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MECHANISTIC ASPECTS OF THE INHIBITION OF MICROSOMAL DRUG OXIDATION BY PRIMAQUINE

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Abstract—The kinetics of inhibition of microsomal drug oxidation (as aminopyrine *N*-demethylase activity) by the antimalarial agent primaquine were found to be concentration-dependent Lower concentrations of primaquine (0–40 μM) elicited slope-hyperbolic, intercept-hyperbolic noncompetitive (mixed) inhibition with an inhibitor equilibrium-dissociation constant (K_i) of 21 μM On the other hand, primaquine concentrations greater than 40 μM elicited essentially simple competitive inhibition as judged from Lineweaver–Burk and Dixon analysis with appropriate replots (K_i = 23 μM) The coincident K_i values suggest that the same enzyme–inhibitor complex is involved in inhibition over all concentrations of primaquine tested The apparent change in kinetics was accounted for in terms of a four-step interaction scheme involving a ternary enzyme–substrate–inhibitor complex that catalyses substrate oxidation at a slower rate than the binary enzyme–substrate complex Competitive inhibition reflects the likelihood that the ternary complex does not form at all, presumably due to reduced accessibility of the active site to substrate A good correlation was found between the K_i values for the inhibition of aminopyrine *N*-demethylase activity (21 or 23 μM) and the modulation of aminopyrine binding (26 μM) by primaquine.

These findings suggest that the inhibition of aminopyrine *N*-demethylase activity by primaquine is mediated *via* an interaction with the oxidised form of cytochrome P-450 In addition, the apparent change in inhibition kinetics suggests a concentration-dependent change in the capacity of primaquine to modulate substrate binding to cytochrome P-450 as well as the formation of a P-450-aminopyrine–primaquine ternary complex

The antimalarial agent primaquine (PQ; 6-methoxy-8-(4-amino-1-methylbutylamino)quinoline) is active against the exoerythrocytic forms of *Plasmodium vivax* and *Plasmodium falciparum* [1]. However, the use of PQ in the prophylaxis of malaria has been restricted by its toxic side effects, especially in those individuals with erythrocytes that are deficient in glucose-6-phosphate dehydrogenase [2]

Despite the use of PQ in humans relatively few studies have been concerned with its effects on hepatic drug metabolising enzymes. Recent studies have demonstrated that PQ interacts significantly with rat hepatic cytochrome P-450 (P-450), the principal component of the microsomal mixed-function oxidase (MFO) system [3–5]. Back *et al.* reported that PQ inhibits microsomal drug oxidation in rats both *in vitro* and *in vivo* [3]. These findings were rationalised in terms of the type II spectral binding interaction produced by PQ in microsomal fractions from untreated and phenobarbitone-pretreated rat liver [4]. It has also been shown that PQ is itself subject to oxidative metabolism *in vitro* in microsomal fractions [6] and *in vivo* in man [7] and experimental animals [8, 9]. Thus it is apparent that PQ is a substrate, as well as an inhibitor, of the microsomal MFO system.

A recent study demonstrated that a linear relationship exists between spectral binding affinity (log K_D) and potency of aminopyrine *N*-demethylase inhibition (log I_{50}) for a series of quinoline-based antimalarial drugs [4]. However, the binding constants were generally an order of magnitude lower (higher affinity) than the corresponding inhibitory data. In this report detailed spectral studies were conducted in which modulation of the aminopyrine type I interaction with ferric P-450 by PQ was studied. In addition, inhibition kinetics were evaluated over relatively wide substrate and inhibitor concentrations in order to determine a more precise mechanistic interpretation of the effects of PQ on drug oxidation.

MATERIALS AND METHODS

Chemicals Primaquine diphosphate and biochemicals were obtained from Sigma Chemical Co (St Louis, MO). All solvents and reagents were at least analytical reagent grade

Animals Male wistar rats (250–300 g) were obtained from the Institute of Clinical Pathology and Medical Research at the Westmead Hospital Rat hepatic microsomes were prepared from untreated animals as described before [10]. Microsomes were stored as frozen pellets until required for use (−20°)

Aminopyrine N-demethylase. Aminopyrine *N*-demethylase (APDM) activity in hepatic microsomal fractions from untreated rats was determined as described previously [11], except that 1 mM NADPH was used in place of an NADPH-generating system and 5 mg of microsomal protein was used in each incubation (vol 3.0 ml). The substrate (aminopyrine; AP) concentration was varied between 0 25 and 2 00 mM and the inhibitor (PQ) concentration was varied between 0 and 100 μM

Binding experiments Microsomal pellets were resuspended in 0 1 M potassium phosphate buffer containing 1 mM EDTA (pH 7.4) to a protein concentration of 1 67 mg/ml so that binding studies would more closely conform with inhibition studies The suspension was divided equally between two cuvettes and a baseline of zero light absorbance was established in an Aminco DW-2a spectrophotometer operating in the split beam mode Type I optical difference spectra elicited by aminopyrine were obtained in the presence of fixed concentrations (0–60 μM) of the modifier PQ essentially as described by Leibman *et al* [12] Apparent spectral dissociation constants (K_D) and maximal absorbance changes (ΔA_{max}) were determined from the x-axis and y-axis intercepts, respectively, of double reciprocal plots of ΔA (peak to trough) versus aminopyrine concentration (four to six different concentrations) The effects of the modifier AP on PQ type II binding to ferric P-450 was assessed in analogous fashion. In other experiments, PQ binding in reduced microsomes was evaluated after the addition of either 0 6 mM NADPH or several crystals of sodium dithionite

Cytochrome P-450 was measured by the method of Omura and Sato [13] and protein was determined by the method of Lowry *et al.* [14]

Graphical methods Inhibitory data from catalytic and binding experiments were plotted according to the method of Lineweaver and Burk [15] and, in some instances, the method of Dixon [16] Lines of best fit were calculated by least squares Replots of Lineweaver–Burk slopes and y-intercepts versus PQ concentration were also constructed in order to further characterise the apparent kinetics

In the case of APDM inhibition at low PQ concentrations, a secondary replot was constructed in which reciprocal Δ slope and reciprocal Δ intercept were both presented as a function of reciprocal inhibitor concentration [17] Δ Slope was calculated as the difference between the slopes of two lines from the initial Lineweaver–Burk plot, e g Δ Slope when PQ = 40 μM was calculated as the slope of the line when PQ = 40 μM minus the slope of the line when PQ = 0 μM Δ Intercept was derived in analogous fashion using the Lineweaver–Burk plot y-intercepts This secondary replot was used to determine values for the variables a and b and the inhibitor equilibrium constant, K_i, as follows (K_s and V_{max} were obtained from the Lineweaver–Burk plot of the uninhibited reaction)

The plot of (Δ intercept)$^{-1}$ versus [PQ]$^{-1}$ in Fig 4 has a y-intercept equal to b $V_{max}/1 - b$. This b value is then used in the calculation of a from the (Δ slope)$^{-1}$ replot y-intercept which equals b $V_{max}/K_s(a - b)$ Finally, K_i is determined from the slope of the latter replot which is equal to a K_i $V_{max}/K_s(a - b)$ [17]

RESULTS

APDM inhibition at low PQ concentrations

The general scheme presented in Fig. 1 was used to express APDM inhibition by PQ Four equilibria are involved

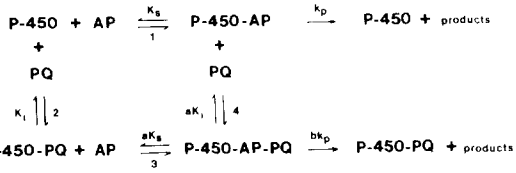

Fig 1 General scheme of the equilibria between enzyme, substrate and inhibitor in relation to the effects of primaquine on aminopyrine N-demethylase activity

$$K_s = \frac{[P\text{-}450]\,[AP]}{[P\text{-}450\text{-}AP]} \quad \text{step 1}$$

$$K_i = \frac{[P\text{-}450]\,[PQ]}{[P\text{-}450\text{-}PQ]} \quad \text{, step 2}$$

$$aK_s = \frac{[P\text{-}450\text{-}PQ]\,[AP]}{[P\text{-}450\text{-}AP\text{-}PQ]} \text{, step 3}$$

$$aK_i = \frac{[\text{P-450-AP}]\,[\text{PQ}]}{[\text{P-450-AP-PQ}]} \text{, step 4}$$

In this scheme the variable a is the factor by which K_s and K_i are altered in the presence of inhibitor (PQ) and substrate (AP), respectively The variable b is the factor by which the rate constant (k_p) of the breakdown of the enzyme–substrate complex to product is altered by the presence of PQ

Lineweaver–Burk analysis of inhibition data indicated that PQ exerts a concentration-dependent effect on APDM kinetics Lower concentrations of PQ (0–40 μM) exerted a mixed-type inhibition of microsomal APDM activity (Fig 2) The primary replots of slopes and y-axis intercepts of the Lineweaver–Burk lines versus PQ concentration were not linear (Fig 3) which indicates that the mixed-type inhibition may be more accurately expressed as slope-hyperbolic intercept-hyperbolic non-competitive Segel [17] has used a secondary replot of

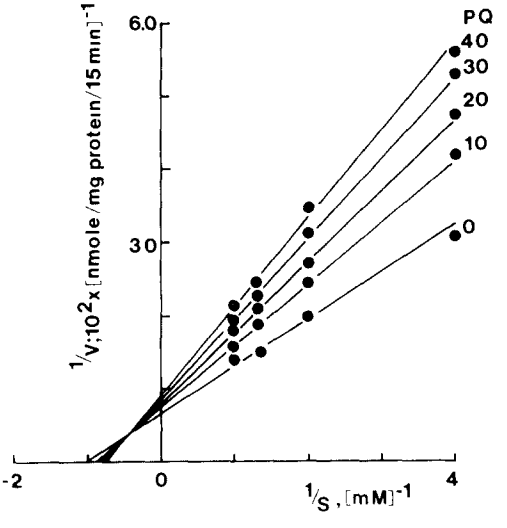

Fig 2 Lineweaver–Burk plots (reciprocal substrate concentration *versus* reciprocal reaction velocity) of the inhibition of aminopyrine N-demethylase activity at lower primaquine concentrations (between 0 and 40 μM)

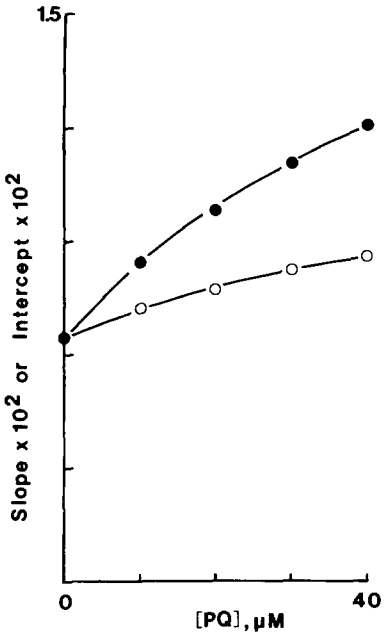

Fig 3 Primary replots of the slopes (●) and y-intercepts (○) of the lines in the Lineweaver–Burk plots from Fig 2 versus inhibitor (primaquine) concentration The non-linearity of these replots indicates hyperbolic mixed-type inhibition

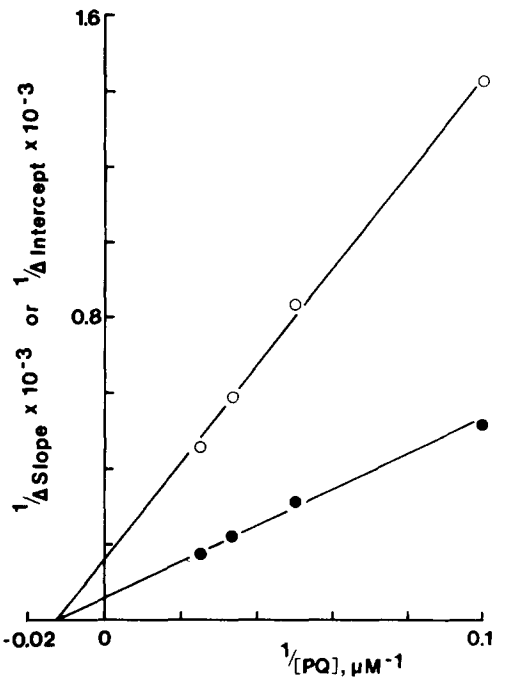

Fig 4 Secondary replots of the Lineweaver–Burk data from Fig 2 In this replot reciprocal Δ slope (●) and reciprocal Δ intercept (○) are plotted against reciprocal inhibitor concentration Δ Slope was calculated as the difference between the slopes of two lines from the initial Lineweaver–Burk plot, Δ intercept was calculated in analogous fashion Refer to Materials and Methods section for complete definition of these parameters

reciprocal Δ slope and Δ intercept versus reciprocal inhibitor concentration to obtain a linear relationship in order to determine a, b and K_i (refer to Materials and Methods for definitions). By this procedure a value of 21 μM was calculated for K_i, the inhibitor equilibrium dissociation constant (Fig. 4).

APDM inhibition at high PQ concentrations

Although lower concentrations of PQ produced a type of mixed inhibition kinetics, concentrations of 60 μM and above appeared to exhibit purely competitive inhibition. This finding was noted from Lineweaver–Burk (not shown) and Dixon analysis (Fig. 5) as well as from the primary replot (Dixon plot slopes versus reciprocal substrate concentration, Fig 6) which is a straight line through the origin This behaviour is characteristic of a simple competitive inhibition mechanism. K_i was estimated to be 23 μM from the Dixon plot intersection point, and was therefore close to that obtained from inhibition data

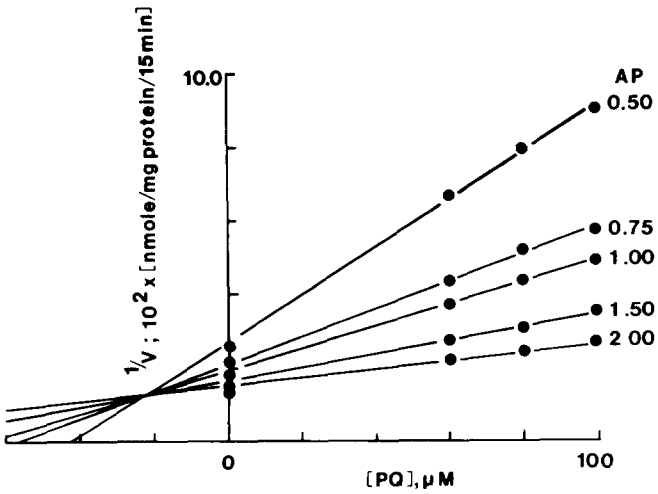

Fig 5 Dixon plots (inhibitor concentration versus reciprocal reaction velocity) of the inhibition of aminopyrine N-demethylase activity at higher primaquine concentrations (between 60 and 100 μM)

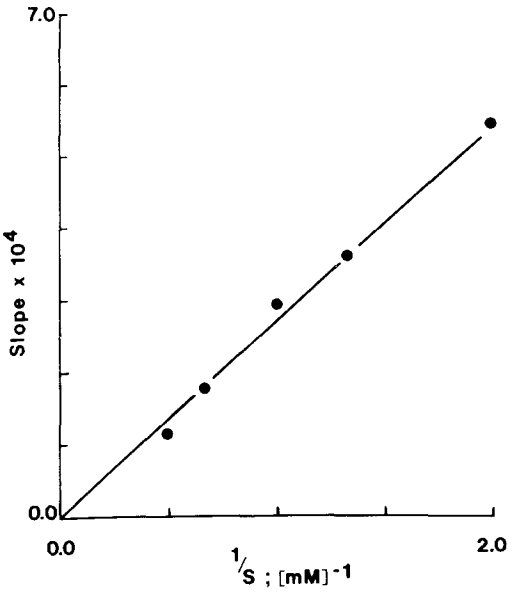

Fig 6 Primary replot of the slopes of the lines in the Dixon plots from Fig 5 versus reciprocal substrate concentration Intersection of the replot line with the origin indicates competitive-type inhibition

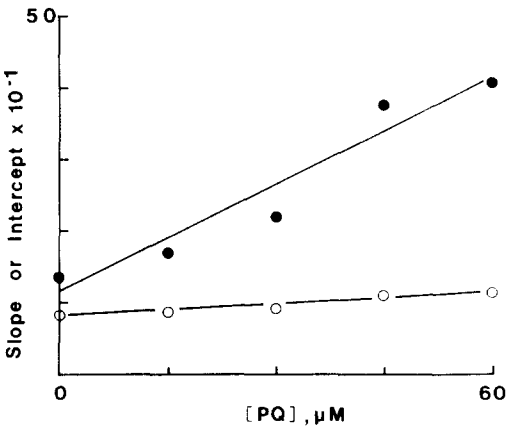

Fig 8 Primary replots of the slopes (●) and y-intercepts (○) of the lines in the Lineweaver–Burk type plots from Fig 7 versus primaquine concentration

at low PQ concentrations. This finding is not surprising, however, as K_i is simply a measure of the affinity of an inhibitor for a particular enzyme and is independent of the nature of inhibition Although the apparent inhibition kinetics are concentration dependent the finding that the two K_i values are coincident suggests that the principal enzyme-inhibitor interaction is constant over all concentrations of PQ tested.

Spectral binding studies

The capacity of PQ to affect AP type I spectral binding parameters was assessed PQ was found to modify the type I binding of AP to ferric P-450 in mixed fashion (Fig. 7). The straight line observed in the primary replot (Fig. 8) is indicative of linear noncompetitive (mixed) inhibition and, from the x-axis intercept of this plot, the K_i of the spectral interaction was determined to be 26 μM. In general terms PQ was found to decrease the affinity (increased spectral dissociation constant, K_D) and the capacity (decreased maximal absorbance change, ΔA_{max}) of the AP type I interaction with ferric P-450 Figure 9 shows these findings and also indicates that the binding efficiency parameter ($\Delta A_{max}/K_D$) [18] is greatly decreased in the presence of PQ

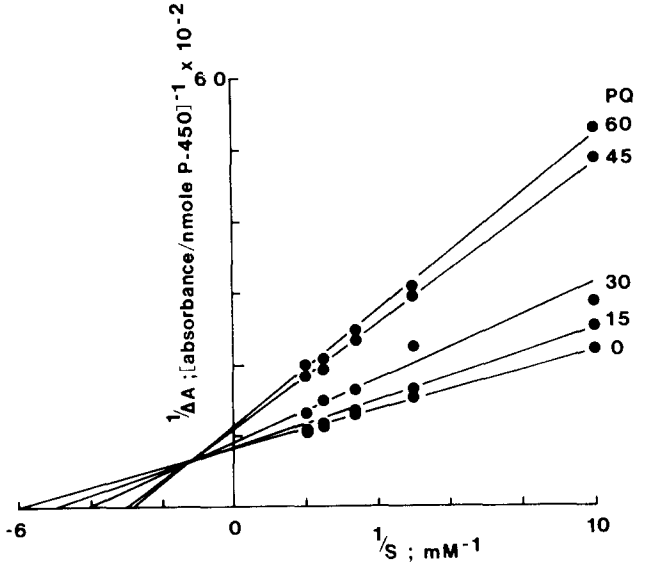

Fig 7 Lineweaver–Burk type plots (reciprocal aminopyrine concentration versus reciprocal spectral change) of the inhibition of the aminopyrine type I spectral interaction with ferric cytochrome P-450 at several fixed concentrations of primaquine

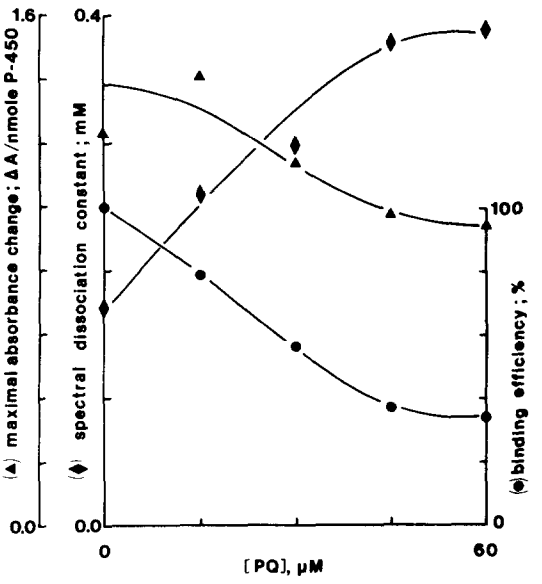

Fig 9 Changes in binding parameters of the aminopyrine type I spectral interaction with ferric cytochrome P-450 in the presence of several fixed concentrations of primaquine

The substrate AP had a much less pronounced effect on the binding of the inhibitor PQ to ferric P-450. From Fig. 10 it is apparent that only a slight decrease in both K_D and ΔA_{max} for the type II interaction of PQ occurred in oxidised microsomes. Indeed even 2 mM AP failed to significantly decrease the binding efficiency of PQ in these fractions.

Interaction of PQ with reduced P-450

The reduction of P-450 in microsomal suspensions with sodium dithionite was found to abolish the type II optical difference spectrum that PQ elicits in oxidised microsomes (not shown). On the other hand, 0.6 mM NADPH produced a time-dependent decrease in the ΔA (peak to trough) of the PQ type II spectrum. After the apparent minimum (ΔA = 0.06 absorbance units per nmole P-450) was reached the spectral trough shifted to ~424 nm (from the type II trough position near 400 nm) and intensified These changes could be accounted for by the exhaustion of NADPH in the sample cuvette and the superimposition of a residual PQ type II spectrum (in the sample cuvette) with an inverted cytochrome b_5 redox spectrum (in the reference cuvette). Exhaustion of NADPH occurred more rapidly with higher concentrations of PQ which therefore implies its utilisation in the metabolism of PQ. However, it is not possible to rule out a direct effect of PQ on NADPH oxidation such as that reported elsewhere [19].

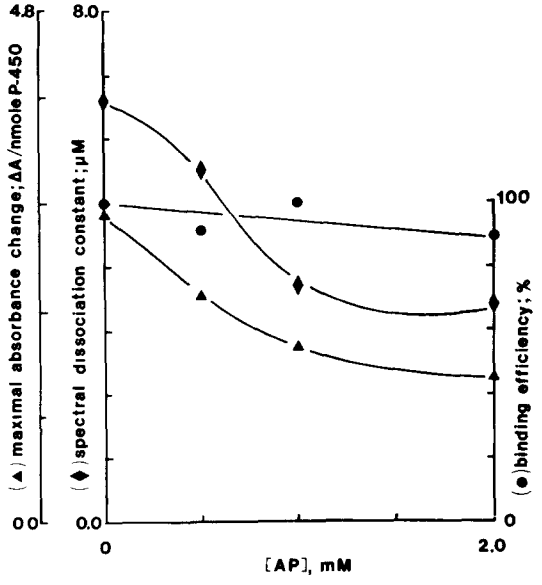

Fig 10 Changes in binding parameters of the primaquine type II spectral interaction with ferric cytochrome P-450 in the presence of several fixed concentrations of aminopyrine

DISCUSSION

The kinetics of APDM inhibition by PQ were found to be dependent upon inhibitor concentration At low PQ concentration the kinetics of inhibition were slope-hyperbolic intercept-hyperbolic noncompetitive (a specific type of mixed inhibition). Between 60 μM and 100 μM PQ the apparent kinetics became essentially competitive. These findings can be rationalised in terms of the general inhibition scheme presented in Fig. 1 The calculated value of a in Table 1 indicates that the dissociation constants of the P-450-AP (K_i = 0.81 mM) and P-450-PQ (K_i = 21 μM) complexes are increased by a factor of 2.05 (decreased affinity) in the presence of inhibitor and substrate, respectively. The resultant ternary complex (P-450-AP-PQ) yields product but, as b = 0.54 (Table 1), this occurs at only 54% of the rate of product formation from P-450-AP. A change to competitive inhibition would result if the value of a became very much larger than 2. Such a change would have the effect of preventing the formation of a ternary complex and, consequently, b = 0 Presumably, therefore, high concentrations of PQ (≥60 μM) are capable of producing this change in the affinity of substrate for the P-450-PQ complex, with the result that the steps 3 and 4 become essen-

Table 1 Kinetic parameters of aminopyrine *N*-demethylase activity and its inhibition by primaquine

Aminopyrine *N*-demethylase activity	
K_s	0 81 mM
V_{max}	136 nmole formaldehyde/mg protein/15 min
Inhibition at low primaquine concentrations	
a	2 05
b	0 54
K_i	21 μM
aK_i	43 μM
aK_s	1 7 mM
Inhibition at high primaquine concentrations	
K_i	23 μM
Inhibition of aminopyrine type I interaction by primaquine	
K_i	26 μM

tially inconsequential and the scheme in Fig. 1 reduces to a two-step process (of competitive inhibition)

In mechanistic terms the apparent change in the kinetics of inhibition of APDM activity must reflect a concentration-dependent change in the nature of PQ binding within the active centre of P-450. It is widely held that type II ligands, including PQ, have an affinity for the sixth axial co-ordination position of the haem iron of ferric P-450 [20, 21] Type II binding is associated with a shift in the spin equilibrium of oxidised P-450 to the low spin component [22]. In contrast, substrates of the MFO system are usually type I compounds [20] This interaction, seen as an increase in absorbance near 390 nm and decrease in absorbance near 415 nm in the optical difference spectrum, reflects a shift in the spin equilibrium favouring the high spin form of ferric P-450 [23] This spin state is more readily reduced by an electron from NADPH via NADPH-cytochrome *c*(P-450) reductase, and is therefore associated with accelerated drug oxidation [24]

Several authors have suggested that multiple binding sites may exist at the catalytic centre of P-450 [25–27]. Within the catalytic centre the type II site, type I site, and other hydrophobic regions that may function in the binding of some substrates, are in close proximity Consequently, few inhibitors are small enough to interact exclusively at the type II site without influencing, or even overlapping, the substrate binding regions In the present situation PQ is known to at least interact at the type II site At low concentrations it is likely that the PQ will distribute to P-450 proteins that are not active in the *N*-demethylation of AP, as well as to those isozymes that catalyse APDM activity Therefore it is possible that AP still has some capacity to bind within the active centre of P-450 prior to metabolism In this way the formation of a ternary complex is feasible and, indeed, is consistent with the kinetic analysis. At high PQ concentration it is apparent that the ternary complex does not form, presumably as a consequence of reduced accessibility of the substrate binding site to AP Certainly PQ has a much greater affinity for binding to P-450 (K_D = 6.6 μM) than AP (K_D = 0.17 mM) and the competitive binding studies in Figs 9 and 10 indicate the preferential binding of the antimalarial agent to the oxidised cytochrome Thus it is very likely that high concentrations of PQ preclude effective substrate binding to P-450 An additional possibility is that PQ could bind to P-450 in other than type II fashion when it is present at high concentration. In fact PQ must be able to undergo a substrate interaction with certain isozymic forms of P-450 as it has been shown to be metabolised by *O*-dealkylation and ring hydroxylation in microsomal systems [6]

The data in the present study strongly suggest that the effectiveness of PQ as an inhibitor of microsomal MFO activity resides in its capacity to interact with oxidised P-450 Inhibition of APDM activity and AP binding to P-450 led to the K_i estimates of 21 (or 23) and 26 μM, respectively That is, the closeness of these two values suggests that inhibition of metabolism is a direct consequence of the modulation of substrate binding No evidence was found for an interaction between PQ and reduced P-450 either by a reversible ligand mechanism such as that observed for isocyanides [28, 29] or after NADPH-mediated formation of a metabolite complex with P-450 such as those observed for SKF 525-A congeners [30, 31] and methylenedioxyphenyl derivatives [32–34]

A number of studies have demonstrated linear correlations between structure and either MFO inhibition potency or P-450 binding affinity [4, 11, 34, 35] Fewer studies have successfully correlated MFO inhibition potency and P-450 binding affinity For example, Dickins and Bridges [36] found that, in an homologous series of 2-*n*-alkylbenzimidazoles, inhibition potency towards MFO activity increased to about the 2-*n*-heptyl derivative In contrast, spectral binding behaviour was less predictable Lower homologues elicited reverse type I spectra with millimolar spectral dissociation constants whereas the higher homologues elicited high affinity (~1 μM) type I or mixed type I/reverse type I spectral changes. Hence no definitive conclusions could be drawn regarding the relationship between MFO inhibition and binding to P-450 As pointed out by these workers, it is possible that binding to various sites within the catalytic centre of the enzyme could occur, and therefore studies of the type described in the present paper may prove useful In view of the present findings it may eventually be possible to relate the concentration-dependent change in the kinetics of MFO inhibition by PQ to the propensity of the antimalarial to undergo oxidative metabolism

The present study also suggests that the capacity of an inhibitor to influence substrate binding to P-450 could well be a more reliable indicator of inhibition potency than binding of the inhibitor in the absence of substrate Another advantage of this approach is that problems resulting from the binding of an inhibitor to more than one region in the P-450 active centre, or a change in apparent spectral binding characteristics along an homologous inhibitor series, may be obviated

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