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Kinetic and substrate binding characterization of hepatic mixed function oxidase system in monkeys with primaquine and (N^1-3-acetyl-4-5-dihydro-2-furanyl)-N^4-(methoxy-8-quinolinyl) 1,4-peptane-diamine

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Recently, our Institute developed a schizontocide named CDRI compound 80/53 [(N^1-3-acetyl-4-5-dihydro-2-furanyl)-N^4-(methoxy-8-quinolinyl) 1,4-peptane-diamine] (80/53*) (Fig. 1). This is an enamine analogue of the well known tissue schizontocidal PQ. Although PQ is the first

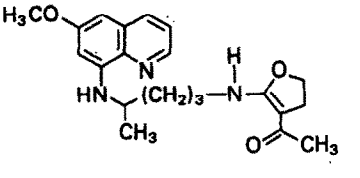

Fig. 1. Compound 80/53.

* Abbreviations: 80/53, CDRI Compound 80/53, N^1-3-acetyl-4,5-dihydro-2-furanyl)-N^4-(methoxy-8-quinolinyl)1,4-peptane-diamine; PQ, primaquine; AH, aniline hydroxylase; AND, aminopyrine-*N*-demethylase.

Table 1. Dose-dependent inhibition by 80/53 and PQ of hepatic drug-metabolizing enzymes

Parameters	Control values	80/53 (M) 10^{-3}	10^{-6}	10^{-9}	PQ (M) 10^{-3}	10^{-6}	10^{-9}
AH*	66.98 ± 8.77	32.86 ± 4.11 (50.94)	40.17 ± 2.81 (40.03)	54.08 ± 5.74 (19.26)	24.32 ± 2.73 (63.69)	37.92 ± 3.74 (43.39)	51.87 ± 3.84 (22.56)
AND†	1.86 ± 0.34	0.41 ± 0.08 (77.96)	0.76 ± 0.14 (59.14)	1.41 ± 0.34 (24.19)	0.32 ± 0.09 (82.79)	0.49 ± 0.11 (73.66)	1.44 ± 0.48 (22.58)

Values are expressed as means ± SD of three separate observations.
* Pmol of *p*-amino phenol formed/min/mg protein.
† Nmol of product formed/min/mg protein.
Values in parentheses denote percentage inhibition.

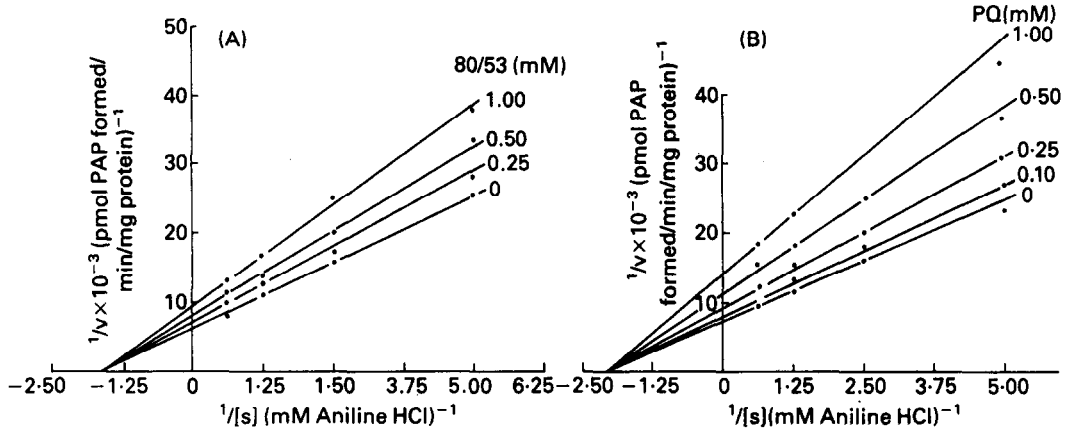

Fig. 2. Lineweaver–Burk plots illustrating the effect of 80/53 and PQ on hepatic AH activity of monkey *in vitro*. (A) 80/53 (B) PQ.

choice as a tissue schizontocide and has a wide application in curing *Plasmodium vivax*, it causes multiple side effects like dizzyness, nausea, anaemia etc. [1]. The system most severely affected by the drug is the drug metabolizing system of the host [2, 3]. Reports exist that 80/53 is less hepatotoxic than PQ [4], but no kinetic analyses or substrate binding spectral studies on mixed function oxidase system have been published. The present study reports on a kinetic analysis of cytochrome P450-specific reactions (hydroxylation and N-demethylation) and the change in substrate binding capacity of cytochrome P450 in the presence of 80/53 and PQ.

Materials and Methods

The maintenance of experimental animals and preparation of samples were essentially the same as described by Pandey *et al.* [4]. AH [5] and AND [6] were determined in 11,000 *g* supernatant while cytochrome P450 [7] was estimated in microsomal fractions.

To study the inhibition of each of the two drug-metabolizing enzymes by PQ or 80/53, 4–5 mg of enzyme protein and a complete supporting system [4] were preincubated with concentrations of the compound ranging from 10^{-3} to 10^{-9} M for 10 min before adding the substrate to start the reaction. (Control incubation mixtures containing equivalent components but with the exception of the compound were run simultaneously). Optical difference spectroscopy was employed to monitor the spectral perturbations in hepatic microsomes produced by 80/53 or PQ *in vitro* [8]. Double reciprocal plots of absorbance (peak to trough of the aniline type II spectrum) as a function of ligand concentration were constructed. Spectral dissociation constants (K_s) and maximal absorbance changes (ΔA_{max}) were calculated from *x*- and *y*-axis intercepts of the double reciprocal plots. Pooled data from three separate optical titrations, each using five ligand (aniline-HCl) concentrations were used for the calculations. Inhibitory observations were plotted according to the method of Lineweaver and Burk [9]. K_s, V_{max} and K_i values were obtained from the Lineweaver–Burk plot of the uninhibited and inhibited reactions [10], respectively.

Protein assay. Protein was determined according to the method of Lowry *et al.* [11] using bovine serum albumin as standard.

Statistical analysis. The data were analysed for statistical significance using Student's *t*-test. P values less than 0.01 were considered significant.

Results and Discussion

The present findings demonstrate that 80/53 and PQ inhibit the activities of AH and AND in a concentration-dependent manner (Table 1). The inhibition of AH activity was characterized as non-competitive since the point of intersection of the lines lies on the $1/S$ axis (Fig. 2 A and B). This observation is in agreement with the earlier findings of Sukhumanan *et al.* [12] who showed that PQ exhibited a non-competitive type of inhibition of AH activity. From the K_i values ($K_{i_{PQ}} = 0.07$ mM; $K_{i_{80/53}} = 0.13$ mM), PQ can be seen to cause more inhibition than 80/53 (Table 2). For AND the inhibition was characterized

Table 2. Kinetic parameters of AH and AND with 80/53 and PQ

	AH		AND	
	80/53	PQ	80/53	PQ
K_s (mM)	0.64 ± 0.06	0.49 ± 0.07	0.38 ± 0.05	0.59 ± 0.06
V^*_{max}	166 ± 11	139 ± 22	2.5 ± 0.12	3.5 ± 0.03
K_i (mM)	0.13 ± 0.01	0.07 ± 0.002	0.48 ± 0.04	0.31 ± 0.01

* Nmol of product formed/min/mg protein.
Values are means ± SD of three separate observations.

Table 3. Effect of compound 80/53 and PQ on substrate binding capacity of hepatic microsomal cytochrome P450 of rhesus monkey

Groupings	K_D (mM)	$A_{abs_{max}}$	% Decrease in binding efficacy
Normal	0.57 ± 0.07	6.25 ± 0.31	—
80/53 (10^{-6} M)	0.57 ± 0.07	5.26 ± 0.35	15.82
PQ (10^{-6} M)	0.57 ± 0.07	4.17 ± 0.29	33.30
80/53 (10^{-3} M)	0.57 ± 0.07	3.70 ± 0.14	40.72
PQ (10^{-3} M)	0.57 ± 0.07	3.13 ± 0.17	50.02

Data are the means ± SD of three separate observations.

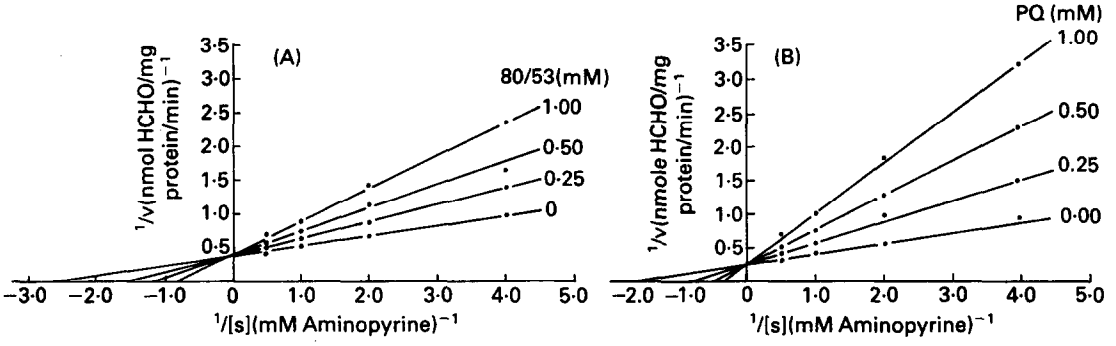

Fig. 3. Lineweaver–Burk plot illustrating the effect of 80/53 and PQ on hepatic AND activity *in vitro*. (A) 80/53 (b) PQ.

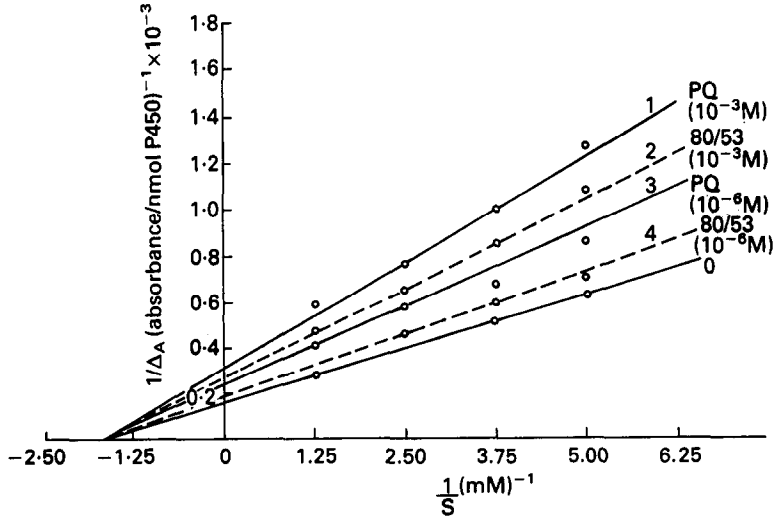

Fig. 4. Lineweaver–Burk-type plots (reciprocal aniline-HCl concentration vs reciprocal spectral changes) of the inhibition of the aniline type II spectral interaction with ferric cytochrome P450 at several fixed concentrations of 80/53 and PQ.

as competitive on the basis of fluctuating K_s values (Fig. 3 A and B). That 80/53 compares favourably with PQ is well marked ($K_{i_{80/53}} = 0.40$ mM, $K_{i_{PQ}} = 0.31$ mM). The other important aspect which is evident from the kinetic analysis is the mutual affinity of compound/drug for demethylation and/or hydroxylation reactions of cytochrome P450. The study pointed out that the affinity of 80/53 is approximately 2-fold lower while that of PQ is 5-fold greater, for P450-mediated hydroxylation reactions when compared to the affinity of substrate for the same. Likewise 80/53 and PQ have a 1.3-fold lower and 1.9-fold greater affinity, respectively, for P450 mediated N-demethylation.

80/53 and PQ display a concentration-dependent decrease in the binding capacity of cytochrome P450 (Table 3 and Fig. 4). Both the antimalarials were found to inhibit the type II binding of aniline HCl with oxidized cytochrome P450 in a non-competitive manner (Fig. 4). The K_D value (0.57 mM) remained the same and was closely related to that of K_s values of AH with 80/53 and PQ (Figs 2 and 4, Table 3), thus pointing towards non-competitive inhibition. The authors carried out type II binding because in the presence of compound/drug only type II binding could be visualized prominently. PQ is known to interact at the type II binding site thereby diminishing complex formation between aniline and P450. Strother *et al.* [13] confirmed that PQ is able to undergo a substrate type II interaction with certain isoenzymic forms of P450 (as it has been shown to be metabolized by O-dealkylation and aromatic hydroxylation in microsomal systems) [14, 15]. The percentage binding efficacy of cytochrome P450 was decreased by supplementing the system with 80/53 or PQ. However the decrease was more pronounced with PQ as compared with 80/53 (Table 3). These binding values are in accordance with the kinetic analyses and show that 80/53 compares favourably with PQ. Being an enamine analogue of PQ, 80/53 causes less inhibition in hepatic mixed function oxidase system [4] and reduced Met-hemoglobin production compared with PQ [16].

In summary, kinetic and substrate binding studies of AH, AND and cytochrome P450 with 80/53 and PQ revealed that 80/53 compares favourably with PQ and shows less interference in the biotransformation system.

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