Pergamon

Bioorganic & Medicinal Chemistry Letters 8 (1998) 2747–2750

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

CONFORMATIONALLY RESTRICTED CARBAMATE INHIBITORS OF HORSE SERUM BUTYRYLCHOLINESTERASE

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Received 22 June 1998; accepted 21 August 1998

Abstract: Conformationally restricted carbamate inhibitors, *exo*-2-norbornyl-*N*-butylcarbamate (**1**), *endo*-2-norbornyl-*N*-butylcarbamate (**2**), 1-adamantyl-*N*-butylcarbamate (**3**), and 2-adamantyl-*N*-butylcarbamate (**4**) as active site-directed irreversible inhibitors of horse serum butyrylcholinesterase are investigated for values of the dissociation constant (K_I), the carbamylation constant (k_2), and the bimolecular rate constant (k_i). Compound **1** is the most potent inhibitor of the enzyme and the values of K_I and k_i are 20 nM and 1.1×10^5 $M^{-1}sec^{-1}$, respectively.

Human serum butyrylcholinesterase (BChE, EC 3.1.1.8) is primarily synthesized in the liver and secreted into the plasma.[1] Although acetylcholinesterases (AChEs, EC 3.1..17) are most efficient at hydrolyzing acetylcholine, BChEs exhibit less specificity toward their substrate and efficiently hydrolyze butyryl- and acetylcholine as well as benzoylcholine. BChE is a tetrameric glycoprotein of four identical subunits with four active sites per molecule.[2-5] The complete amino acid sequence of BChE is known and it shows 53.8 % amino acid sequence identity with *Torpedo californica* AChE (TcAChE).[5,6] The amino acid sequence at the active site serine of AChE and BChE is well conserved.[7,8] BChE shows similar responses to a number of classical AChE inhibitors but differs from AChE in its substrate preference. Elucidation of the three-dimensional structure of TcAChE confirmed earlier studies,[7,8] and showed that TcAChE contains a catalytic triad similar to that present in other serine hydrolases.[9-11] Along with an active site serine residue, a histidine and a glutamate residues are considered as the catalytic triad of both AChE and BChE.[7,8,12-14] The active sites of both enzymes share a high degree of homology and consist three major domains:[14] (1) an esteratic site (ES), comprised of the active site serine that hydrolyzes acetylcholine or butyrylcholine through charge relay within a catalytic triad of amino acids, to form a high transient acylated enzyme complex which rapidly hydrolyzes to rejuvenate active enzyme; (2) an "anionic" site (AS) that is ≥ 4.7 Å from ES, where the quaternary ammonium group of acetylcholine and of various active site ligands binds and that is involved in the correct orientation of a substrate to the ES for hydrolysis through the cation-π interactions between the quaternary ammonium group of the substrate and aryl amino acid residues of enzymes;[7,8] (3) a hydrophobic site (HS) that was contiguous with or near the ES and AS and that was important in binding aryl substrates and active site ligands. Edrophonium is known to bind at the AS of the AChE and BChE and acts as a competitive inhibitor of both enzymes.[15] A series of carbamate analogues of (-)-physostigmine are known to inhibit both enzymes at ES.[16,17] Although the association rate constants of AChE for the neutral inhibitor *m*-*t*-butyltrifluoroacetophenone are about 2 orders of magnitude slower than *m*-(*N*,*N*,*N*-trimethylammonio)-2,2,2-trifluoroacetophenone,[18] due to the fact that the interaction of

0960-894X/98/$ - see front matter
PII: S0960-894X(98)00484-3

quaternary nitrogens with the π electrons of aromatic groups (Trp) in AS of TcAChE.is stronger than that of *t*-butyl groups,[7,8] the uncharged inhibitors of cholinesterases are still popular due to their easy preparations. To speculate the actual conformation of the inhibitor to which BChE is bound, conformationally restricted carbamate inhibitors such as *exo*-2-norbornyl-*N*-butylcarbamate (**1**), *endo*-2-norbornyl-*N*-butylcarbamate (**2**), 1-adamantyl-*N*-butylcarbamate (**3**), and 2-adamantyl-*N*-butylcarbamate (**4**) (Figure 1) are ready to investigate the values of the dissociation constant (K_I), the carbamylation constant (k_2), and the bimolecular rate constant (k_i). All these compounds bear the *N*-butylcarbamate group that binds to the ES of BChE and the secondary carbon located two carbons away from the *N*-butylcarbamate group, that mimics the quaternary ammonium group of butyrylcholine and may bind to the AS of the enzyme. Furthermore, in these cyclic compounds, the free rotation at the ethylene group next to the *N*-butylcarbamate group is prohibited because of the rigidity of the ring.

As part of an investigation of carbamate inhibitors of serine protease inhibitors, compounds **1-4** were prepared and evaluated for their effects on BChE. As 4-nitrophenyl-*N*-alkyl carbamates to cholesterol esterase,[19] carbamates **1-4** were characterized as the active site-directed irreversible inhibitors of BChE and met some of the criteria proposed by Abeles and Maycock.[20] First, the inhibition was time-dependent and followed first-order kinetics; second, with increasing concentration of inhibitor the enzyme displayed saturation kinetics; third, the enzyme could be protected from inhibition by carbamates **1-4** in the presence of a competitive inhibitor, edrophonium. The mechanism for active site-directed irreversible inhibition in the presence of substrate was shown in Scheme.[19] Because the inhibition of CEase followed first-order kinetics over the observed time period, the rate of hydrolysis of EI' must be significantly slower than the rate of formation of

Scheme. Kinetic scheme for active site-directed irreversible inhibition of CEase in the presence of a substrate

$$E + S \underset{}{\overset{K_m}{\rightleftharpoons}} ES \xrightarrow{k_{cat}} E + P$$

$$E + I \overset{K_I}{\rightleftharpoons} EI \xrightarrow{k_2} EI' \xrightarrow{k_3} E + Q$$

EI'(k_2>>k_3). Therefore, values of K_I and k_2 can be calculated from Equation 1.[19] In Equation 1, k_{app} values are the first-order rate constants which can be obtained according to Hosie's method.[19] Bimolecular rate

$$k_{app} = \frac{k_2 [I]}{K_I(1+\frac{[S]}{K_m}) + [I]} \qquad (1)$$

constant, $k_i = k_2/K_I$, is related to overall inhibitory potency.

The kinetic data of the inhibition of horse serum BChE by compounds **1-4** were summarized (Table). The values of K_I, k_2, and k_i were obtained according to Hosie's method.[19] Compound **1** was the most potent inhibitor of BChE we got and the K_I value was about same magnitude to that of (-)-physostigmine and much lower than that of edrophonium. The inhibitory potency of compounds **2-4** toward BChE was about the same. The k_2 values of all compounds were also about the same. Therefore, the inhibitory potency of the inhibitor was mostly dependent upon the reversible binding between the enzyme and the inhibitor (K_I values). For compound **1**, the lowest K_I value (20 nM) and the highest affinity to BChE were observed. Therefore, the conformation of substrate in the enzyme-substrate complex mimicked that of compound **1** (Figure 2). Thus, butyrylcholine in the ES complex may adapt an eclipsed conformation at the ethylene part with the torsional

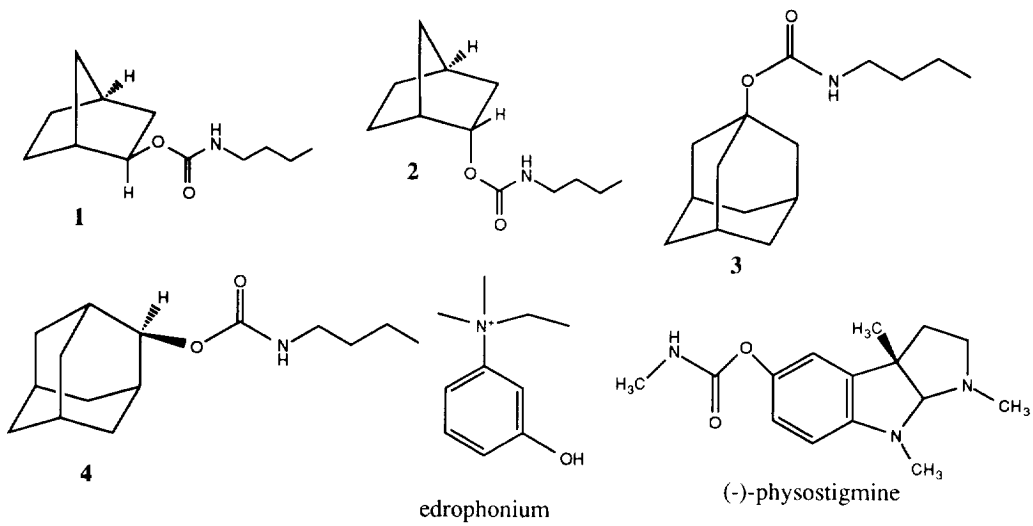

Figure 1. Molecular formulas of compounds 1-4, edrophonium, and (-)-physostigmine

Table. Kinetic Data for the BChE-Catalyzed Hydrolysis of Butyrylthiocholine in the Presence of 5,5'-dithio-bis-2-nitrobenzoate and Carbamates 1-4.[a]

Inhibitors[b]	K_I (nM)[c]	k_2 (10^{-3}sec^{-1})	k_i (10^3M^{-1}sec^{-1})[d]
1	20±6	2.2±0.2	110±30
2	420±80	0.92±0.05	2.2±0.4
3	800±200	1.5±0.3	1.9±0.6
4	800±300	1.4±0.2	1.8±0.7
edrophonium	340,000±30,000[e]		
(-)-physostigmine	16±3[f]		

a. General procedures: The BChE-catalyzed hydrolysis of butyrylthiocholine was followed continuously at 410 nm in the presence and absence of an inhibitor and in the presence of 5,5'-dithio-bis-2-nitrobenzoate by the Ellman method[21] on a UV-visible spectrometer (HP 8452) that was interfaced to a personal computer. Kaleida Graph™ (version 2.0) was used for all least-squares curve fittings. Horse serum BChE was purchased from Sigma. All the other procedures were the same as described by Hosie et al.[19] b. All compounds were prepared from the condensation of the the corresponding alcohol with 1.2 mole equivalents of *n-butylisocyanate* in the presence of catalytic amount of pyridine in dichloromethane at 25°C for 48h (81-90%). c. These values were in excellent agreement with those determined in the absence of substrate (zero time).[22] d. $k_i = k_2/K_I$ e. This value was the competitive inhibition constant and was in excellent agreement with reference 15. f. This value was the IC_{50} value and taken from reference 16.

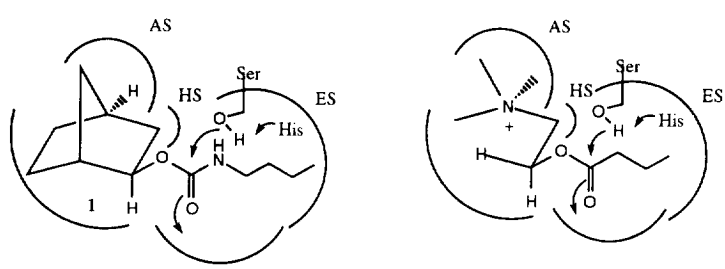

Figure 2. Interactions between BChE and compound 1 and BChE and butyrylcholine

angle of 120 or 240°. In other words, three active sites of BChE, ES, AS, and HS looked like an L letter. Further, BChE preferred flat inhibitors, such as compound **1** and (-)-physostigmine to bulky inhibitors such as compounds **2-4**. Thus, the shape of the catalytic active site of BChE was relatively flat when we compared to that of AChE. Besides ES, AS, and HS, the active site of AChE also consisted a fourth domain: the peripheral anionic site (PAS) that bound cationic ligands, such as gallamine, *d*-tubocurarine, and decamethonium and that was >20 Å from the active site.[14] From our present data, the presence of PAS in the active site of BChE seemed less possible.

Further studies on the pure enantiomer of compound **1** will be communicated in due course.

Acknowledgement: We thank the National Science Council of Republic of China for financial support.

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