Bioorganic Chemistry **27,** 197–205 (1999)
Article ID bioo.1998.1121, available online at http://www.idealibrary.com on IDEAL®

The Kinetic Inhibition of Acetylcholinesterase from Human Herytrocyte by Tacrine and Some Tacrine Derivatives[1]

Fernando Porcelli,[2] Maurizio Delfini, and Maria Rosaria Del Giudice

Cattedra di Chimica Fisica, Dipartimento di Scienze Ambientali, Università della Tuscia, Via S. Camillo de Lellis-01100, Viterbo, Italy; Dipartimento di Chimica, Università "La Sapienza," P.le Aldo Moro 5, 00185, Roma, Italy; and Laboratorio Chimica del Farmaco, Istituto Superiore di Sanità, V.le Regina Elena 299-00161, Roma, Italy

Received June 23, 1998

The kinetics of the reaction catalyzed by human erythrocyte Acetylcholinesterase (AChE) is studied in the presence of its inhibitor Tacrine (1,2,3,4-tetrahydro-9-acridinamine), and of two newly synthesized compounds, 6-metoxy-Tacrine and *N*-eptyl-Tacrine. The proposed kinetic model describes the rate of the enzymatic reaction in terms of competitive and uncompetitive mixed inhibition of the three different inhibitors. The kinetic parameters describing the rate of the reaction are obtained. The competitive and uncompetitive inhibition constants of the different inhibitors are reported and the mixed competitive–uncompetitive inhibition is discussed.

Key Words: kinetics; enzyme inhibition; acetylcholinesterase; tacrine; Alzheimer's disease.

INTRODUCTION

Acetylcholinesterase (acetylcholine acetylhydrolase, EC 3.1.1.7, AChE) is an extrinsic membrane-bound enzyme projecting into the synapse. It promotes the hydrolysis of the neurotransmitter acetylcholine (ACh) at the cholinergic synapses with liberation of choline (*1–7*). Because of its central role in the neurotransmission system, the AChE, has been an attractive target for the rational design of mechanism-based inhibitors (*4*). The inhibition of AchE *via* phosphorylation of the serine at the active site has been used in chemical warfare agents such as "sarin" and "soman." Also, other AchE inhibitors such as physostigmine and neostigmine have been used as therapeutic agents in the treatment of glaucoma and "myasthenia gravis." Over the last decade inhibitors of AChE have been employed in allaying the symptoms of Alzheimer's disease. In fact, a topic of the disease is a developing cholinergic deficit that severely impairs the neuromodulatory function of this transmitter. Thus, it may

[1] This research was supported by Grant COFIN MURST 97 CFSIB.
[2] To whom correspondence and reprint requests should be addressed. Fax: +39-0761-357179. E-mail: porcelli@unitus.it.

0045-2068/99 $30.00

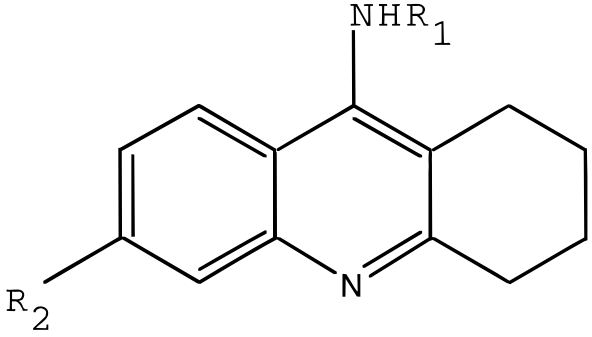
be possible to increase ACh levels in affected regions of the brain by inhibiting AChE. Tacrine, 1,2,3,4-tetrahydro-9-aminoacridine (for a Pharmacological review see (*8*)) is a potent inhibitor of AChE and has become the first drug specifically approved by FDA (Food and Drug Administration) in 1993 for the treatment of Alzheimer's symptoms. Therefore, Tacrine derivatives have become the main scaffold to obtain more active and better tolerated drugs for Alzheimer treatment by modulating their pharmacokinetics and pharmacodynamics.

Although several kinetic models describing the hydrolysis of Ach in the absence and presence of inhibitors have been proposed (*9–16*), there is a fundamental lack of agreement in the literature on the type of inhibition caused by Tacrine.

In this paper the inhibition of AChE from human erythrocytes by three different inhibitors, Tacrine and two recently synthesized Tacrine derivatives (Fig. 1), is studied. A new kinetic inhibition model is proposed to describe the reaction rate in term of noncompetitive and mixed competitive–uncompetitive behavior of the inhibitors. Further, the kinetic and thermodynamic constants obtained are used to distinguish between the two different categories of inhibition.

MATERIALS AND METHODS

Enzymes and substrates. The lyophilized powder of AChE type XII from human erythrocytes (0.62 units/mg solid) containing phosphate buffer salts was purchased from Sigma Chemical Co. (St. Louis, MO; by definition, one unit of AChE hydrolyzes



1a $R_1 = H$; $R_2 = H$

1b $R_1 = H$; $R_2 = OCH_3$

1c R_1 = eptyl; $R_2 = H$

FIG. 1. Molecular structures of synthesized Tacrine darivatives: 1a, Tacrine; 1b, 6-metoxyacrine; 1c, *N*-epthltacrine.

1 mM of acetylcholine to choline and acetate per minute at pH 8.0 and 37°C. Acetylthiocholine iodide, Dithiobisnitrobenzoic acid (DTNB), and sodium phosphate were reagent grade also from Sigma Chemical Co. The inhibitors Tacrine, 6-metoxytacrine, and *N*-eptyltacrine were prepared by M. R. Del Giudice according to a procedure already described elsewhere (*23*).

Spectrophotometric measurements were performed by a Varian DMS 90 UV-VIS spectrophotometer, equipped with a thermostated cell holder. Stock enzyme-buffered solutions (2.0 units per milliliter) were stored at 5°C and prepared daily to avoid the loss of enzyme activity after a freeze–thaw cycle (*24*). The amount of enzyme used in the kinetic assays was in the order of 0.02 units per milliliter.

Initial velocity measurements. The reaction rate was determined according to the modified Ellmann's method (*25*), which assumes the following reactions:

$$\text{Acetylthiocholine} \xrightarrow{AChe} \text{Thiocholine} + \text{Acetate}$$

$$\text{Thiocholine} + \text{DTNB} \xrightarrow{fast} \text{Yellow color}, \qquad [1]$$

where the formation of the yellow anion of 5-thio-2-nitro-benzoic anion is a function of hydrolase activity ($\varepsilon = 13600\ \text{M}^{-1}\ \text{cm}^{-1}$ at $\lambda = 412$ nm).

Reaction mixtures were made up to a 1.00 ml containing sodium phosphate buffer 0.1 M, pH 8.00, Acetylthiocholine $1.0 \times 10^{-4} - 5.0 \times 10^{-4}$ M, DTNB 5.0×10^{-4} M, and inhibitors, Tacrine, 6-metoxytacrine, and *N*-eptyltacrine $1.5 \times 10^{-7} - 7.0 \times 10^{-7}$ M.

The reaction was initiated by adding 0.010 ml of standardized enzyme solution. Initial velocities were based upon the early linear portions of the reaction curves usually involving the first few percent of the total reaction and were corrected for background spontaneous hydrolysis of acetylthiocholine.

The experiments of inhibition kinetics were performed after constant preincubation time (15 min) and inhibitor concentration while varying substrate concentration.

KINETIC EQUATIONS

The rate equations. In presence of inhibitor like Tacrine and in absence of substrate inhibition, the kinetic mechanism can be described by the following equations:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} ES \xrightarrow{k_3} \underset{C}{EA} \xrightarrow{k_p} E + A \qquad [2a]$$

$$E + I \underset{k_{-2}}{\overset{k_2}{\rightleftarrows}} EI \qquad [2b]$$

$$ES + I \underset{k_{-4}}{\overset{k_4}{\rightleftarrows}} ESI \qquad [2c]$$

$$EA + I \underset{k_{-5}}{\overset{k_5}{\rightleftarrows}} EAI. \qquad [2d]$$

The symbols *S*, *C*, and *A* refer to concentrations of acethylcholine, choline, and acetic acid, respectively. A similar approach, although with different enzyme and substrates, has been successfully used (*26*) to describe the kinetics of the GABA (γ-aminobutyric acid)—glutamate transformation in presence of inhibitors of the γ-aminobutyrate aminotransferase.

The Eq. [2a] involves two enzyme–substrate intermediates: the first one, *ES*, may be considered a Michaelis addition complex, while the second, *EA*, may be considered the acylenzyme derived from the Michaelis complex when the acetyl group of the acetylcholine is transferred from the substrate to a group of the active site of the enzyme (E). Since we are interested in studying the competitive and uncompetitive inhibitions by Tacrine and its derivatives, Scheme 2 does not account for the inhibition by the substrate. Of course the proposed equations are then valid for substrate concentrations below the range of substrate inhibition.

The total enzyme concentrations $[E_o]$ is given by:

$$[E_o] = [E] + [EA] + [ES] + [EI] + [ESI] + [EAI]. \qquad [3]$$

Because of the extremely fast acylation and deacylation rates, the kinetic analyses involve the steady-state approximation, that is $(d[E]/dt) = 0$ during initial velocity measurements, under the condition $[E_o] \ll [S]$. The reciprocal form of the forward rate equation is then given by:

$$\frac{v_m}{v} = 1 + \frac{K_{\text{app}}}{[S]}\left(1 + \frac{[I]}{K_{\text{I}}}\right) + K_{\text{unc}}\,[I], \qquad [4]$$

with

$$v_m = k_p[E_o], \qquad [5]$$

$$K_{\text{app}} = \frac{(k_{-1} + k_3)k_p}{k_1 k_3}; \qquad K_{\text{I}} = \frac{k_{-2}}{k_2}; \qquad K_{\text{unc}} = \left(\frac{k_p}{k_3 K_{\text{I}}} + \frac{1}{K_{\text{A}}}\right), \qquad [6]$$

being

$$K'_{\text{I}} = \frac{k_{-4}}{k_4}; \qquad K_A = \frac{k_{-5}}{k_5}. \qquad [7]$$

In the equations K_{I}, K'_{I}, and K_A are the dissociation constants of the various enzyme–inhibitor complexes and the different quantities are expressed in the following units: $(1/v_m)$ in [min/mol], k_{-1}, k_{-2}, k_{-4}, k_{-5}, k_3, k_p in [min^{-1}], k_1, k_2, k_4, k_5, in [1/mol min], K_{I}, K'_{I}, and K_A in [mol/1] (i.e., M), K_{unc} in [1/mol] (i.e., M^{-1}), $[E_o]$ and $[S]$ in [mol/1] (i.e., M). The constant K_{app} expressed in [mol/1] can be considered as the apparent Michaelis constant. Rate Eq. [4] assumes the deacylation as the rate determining step and is then formally similar to that proposed by Rosenberry (*3,15*). However, in