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PROTEIN LIGAND INTERACTIONS. PART 5: ISOQUINOLINE ALKALOIDS AS INHIBITORS OF ACETYLCHOLINESTERASE FROM *ELECTROPHORUS ELECTRICUS*

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Kinetic analysis has shown that papaverine, berberine and isoquinoline alkaloids act as reversible competitive inhibitors of acetylcholinesterase with respect to the substrate, acetylthiocholine chloride. The inhibitor constants (Ki) vary from 3.5 μM to 88 μM. With time they act as irreversible covalent inhibitors with papaverine producing 85% inactivation after 40 min. Pseudo first-order kinetics are observed with the rate constant being proportional to the concentration of the ligand and the order of reaction being equal to one. Spectrophotometry was used to study the binding of the ligands with acetylcholinesterase and Scatchard analysis used to calculate the respective dissociation constants and the number of binding sites.

KEY WORDS: Acetylcholinesterase, competitive inhibition, isoquinoline alkaloids

INTRODUCTION

The principal biological role of acetylcholinesterase (AChE) is the termination of nerve impulse transmission by hydrolysis of acetylcholine and subcellular fractionation and cytochemical studies indicate that the enzyme is associated with the surface membrane of the excitable cell. The precise relationship of AChE to the excitable membrane is, however, not clearly understood and various studies indicate that a significant part of the enzyme is not tightly associated with the plasma membrane.[1]

Studies of the properties of AChE are important, not only for elucidating the mechanisms of action of the enzyme but also for clarifying the nature of its association with the excitable membrane, a necessary condition for understanding the way AChE fulfils its biological role.

The existence in excitable tissue such as nerve, muscle and electrogenic tissue of nicotinic acetylcholine receptor (nAChR) and AChE both with an affinity for acetylcholine raises the question as to whether, and to what extent, the available neurotransmitter is shared between the two macromolecules. With the exception of polypeptide neurotoxins all ligands of the enzyme are also ligands of the receptor.

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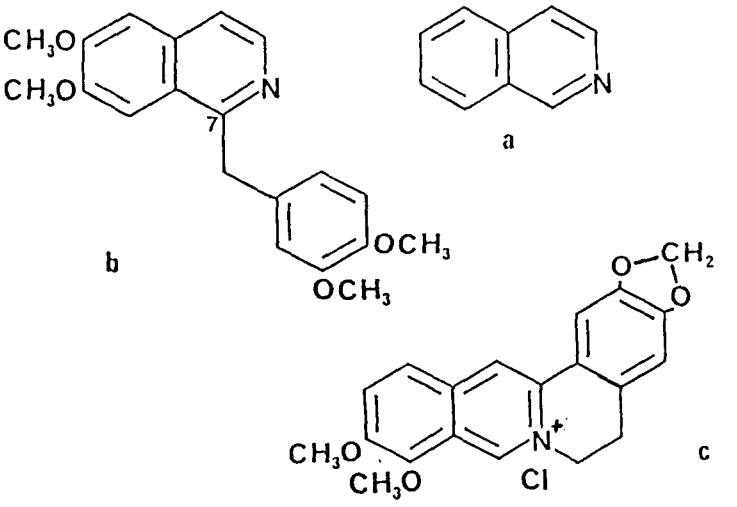

FIGURE 1 (a) Isoquinoline (b) Papaverine (c) Berberine.

The ligands of the enzyme can be subdivided into substrates and competitive inhibitors while those of the receptor can be subdivided into agonists and antagonists (competitive blockers). Since it had been found in these laboratories that the isoquinoline alkaloids acted as agonists to nAChR it was reasonable to suppose that they would interact with the enzyme as well. A detailed description of the interaction of these molecules with the enzyme might provide insights not only into the molecular mechanism of functional excitation and permeability change, but also into the principles on which more complex neural functions are based. With this in mind we have undertaken a systematic investigation into the kinetics of interaction and binding of various isoquinoline alkaloids including papaverine and berberine with AChE (Figure 1).[2]

Binding and kinetic studies critically depend on the specificity of the ligands applied and the properties of the observed changes in optical properties. Careful selection of the ligands and a study of the kinetics and changes in optical properties observed upon their binding to the enzyme may provide information on the basic principles of intracellular communication and on the functional properties of integral membrane proteins.

The purpose of the present work is to use a ligand/inhibitor with one or more features present that mimic the key parameters of enzyme-inhibitor function. Such a molecule must (a) fit the active site of the enzyme; (b) have a rigid and fixed conformation; (c) possess a quaternary nitrogen to simulate the natural substrate (acetylcholine); (d) possess additional functional groups to act as electron donors for specific amino acids on the enzyme.

MATERIALS AND METHODS

Materials

Sephacryl S-400 was purchased from Pharmacia; bovine serum albumin, acetylcholine, acetylthiocholine chloride, and 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) were obtained from Sigma. Alkaloids were bought from Aldrich Chemical Co., and dialysis membranes obtained from Spectropor. All other inorganic and buffer materials were of reagent grade. Acetylcholinesterase type III, salt free, 1000 U mg^{-1} lyophilisate from *Electrophorus electricus* was purchased from Boehringer-Mannheim.

Methods

Spectrophotometric Analysis. Spectrophotometric measurements were made using a Shimadzu UV-160A spectrophotometer. All titrations were carried out at 20°C in 0.1 M phosphate buffer (pH 7.2).

For studies involving binding of the alkaloids to the enzyme increasing concentrations of ligand (0–45 μM) were added to a sample of enzyme (12 $ng.ml^{-1}$). Absorbances were monitored at respective wavelengths and concentrations of the alkaloids obtained using $E_{220}=6.31\times10^4$ $M^{-1}.cms^{-1}$ (isoquinoline, $M_R=129$); $E_{240}=7.4\times 10^4$ $M^{-1}.cms^{-1}$ (papaverine, $M_R=376$); $E_{263}=5\times10^4$ $M^{-1}.cms^{-1}$ (berberine, $M_R=353$).

The data were used to determine the dissociation constant (Kd) of the ligands with the complexes formed with the enzyme. The results were analysed according to Equations (1) and (2).

$$Kd/(1-\$) = (L_t)/\$ - p(A)_t \quad (1)$$

$$\$ = \Delta A/\Delta A_{max} \quad (2)$$

where $(A)_t$ is the total concentration of acceptor in the system; p is the total number of binding sites; \$ is the fractional occupancy of total acceptor sites by ligand; ΔA is the change in absorbance in the presence of a known amount of ligand; ΔA_{max} is the change in absorbance at full saturation with ligand; and (L_t) is the total concentration of ligand.

Acetylcholinesterase Assay. Hydrolysis of acetylthiocholine by acetylcholinesterase was recorded at 410 nm and room temperature following the method according to Ellman.[3] The assay mixture (3.14 ml) contained phosphate buffer (0.1 M, pH 8), buffered Ellman's reagent (5,5′-dithiobis-2-nitrobenzoic acid) (10 mM), $NaHCO_3$ (17.85 mM, pH 7.0), and acetylthiocholine chloride (200 μM). Aliquots were made up to 3.12 ml with phosphate buffer (pH 8) and the reaction was initiated by the addition of 0.02 ml of acetylcholinesterase. The enzyme concentration was determined using an absorption coefficient ($A_{1\,cm}$) of 16.1 at 280 nm and a M_R of 260,000.[4]

Irreversible and reversible inhibition of acetylcholinesterase by incubation with various isoquinoline alkaloids was studied in the presence of phosphate buffer (0.1 M,

pH 8), acetylthiocholine chloride (0–250 μM) and ligand (0–40 μM, reversible inhibition, 0–100 μM, irreversible inhibition). Aliquots (3.12 ml) of such reaction solutions were initiated by the addition of enzyme (0.02 ml).

The test of the reversibility of the interaction was carried out by removal of the ligand by dialysis. A mixture of the alkaloid (20 μM) and the enzyme preparation (0.2 ml) was incubated at room temperature for 1 h. The solution was dialysed against deionised water at 4°C, pH 7.5 for 48 h. The enzyme activity was determined at the beginning of dialysis and after 24 h and 48 h.

Equilibrium Dialysis. These experiments were carried out as suggested by McPhie.[5] Routinely, acetylcholinesterase (6 $\mu g.cm^{-3}$, 3.0 ml) was dialysed at 4°C against phosphate buffer (20.0 mM, pH 7.6) containing ligand (0–40 μM) under constant agitation for 16 h. After equilibrium the concentration of ligand outside the dialysis bag was determined. Data were analysed according to Scatchard[6] (Equation 3).

$$\nu(L_f) = p - \nu/Kd \tag{3}$$

where ν is the concentration of bound ligand; p is the number of binding sites per mole; (L_f) is the concentration of free ligand; and Kd is the dissociation constant of ligand-enzyme complex.

RESULTS

Spectrophotometric Studies

Typical binding curves and the analysis as a plot of 1/(1 – $) versus (Ligand)/$ are shown for the effect of the alkaloids on acetylcholinesterase (Figure 2). From the plots the dissociation constant (Kd) for a single homogeneous class of binding sites for the alkaloids can be estimated and represented (Table 1). In each case the number of binding sites was 2 (Figure 2). Papaverine had a Kd of 18 μM, the simple isoquinoline showed a value of 79 μM while the pentacyclic more rigid structure of berberine appeared to bind relatively tightly with a Kd value of 4.6 μM.

Equilibrium Dialysis

As seen in Figure 2 the interaction of the alkaloids with acetylcholinesterase has all of the characteristics of a reversible equilibrium and this equilibrium has been characterised by equilibrium dialysis. The saturation binding curves and Scatchard analyses[6] of these results indicates that the enzyme contains a single class of binding sites (Figure 3). The calculated dissociation constants of the various alkaloid-AChE complexes are presented (Table 1). The Kd values for the titration of the alkaloids with the enzyme showed a decrease from 86 μM (isoquinoline) to 20.4 μM (papaverine) to 5.7 μM (berberine).

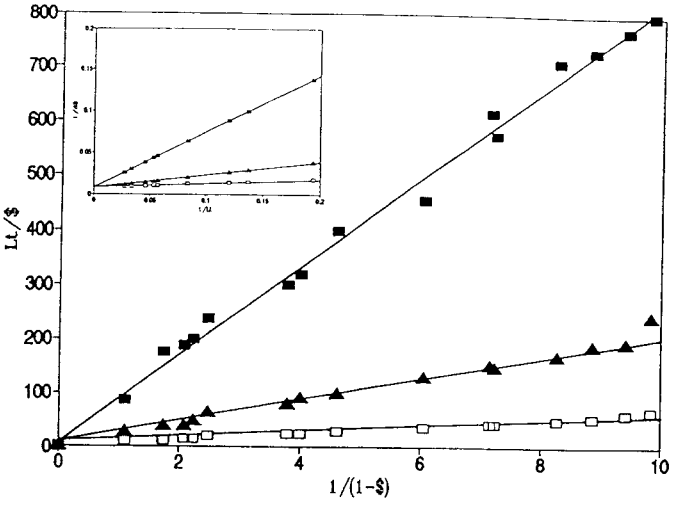

FIGURE 2 Analysis data and double reciprocal plots of absorbance change (inset) for the binding of isoquinoline (■), papaverine (▲) and berberine (□) to acetylcholinesterase in 100 mM phosphate buffer, pH 7.2.

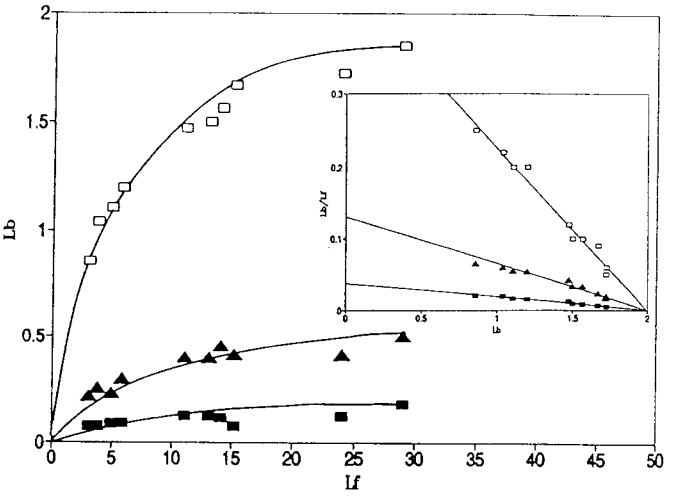

FIGURE 3 Saturation binding curves and Scatchard plots for the binding of isoquinoline (■), papaverine (▲) and berberine (□) to acetylcholinesterase as determined by equilibrium dialysis. L_b is the number of moles of bound alkaloid L_f is the concentration of free alkaloid.

TABLE 1
Kinetic parameters and binding constants for isoquinoline alkaloids with acetylcholinesterase.

			Spectrophotometry			Equilibrium dialysis	
Inhibitor	Conc(M)	$K_{m.app.}(\mu M)$	Ki(μM)	Kd(μM)	p	Kd(μM)	p
Berberine	10	173.3	3.2(3.8*)	4.6	1.98	5.7	2.07
	20	304.5	3.3				
	40	567	3.2				
Papaverine	10	64.15	19(23.4*)	18	2.02	20.4	1.99
	20	86.21	18.5				
	40	130.42	19.2				
Isoquinoline	10	46.83	87.4(88*)	79	2.0	86	2.0
	20	51.66	86.2				
	40	61.31	89.1				

*From extrapolation of slope vs (ligand) plot (Figure 5, inset).

Irreversible Inhibition Studies

Incubation of acetylcholinesterase with papaverine resulted in a progressive loss of enzyme activity. As shown (Figure 4a) the inactivation of the enzyme by papaverine was dependent on the concentration of the ligand and on the incubation time. When the enzyme was mixed with 50 μM papaverine, 14% of the control activity remained after 40 min. While in the presence of 100 μM papaverine it took only 20 min to reach the same activity. The inactivation followed pseudo first-order kinetics as indicated by typical semilogarithmic plots of enzyme activity *vs* time. Double log plots of k_1 as a function of alkaloid concentration (Figure 4c) were also linear, yielding a slope of 1 and hence a reaction order (n) of 1 with respect to papaverine (Equation (4)). The second-order rate constant (k_2) was obtained from the slope of the linear plots of pseudo first-order rate constant (k_1) vs papaverine concentration (Figure 4b) and was 43 $M^{-1}.min^{-1}$. The linearity of secondary plots implies that the interaction is bimolecular, in which reversible ligand-AChE complex is not formed before inactivation.

Reversible Inhibition Studies

The evidence of a reversible inhibitory mechanism was realised after characteristic Lineweaver-Burk plots (Figure 5) were produced, indicating that all of the alkaloids studied were competitive inhibitors of the enzyme with respect to acetylthiocholine chloride. A replot of the slopes of this plot versus ligand concentration is linear (Figure 5; inset). The relevant kinetic parameters for the ligands are represented

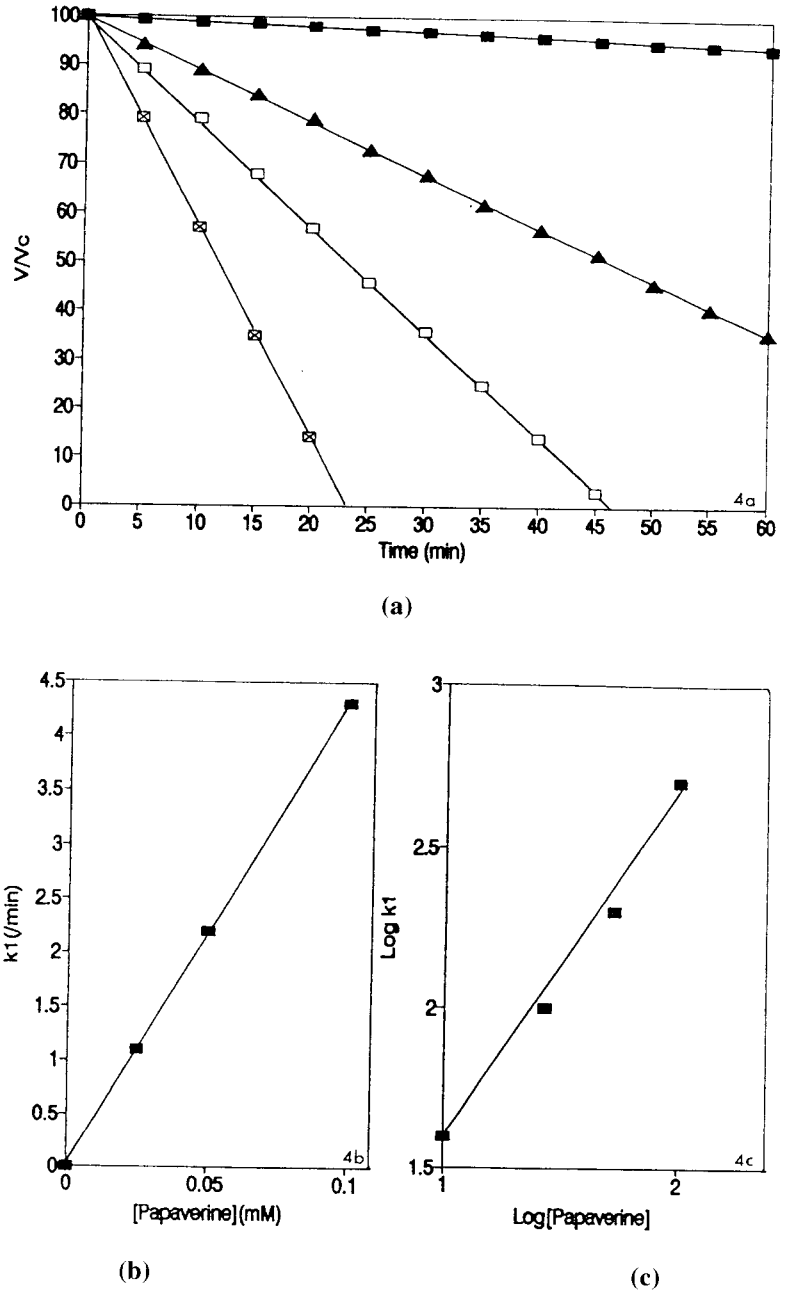

FIGURE 4 Inactivation of acetylcholinesterase by papaverine at pH 7.2. (a) Concentration of papaverine is ■, 0 μM; ▲, 25 μM; □, 50 μM; ⊠, 100 μM. V and V_c are enzyme activities of experimental and control. (b) Plot of k_1 obtained at various concentrations of papaverine against papaverine concentrations. (c) Apparent order with respect to papaverine concentration.

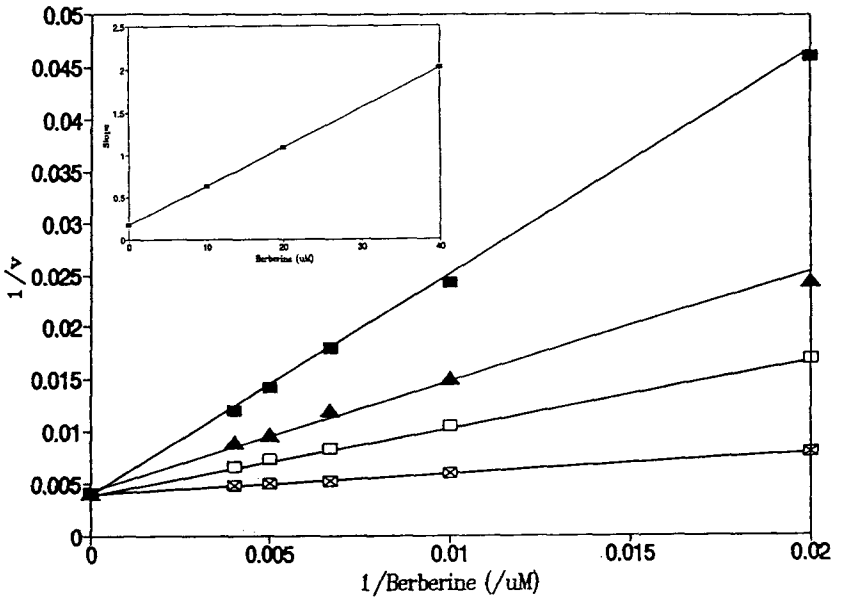

FIGURE 5 Competitive inhibition of acetylcholinesterase by berberine at pH 7.2. Concentration of berberine is ■, 0 μM; ▲, 10 μM; □, 20 μM; ⊠, 40 μM. V represents the rate of the enzymatic reaction. The secondary plot represents the slopes of the Lineweaver-Burk lines versus berberine concentration.

(Table 1). The $K_{m.app.}$ are the respective Michaelis constants and Ki is the inhibitor constant of the enzyme-inhibitor complex calculated from Equation (5).

$$\log k_1 = n \log[\text{Papaverine}] + \log k_2 \quad (4)$$

$$K_{m.app.} = K_m (1 + Ki^{-1}.I) \quad (5)$$

where I is the concentration of the particular ligand. With variable substrate concentration, values for K_m, and V_{max}, for AChE were determined as 42 μM and 240 μM.min^{-1} respectively.

Berberine showed a marked inhibition of the enzyme with a Ki of 3.2 μM. The other alkaloids were less potent as inhibitors with Ki values of 87 μM (isoquinoline) and 19 μM (papaverine).

DISCUSSION

This work has shown that initially isoquinoline alkaloids inhibit AChE reversibly and competitively with respect to the substrate acetylthiocholine chloride. A kinetic analysis of the inhibition of the enzyme by papaverine is presented (Figure 5) using the standard graphical technique of Lineweaver and Burke.[7] The lines are drawn by

computer calculation for linear competitive inhibition (Equation (6)) and least square analysis.

$$1/V = 1/V_{max}(K_m(1 + I.Ki^{-1}))1/S + 1/V_{max} \quad (6)$$

The data demonstrate reversible competitive inhibition for the system, implying that the alkaloids bind at the substrate binding site of the enzyme.

The potency of a particular ligand in the interaction with acetylcholinesterase is determined by the dissociation constant for the enzyme-inhibitor complex. The fit into the active site of the enzyme, reflected by the values of Kd (or Ki) is largely determined by the size, structure, and configuration of the inhibiting molecule. The capability of the inhibitor to bind non-covalently at, or close to, the active site could also influence these values.

Detection of spectroscopic changes in a protein upon binding with ligands is one of the simplest methods to study ligand-induced conformational changes. In the present investigation the changes in optical properties of the enzyme upon binding with the alkaloids may be due to the presence of non polar regions around the acetylcholine binding site of the enzyme. The alkaloids themselves cannot however increase the basicity of the microenvironment of the binding locus. It is assumed that the binding of ligands in the active site leads to exposure of new groups and a transmission of conformational changes from one subunit to another. The isoquinoline alkaloids bind strongly to a hydrophobic domain on the enzyme surface increasing the rigidity of the molecule and, at the same time, influencing the solvating properties of the catalytic site and hence accelerating the binding process.

The linearity of the Scatchard plot (Figure 4) from the binding of the isoquinoline alkaloids to the enzyme supports evidence of independent and indistinguishable subunit interactions.

Since the inhibitors tested in this investigation gave competitive kinetic plots it is assumed that they all interact exclusively at the same binding site of the enzyme. The necessary structures which the alkaloids must have in order to be able to exert an inhibitory effect may be defined as follows. A polar or ionic locus must be present which interacts with the positive quaternary nitrogen in a planar arrangement ($=N^+$). Several lines of evidence indicate hydrophobic interactions. There was an increase in inhibitory potency upon systematic increases in the hydrophobicity of the alkaloids studied. The simple isoquinoline molecule has limited hydrophobic groups when compared to papaverine and berberine and therefore has a greater dissociation constant (Kd).

The aromatic character of rings A and B is not entirely necessary for the inhibition since the simple isoquinoline molecule has a very poor inhibitory effect. The angle between rings A and D (approximately 20° from Dreiding models) seems to be crucial for inhibition since berberine, possessing such a structure, is a powerful inhibitor of the enzyme. Papaverine is a weaker inhibitor than berberine as it has two methoxy groups in place of the methylenedioxy substituent of berberine. This may be ascribed to a decrease in energy on binding which is offset by freezing the conformation from freely rotating; a phenomenon which does not apply to

berberine having a fixed non-rotational system. Furthermore papaverine (pKa=8) would be 50% ionised, while berberine is fully ionised at physiological pH. Since the inhibitor and binding power of the alkaloid increases as the polarity of substituents on C_7 increases it can be assumed that the binding site of the enzyme for these alkaloids has hydrophobic character and that this hydrophobic group of the enzyme comes into the neighbourhood of C_7 during the interaction of the enzyme with the inhibitor.

Substituents on C_7 with a negative inductive effect remove electrons from the C and D rings and thus increase the positive charge on the nitrogen. This is reflected by the participation of mobile electrons of these substituents (free electron pairs of oxygen or electron contribution of alkyls by hyperconjugation) in conjugation with π-electrons or ring D.[9] This in turn leads to an extension of the conjugated system which may facilitate the interaction of the alkaloids with the electron donor parts of the enzyme.

Absorbance spectroscopy, equilibrium dialysis and steady-state kinetics supports the absence of dependent subunit interactions since the same Kd values were obtained in each case. The isoquinoline alkaloids are rigid, they bind at the substrate binding site and they possess quaternary nitrogen functional groups. Thus they have adequately satisfied all the necessary features and parameters for a ligand described above.

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