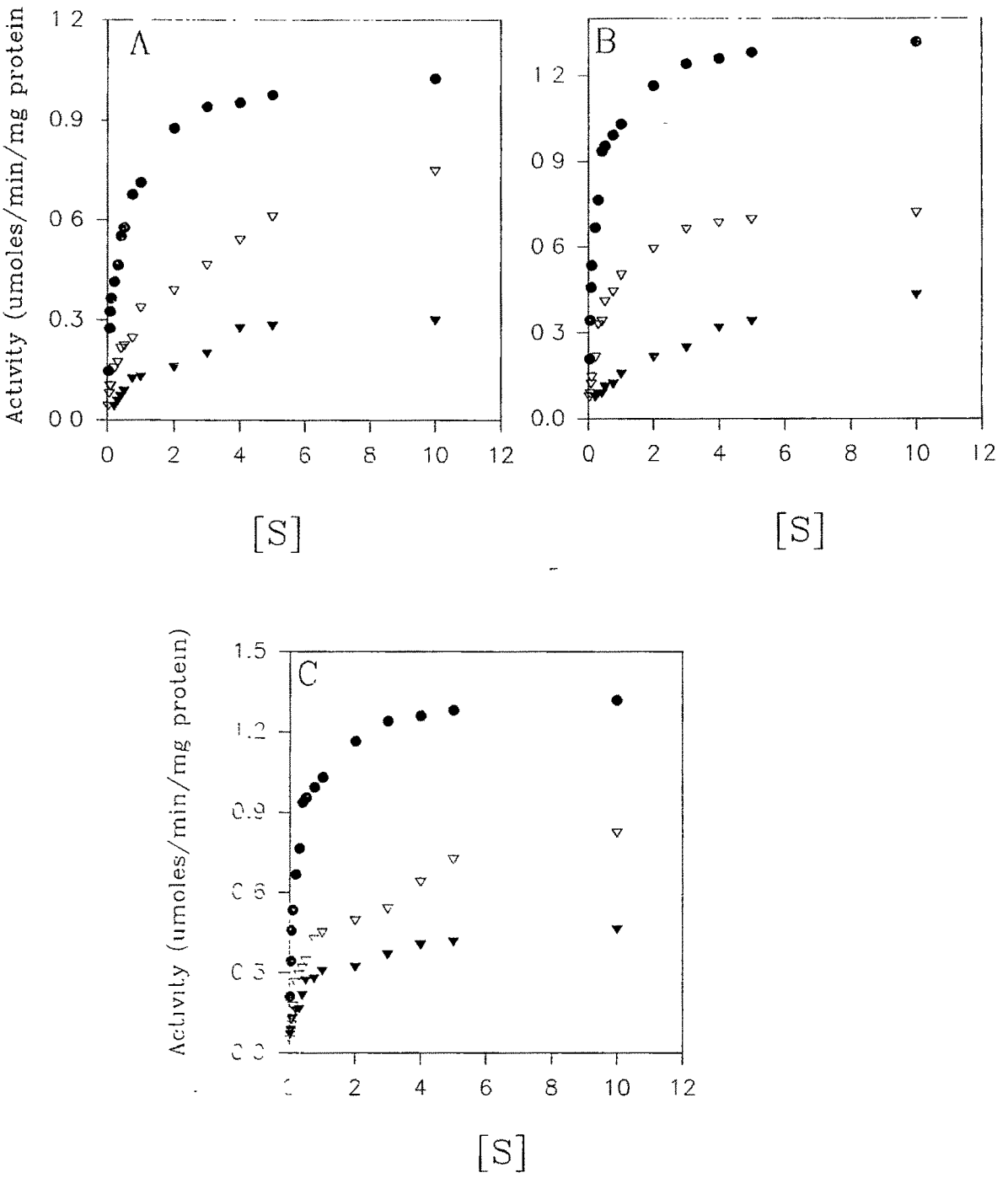

A
B
C
Activity (umoles/min/mg protein
[S]
0
2
4
6
8
10
12
0.0
0.3
0.6
0.9
1.2
1.5

Fig. 4 Substrate kinetic analysis of uninhibited AChE (A) substrate saturation, (B) lineweaver-Burk plot, (C) Eadie-Hofstee plot and (D) Eisenthal Cornish-Bowden plot. The results are typical of 8 independent observations for each group.

Fig.4

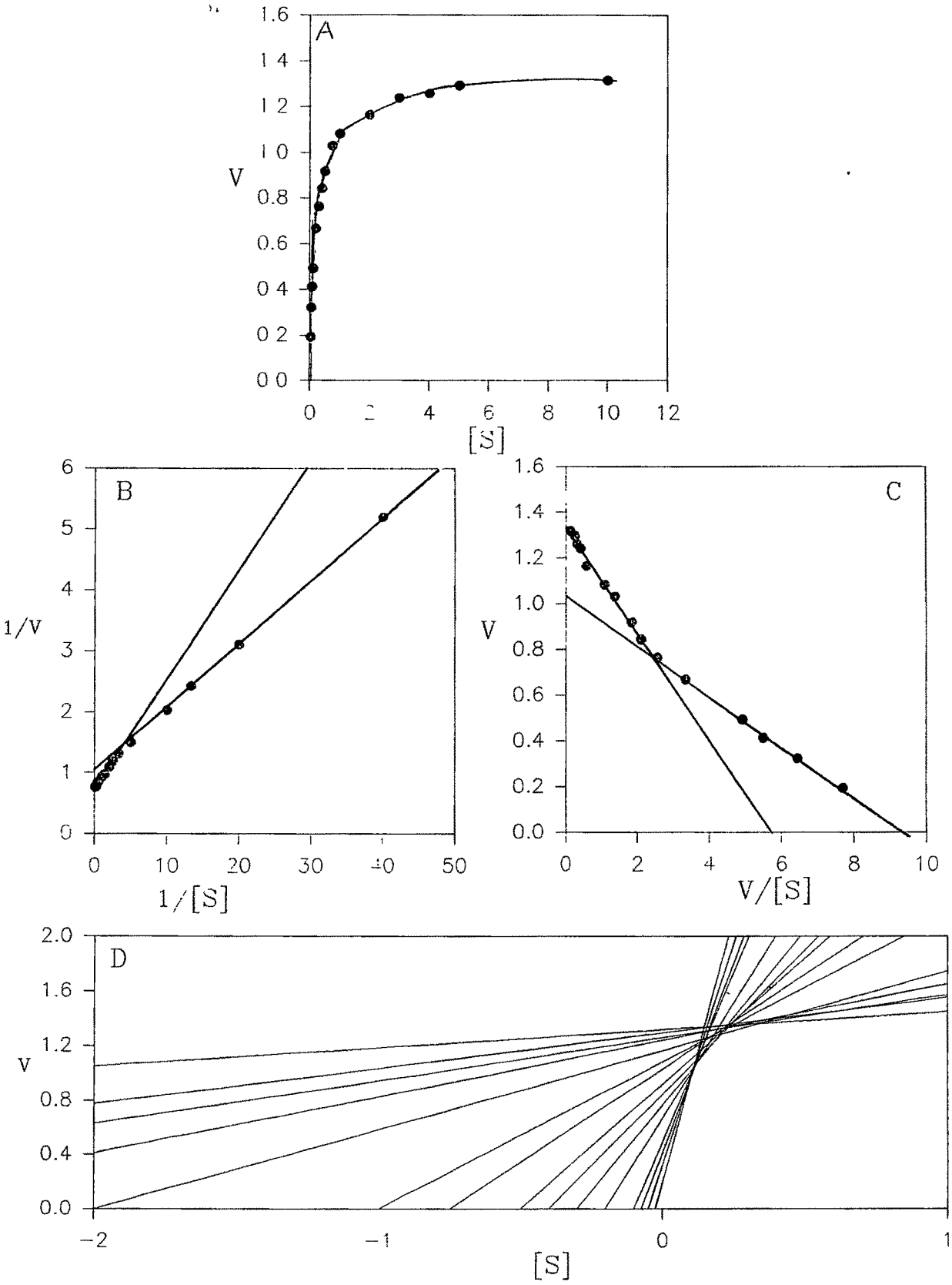


Fig. 5 Lineweaver-Burk plots of AChE in the presence IC33 and IC67 concentration of Quinine(A,B), Chloroquine (C,D) and Primaquine (E,F). The results are typical of 8 independent observations for each group

Fig. 5

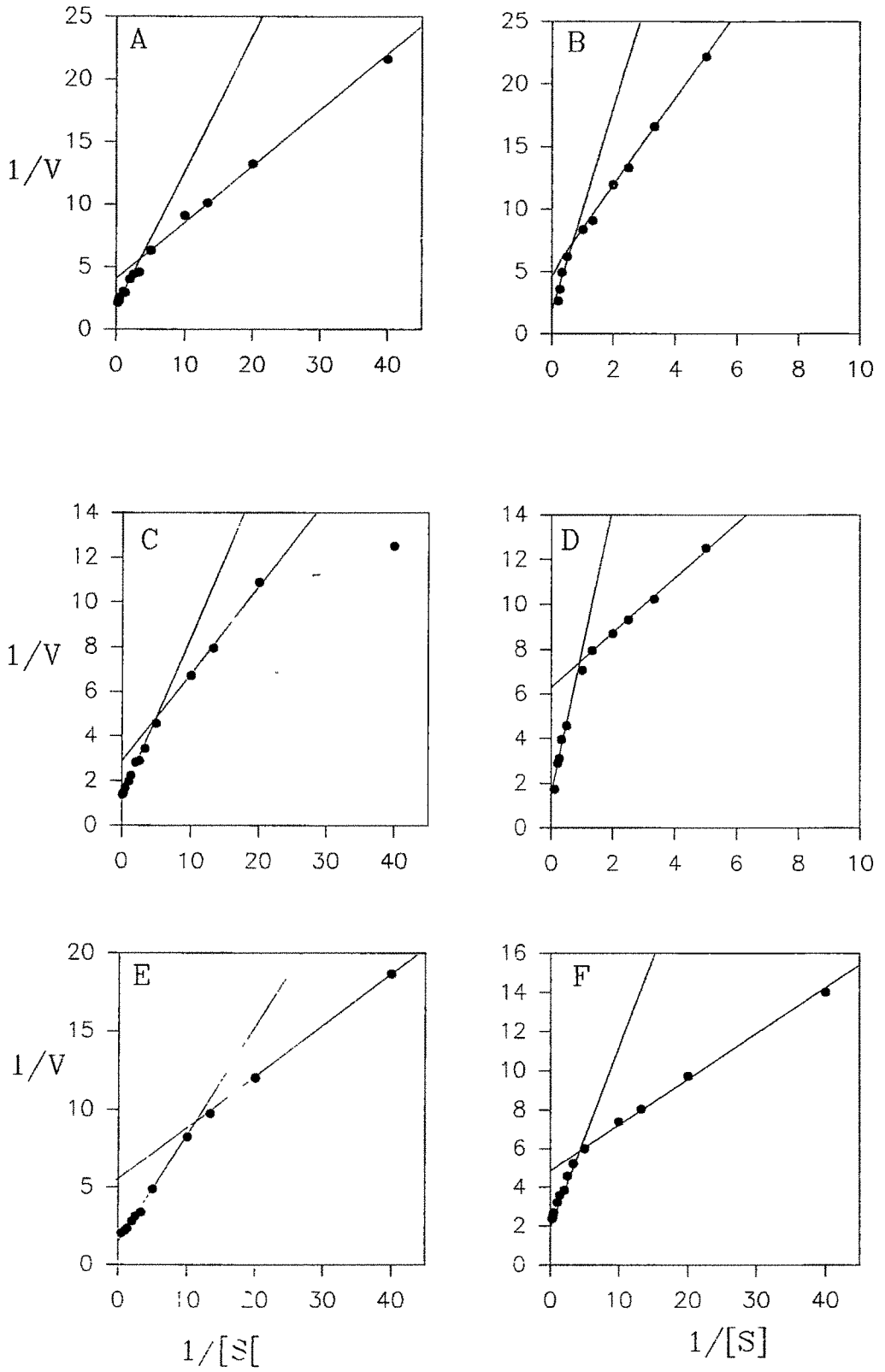


Fig. 6 Lineweaver-Burk plots for component I (A,C,E) and component II (B,D,F) of AChE in the presence of IC33 and IC67 concentration of Quinine(A,B), Chloroquine (C,D) and Primaquine (E,F) --■-- represents the uninhibited enzyme, --Δ-- represents the IC33 and --▲-- represents the IC67 group. The results are typical of 8 independent observations for each group.

Fig..6

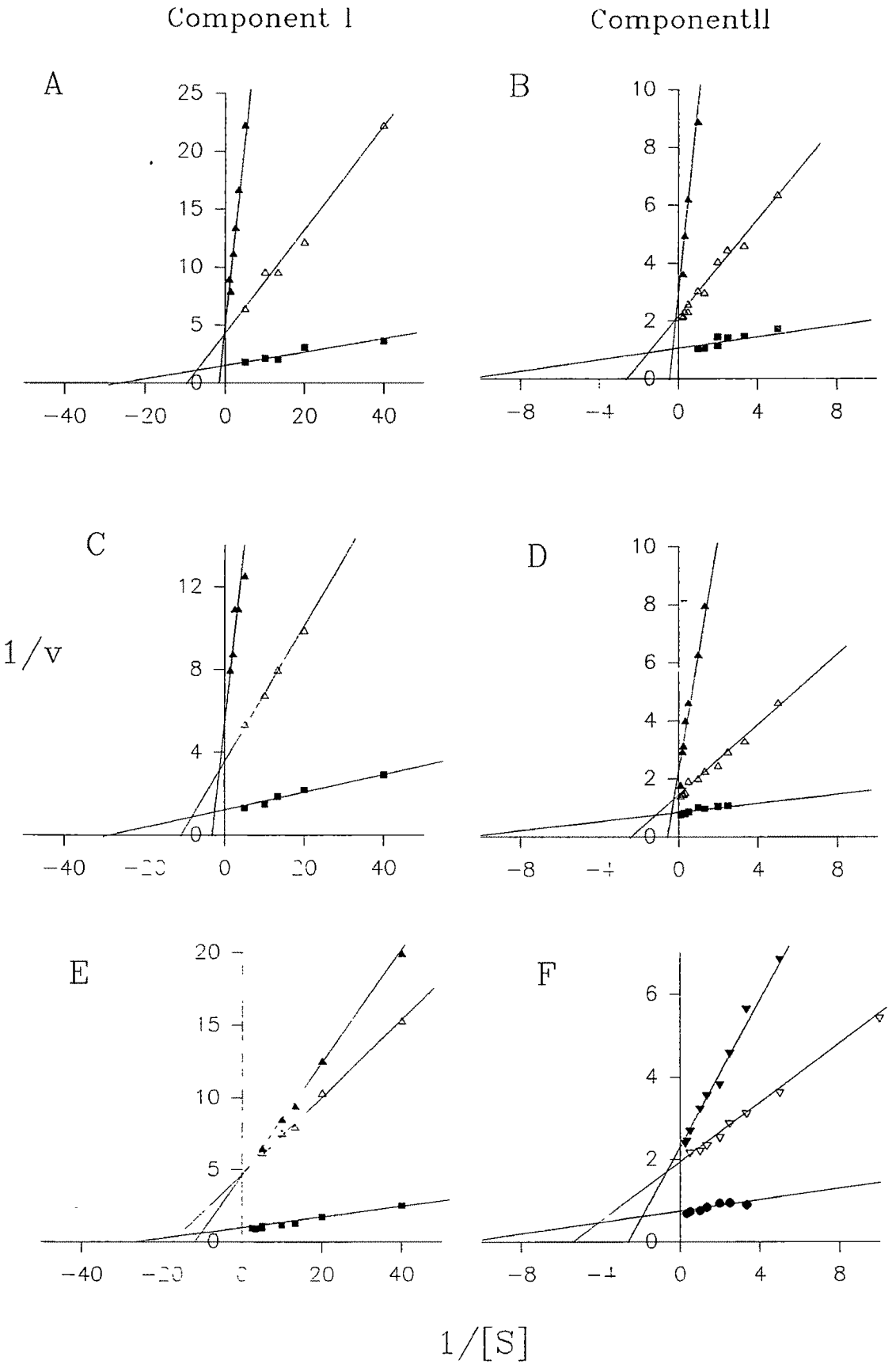

Component I
ComponentII
A
B
C
D
E
F
1/v
1/[S]
25
20
15
10
5
0
−40
−20
0
20
40
10
8
6
4
2
−8
−4
4
8
12
20

Table 3 Effect of quinine on substrate kinetics of AChE.

Enzyme	Component I		Component II	
	Km	Vmax	Km	Vmax
Uninhibited enzyme	0.05±0.01	0.69±0.09	0.21±0.02	1.11±0.11
+Quinine IC33	0.28±0.05	0.13±0.03	0.79±0.17	0.79±0.11
+Quinine IC67	0.67±0.02	0.25±0.06	2.70±0.35	0.45±0.04

Experimental details are as given in text. When quinine effect was studied the membranes were pre-incubated with two different concentrations of quinine for 2 min.
Km=μM;
Vmax is given as μmoles/min/ mg protein.
Km and Vmax values were calculated by three different methods and averaged as described in the text.
The results are given as mean ± SEM of eight independent observations.

Table 4 Effect of chloroquine on substrate kinetics of AChE.

Enzyme	Component I		Component II	
	Km	Vmax	Km	Vmax
Uninhibited enzyme	0.05±0.01	0.69±0.09	0.21±0.024	1.11±0.11
+Chloroquine IC33	0.10±0.02	0.28±0.04	0.58±0.07	0.69±0.04
+Chloroquine IC67	0.35±0.10	0.18±0.02	1.75±0.10	0.46±0.02

Experimental details are as given in text. When chloroquine effect was studied the membranes were pre-incubated with two different concentrations of chloroquine for 2 min.
Km=μM;
Vmax is given as μmoles/min/ mg protein.
The Km and Vmax values were calculated by three different methods and averaged as described in text.
The results are given as mean ± SEM of eight independent observations.

Table 5 Effect of primaquine on substrate kinetics of AChE.

	Component I		Component II	
Enzyme	Km	Vmax	Km	Vmax
Uninhibited enzyme	0.05±0.01	0.69±0.09	0.21±0.02	1.11±0.11
+Primaquine IC33	0.08±0.02	0.22±0.03	0.36±0.04	0.53±0.04
+Primaquine IC67	0.10±0.03	0.17±0.03	0.59±0.07	0.44±0.04

Experimental details are as given in text. When primaquine effect was studied the membranes were pre-incubated with two different concentrations of primaquine for 2 min.
Km=μM;
Vmax is given as μmoles/min/ mg protein.
The Km and Vmax values were calculated by three different methods and averaged as described in text.
The results are given as mean ± SEM of eight independent observations.

the Km values increased by 1.8 and 2.8 fold (Table 4) Interestingly, for primaquine the Km values were least effected. The increase amounted to 1.4 and 2.8 folds.

From the Lineweaver-Burk plots shown in Fig 5 and the data in Tables 3-5, it was apparent that with all the antimalarials the inhibition was of mixed type. This type of mixed inhibition is characterized by decrease in Vmax, increase in Km and intersection above the plane (11). Taking into consideration these observations Ki of this type of inhibition was calculated by employing the equation as described in the Materials and Methods The data on the Ki values for the two components are given in Table 6. The Ki for chloroquine was the lowest while the Ki for quinine was of the highest magnitude. Interestingly, the Ki values for both the components were comparable (Table 6).

Finally, the inhibition data in Fig 3 were analyzed to find out if the presence of inhibitor affected the binding of the substrate molecules to the enzymes. The typical Hill plots are shown in Fig 7. As can be noted, in the uninhibited enzyme 1 substrate molecule was bound till 1mM concentration of the substrate; at higher concentration two substrate molecules were bound. Quinine resulted in binding of three substrate molecules beyond substrate concentration of 2.7 mM Chloroquine had no effect on binding pattern, as was the case with primaquine

Discussion

The two cholinesterases AChE and BuChE are known to differ with respect to their substrate specificity and substrate inhibition characteristics (15). Besides, the two enzymes are inhibited by specific inhibitors (15,16). The inhibitors broadly fall in two

Table 6 The Ki values of antimalarials for AChE.

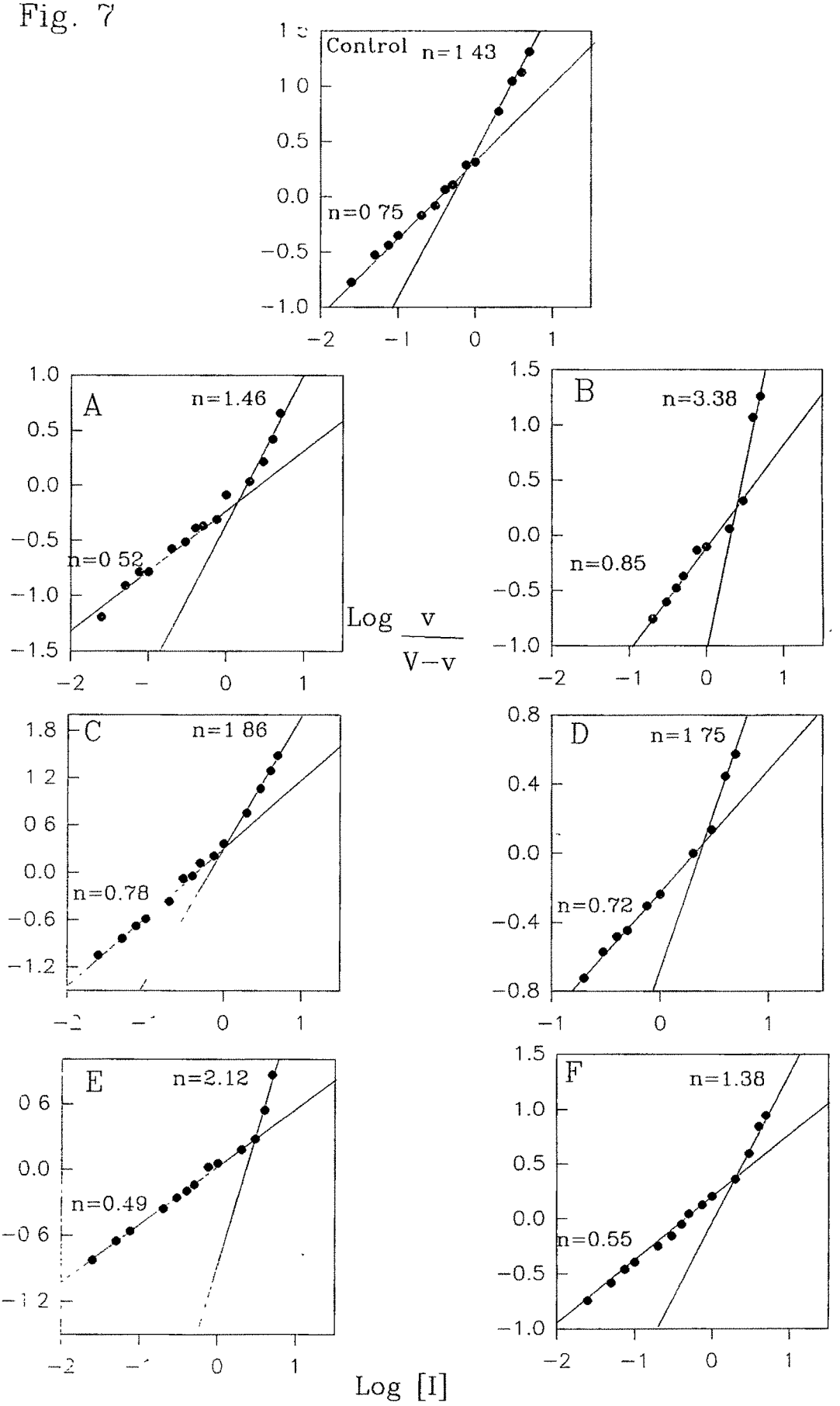
	Ki, uM	
Antimalarial	Component I	Component II
Quinine	594.5±119.8	742.6±148.7
Chloroquine	9.3±1.3	9.8±1.4
Primaquine	30.9±5.5	34.7±5.7

The Ki values were calculated by using the equation given in text.

The results are given as mean ±SEM of eight independent observations.

Fig 7 Hill plot analysis of the data presented in Fig. 4. Quinine (A) IC33, (B) IC67; Chloroquine (C) IC33, (D) IC67 and Primaquine (E) IC33 and (F) IC67. n is the Hill's coefficient The experimental details are as given in the text and in Fig 4

Fig. 7


Control n=1 43
n=0 75
A
n=1.46
n=0 52
B
n=3.38
n=0.85
C
n=1 86
n=0.78
D
n=1 75
n=0.72
E
n=2.12
n=0.49
F
n=1.38
n=0.55
Log v / V−v
Log [I]

groups firstly those compounds which have a quaternary ammonium group or secondly those which belong to the family of organophosphorous compounds (5). Inhibition with the former is considered to be reversible while the inhibition with the later group of inhibitors is of ir-reversible type (5,17). However, reactivation of the enzyme inhibited by organophosphorous compound using suitable thiol compound or derivative of hydroxamic acid, oximes and bis-quaternary oximes has been demonstrated (18).

The antimalarials are quinoline derivatives and are known to inhibit several nicotinamide nucleotide-dependent dehydrogenases by virtue of their property to abstract electrons (19,20) The quaternary nitrogen of the quinoline ring participates in the electron abstraction (19)

The inhibition of AChE by the antimalarials in the first place would therefore seem to be a somewhat remote possibility. However, the observed inhibition can be easily explained on the basis of the basic fact that the antimalarials are quinoline derivative and the quaternary ammonium structure is a common denominator which could qualify these compounds as inhibitors.

The inhibitor specificity of AChE and BuChE has been explained in terms of the difference in the volume of the active site gorge of the two cholinesterases (16). In AChE the active site gorge contain active serine (203), histidine (447) and glutamate (334) which form a charge relay system responsible for ACh hydrolysis (15). The active site gorge is lined by 14 aromatic amino acids, which include W84, Y337 (21). The gorge is narrow having a volume of $302.21(A^{o})^{3}$ and can therefore accommodate only acetylcholine but not higher esters (16). W84 and Y 337 are part of choline binding sites

The W84, which is conserved in all cholinesterases, plays a role in the orientation and stabilization of the quaternary ammonium group of the substrate and is also responsible for releasing the substrate at the active site The Y337 is believed to stabilize binding of ligands such as huperizine A, endrophonium, acridines and one end of bisquaternary compound BW284651 (16). The inhibitors thus acts by interfering with the binding of the substrate with Y337. However, Y337 destabilizes binding of phenathiozine eg. ethopropazine, which has bulky exocyclic substitution (16). The D74 is considered to be the peripheral anionic site, which binds and directs the substrate into the active site gorge (22).

The results of the present study demonstrate that the three antimalarials inhibited the erythrocyte AChE activity in a differential manner. This could possibly relate to the structures of the three antimalarials (Chapter 1). As can be noted quinine has a bulky exocyclic substitution group, in chloroquine the side chain is located on the opposite side of the quinoline nitrogen whereas in primaquine the side chain is on the same side of quinoline nitrogen. Presumably this difference in the structure of the three antimalarials would influence their binding with the D74 residue. Such an assumption is supported by the data in Fig.1 and Table 1, showing that the IC33 and IC67 concentration were the lowest for chloroquine, intermediate for primaquine and the highest for quinine. Hill plot analysis (Fig 2 and Table. 2) indicated that the values of Hill coefficient were more or less comparable for the three antimalarials. The value in fact changed for chloroquine at higher concentrations However, it may be pointed out here that the experiments were performed with erythrocytic membrane and not with purified enzyme. Hence, it is possible that the observations in Fig.1 and Table 2 may relate to the binding of the

antimalarials to the membrane proteins also thereby masking the effect on the enzyme itself.

It has been reported that the binding of the inhibitors at D74 results in the inhibition of enzyme activity either due to the decreased accessibility of the substrate at active site gorge or due to conformational change in the enzyme (22). Additionally, it has been suggested that if the inhibitor is released in the active site gorge it can interfere with the binding of the substrate at the active site without itself getting bound at the active site (22). The acyl pocket of 2 F residues 288 and 290, which is responsible for forming a clamp around methyl moiety of acyl group may have no role in the process. Under the circumstances one may anticipate that the resulting inhibition more likely than not will be of mixed type.

The results of the present studies show that the inhibition by the three antimalarials was indeed of mixed type (Fig. 5 and 6, Tables 3-5). Nevertheless, as is to be expected the potency of the inhibitor in terms of the Ki value was differential and consistent with the observation reported in Fig 1 and Table 1. Thus Ki values for chloroquine were in the range of 9-10 μM while these values for primaquine were 3.5 fold higher; the Ki values were the highest for quinine, being in the range of 600-700 μM The observations are consistent with the structural properties of the three antimalarials referred to above.

In conclusion the results of the present study have shown for the first time that the antimalarials act as potent inhibitors of human AChE. The presence of the protein showing sequence homology with cholinesterases has been reported in protozoans e.g. Dictyostelium crystal protein and Dictyostelium D2 protein (15). It is therefore tempting

to speculate that the antimalarials might afford protection from the malarial parasite by mechanisms involving interaction with similar proteins, which showed structural homology More interesting than this is the implication that they may be exerting beneficial effects in A.D patients directly by inhibiting AChE as seen here and thereby increasing the ACh concentration.

Although several cholinesterase inhibitors such as tachrine, rivastagmine, donepazile etc are being used for management of conditions such as A.D. their side effects have become increasingly noticeable. Compared to these, side effects of the antimalarials are minimum (1,5). Therefore therapy with these drugs could open newer avenues for developing drugs which have fewer side effects and hence could be beneficial to A.D. patients.

The higher vertebrates also contain another cholinesterase i.e. BuChE which shows structural similarity and differences in active site gorge compared to AChE (15,16,23). It is therefore of interest to find out if the antimalarials inhibit even the BuChE activity. These results are described in the next chapter of the thesis.

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Summary

The effects of the antimalarials on acetylcholinesterase (AChE) were examined. It was found that the antimalarials were potent inhibitors of human erythrocyte membrane AChE Chloroquine was found to be the most effective, followed by primaquine and quinine. The IC33 concentrations for chloroquine and primaquine were 22 and 38μM whereas that for quinine was 3.2mM The concentrations required to obtain 67% inhibition (IC67) were about 9 and 7 times higher for chloroquine and primaquine while that for quinine was only about 2.5 times higher. Hill analysis revealed that chloroquine shows de-binding above 40 μM concentration. The two kinetic components of AChE were inhibited by the three antimalarials and the inhibition was of mixed type. Increasing concentrations of antimalarials cause progressive decrease in the Vmax of both the components IC33 concentrations resulted in 1.6-6 fold increase in Km of both the components while IC67 concentration caused 2.8 to 13 fold increase with maximum effect seen with quinine. The Ki values were lowest for chloroquine suggesting that it was the most potent inhibitor. These values were 3.3 and 60 times higher for primaquine and quinine