Kinetic Study on the Inhibition of Acetylcholinesterase by 1-Benzyl-4-[(5,6-dimethoxy-1-indanon)-2-yl]methylpiperidine Hydrochloride (E2020)

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E2020 (1-benzyl-4-[(5,6-dimethoxy-1-indanon)-2-yl]methylpiperidine hydrochloride) is currently being developed as a treatment for senile dementia of the Alzheimer type. Its mechanism of action is to increase central cholinergic activity by inhibiting acetylcholinesterase (AChE) in the brain. In this study, the kinetics of the inhibitory action of E2020 on AChE was examined in comparison with its derivatives (2A1050 and 2A1034) and the reference compound tetrahydroaminoacridine (THA) all of which have a similar action. Analysis of the data using Lineweaver–Burk plots shows that inhibition by the test compounds fell into the category of mixed type. Inhibitor dissociation constants for the free enzyme (K_i) and acetyl-enzyme (K_i^*) of E2020 are one or two orders of magnitude lower than those of 2A1050, 2A1034 and THA. These findings indicate the strong inhibitory effect of E2020 on AChE.

Key words acetylcholinesterase; enzyme kinetics; synthetic inhibitor; electric eel

Some inhibitors of acetylcholinesterase (AChE) are known to be useful for treatment of Alzheimer's disease, senile dementia, ataxia and for improving the long-term memory processes by enhancing cholinergic activity.^{1–6} Consequently, AChE inhibitors are potential candidates for the treatment of Alzheimer's disease.

It has been reported that the sequence of the active-site peptides of electric eel AChE, containing Ser-200 and His-440, respectively, were 87% and 71% homologous with the human brain peptides.⁷ Because of the homology of the active-site peptides, it was supposed that the characteristics of these inhibitors for electric eel AChE were similar to those for human brain AChE. In our search for new synthetic reversible inhibitors of AChE directed at Alzheimer's disease, we found that E2020 (1-benzyl-4-[(5,6-dimethoxy-1-indanon)-2-yl]methylpiperidine hydrochloride) exhibits potent inhibition of AChE.^{8–12} To characterize the AChE inhibitory activity of E2020, we carried out kinetic analysis on E2020 and the related piperidine derivatives, 2A1050 (1-benzyl-4-[(5,6-dimethoxy-1-indanon)-2-yl]piperidine hydrochloride) and 2A1034 (1-benzyl-4-[(5,6-dimethoxy-1-indanon)-2-yl]ethylpiperidine hydrochloride), and the reference compound THA (tetrahydroaminoacridine

hydrochloride) with respect to electric eel AChE. The chemical structures of E2020, 2A1050, 2A1034 and THA are shown in Fig. 1.

It is known that AChE catalysis proceeds in three steps and that some reversible AChE inhibitors show a mixed-type inhibition due to blockade of the deacetylation process.^{13–15} Figure 2 shows a schematic representation of the enzyme inhibition and formation of intermediates. Initially, acetylcholine binds to the active center of AChE. Subsequent acetylation liberates choline to leave the acetyl-enzyme (EA) which can accept an inhibitor at its vacant anionic site to form an EA inhibitor-complex (EAI). Some reversible inhibitors bind to free enzyme (E) and inhibit the formation of enzyme substrate complex (ES) and, at the same time, bind to EA blocking the deacetylation process. Reversible inhibitors generally show mixed-type inhibition.

Kinetic data were analyzed using Lineweaver–Burk plots and the dissociation constants K_i and K_i^* were determined using an equation which was based on the hypothetical reaction scheme shown in Fig. 2, as proposed by Froede *et al.*¹⁶

Fig. 1. Chemical Structures of E2020, 2A1050, 2A1034 and THA

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Fig. 2. Reaction Scheme in Which the Inhibitor Combines with E and EA

E, free enzyme; S, substrate; ES, enzyme substrate complex; EA, acetyl-enzyme; P, product; I, inhibitor; EI, enzyme inhibitor complex; EAI, acetyl-enzyme inhibitor complex; K_i , dissociation constant for enzyme and inhibitor; K_i^* , dissociation constant for EA and inhibitor.

MATERIALS AND METHODS

Enzyme AChE (type V-S) purified from the electric organ of electric eels was purchased as a lyophilized powder from Sigma Chemical Co. (St. Louis, MO, U.S.A.). An enzyme solution was prepared freshly just before use by dissolving the powder in 0.1 M phosphate buffer (pH 8.0) containing 5 mM MgCl_2 and then keeping it in an iced water bath at 5 °C as a stock solution.

Test Compounds E2020 (lot no. 88050901) and 2A1050 (lot no. 870114) were synthesized at Eisai Chemical Co. (Ibaraki, Japan). 2A1034 (lot no. YI1554) and THA (lot no. YITHA1) were synthesized at Eisai Tsukuba Laboratories (Ibaraki, Japan). Fresh solutions of the test compounds were prepared by dissolving these compounds in distilled water and keeping them at room temperature as stock solutions.

Substrate Acetylthiocholine iodide (lot no. WKQ9424) was purchased from Wako Pure Chemical Industries (Osaka, Japan). A substrate solution was prepared by dissolving acetylthiocholine in distilled water and keeping it at room temperature as a stock solution.

Reagents for Enzyme Assay Dithiobisnitrobenzoic acid (DTNB) for SH determination, monopotassium phosphate (KH_2PO_4), disodium phosphate ($\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$), magnesium chloride ($\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$), and sodium hydrogencarbonate (NaHCO_3) were all of reagent grade and purchased from Wako Pure Chemical Industries. DTNB solution was prepared by dissolving 36.9 mg DTNB in 10 ml 0.1 M phosphate buffer (pH 7.0) by sonication, then adding 15 mg sodium hydrogencarbonate.

Buffer for Enzyme Assay Buffer consisted of 0.1 M phosphate buffer (pH 8.0) containing 5 mM MgCl_2 .

Determination of AChE Activity Enzyme activity was measured by a modification of the method of Ellman *et al.*¹⁷⁾ The principle involves the measurement of the rate of production of thiocholine as acetylthiocholine is hydrolyzed by AChE. Hydrolysis is accompanied by a continuous reaction between thiocholine and DTNB which produces the yellow anion of 5-thio-2-nitrobenzoic acid. The rate of anion production is measured by absorbance at 412 nm (Hitachi 330 spectrophotometer, Tokyo, Japan). In this procedure 2.81 ml buffer, 100 μl DTNB solution, 30 μl test compound stock solution, 30 μl enzyme stock solution and 30 μl substrate stock solution were mixed and the change in absorbance at 412 nm was recorded immediately after mixing at 25 °C. The concentration of E2020 used was 0–3.99 $\times 10^{-9}$ M, the concen-

tration of 2A1050 was 0–5.05 $\times 10^{-8}$ M, the concentration of 2A1034 was 0–1.93 $\times 10^{-8}$ M and the concentration of THA was 0–1.97 $\times 10^{-8}$ M. The substrate concentrations in all experiments were between 0.332–2.010 $\times 10^{-4}$ M and the enzyme concentration was 0.0025 units/ml. Phosphate buffer (pH 8.0) containing 5 mM MgCl_2 was used as a blank in all experiments.

Estimation of K_i and K_i^* AChE catalysis proceeds in three steps, *i.e.*, Michaelis complex formation, acetylation and subsequent deacetylation in which stoichiometric formation of the acetyl-enzyme intermediate (EA) occurs. The dissociation constants of the test compounds with E (K_i) and with EA (K_i^*) were estimated according to the theory proposed by Froede *et al.*¹⁶⁾ The values of K_i and K_i^* were estimated by nonlinear regression analysis using an NEC PC-9801RX personal computer and a nonlinear least squares program (MULTI).¹⁸⁾ Analysis was performed on data obtained from the equation:

$$\frac{1}{v} = \frac{1}{V} \left(1 + \frac{I}{K_i^*} \right) + \frac{K_M}{V} \left(1 + \frac{I}{K_i} \right) \frac{1}{S}$$

where v is the enzyme activity (reaction velocity), S is the substrate concentration, I is the inhibitor concentration, K_M is the Michaelis constant between the enzyme and the substrate, and V is the maximum enzyme activity.

RESULTS AND DISCUSSION

The relationship between the concentrations of substrate and test compounds, and enzyme activities were analyzed using Lineweaver–Burk plots. As shown in Fig. 3, E2020, 2A1034 and THA exhibited inhibitory activity between that associated with conventional competitive and noncompetitive inhibitors, and 2A1050 exhibited inhibitory activity between noncompetitive and uncompetitive inhibitors. Consequently, inhibition by all the test compounds fell into the category of mixed type.

The dissociation constants of the test compounds with E (K_i) and EA (K_i^*) are shown in Table 1.

The test compounds strongly inhibited AChE, with K_i and K_i^* ranging from 10^{-7} to 10^{-9} M. The K_i and K_i^* of E2020 are one or two orders of magnitude lower than those of 2A1050, 2A1034 and THA.

All test compounds (E2020, 2A1050, 2A1034 and THA) strongly inhibited AChE and the mode of inhibition fell into the category of mixed type. E2020, 2A1034 and THA exhibited inhibitory activity which was between that exhibited by competitive and noncompetitive inhibitors, and 2A1050 exhibited inhibitory activity which was between that exhibited by noncompetitive and uncompetitive inhibitors. However, the K_i and K_i^* values for E2020 are one or two orders of magnitude lower than those of 2A1050, 2A1034 and THA.

The chemical structure of E2020 can be considered as consisting of two components, *i.e.*, benzylpiperidine and the indanone ring. 2A1050, E2020 and 2A1034 have 0, 1 and 2 methylene groups between benzylpiperidine and the indanone ring, respectively. In a comparison of K_i and K_i^* values, it is clear that E2020 shows the greatest affinity for the active center of AChE of all the test compounds, and the compatibility of E2020 for the active center of

Fig. 3. Kinetics of Inhibition of AChE by E2020 (A), 2A1050 (B), 2A1034 (C), and THA (D) in Lineweaver-Burk Plots

(A) E2020 concentration: (○) 0 M, (△) 2.00×10^{-9} M, (□) 3.99×10^{-9} M. (B) 2A1050 concentration: (○) 0 M, (△) 2.53×10^{-8} M, (□) 5.05×10^{-8} M. (C) 2A1034 concentration: (○) 0 M, (△) 0.97×10^{-8} M, (□) 1.93×10^{-8} M. (D) THA concentration: (○) 0 M, (△) 0.98×10^{-8} M, (□) 1.97×10^{-8} M.

Table 1. Dissociation Constants of E2020, 2A1050, 2A1034 and THA with AChE

Test compound	K_i (M)	K_i^* (M)
E2020	4.27×10^{-9}	9.48×10^{-9}
2A1050	1.22×10^{-7}	5.40×10^{-8}
2A1034	1.87×10^{-8}	2.70×10^{-8}
THA	2.04×10^{-8}	3.83×10^{-8}

AChE is greater than that of 2A1050 and 2A1034. This means that in E2020 the distance between benzylpiperidine and the indanone ring is optimum. Furthermore, E2020 exhibits a stronger inhibitory activity than THA which is a potent AChE inhibitor and which is currently being used in the treatment of Alzheimer's disease.⁴⁾ These findings suggest that E2020 is an exceptionally potent inhibitor of AChE.

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