Design and Synthesis of Phenyl Boronic Acids and Benzothiophenones as Anticholinesterases

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Since cholinesterases (ChE) such as acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) play critical roles for the neurotransmission, the detailed chemical mechanism of the ChE-catalyzed reactions are well documented.[1-3] The physiological role of AChE is hydrolysis of the neurotransmitter, acetylcholine (ACh) to acetate and choline. AChE inhibitors are used as chemical warfare agents, insecticides, and drugs against Alzheimer's disease (AD). Numerous evidences suggest that the neurobiological basis of senile dementia in AD and related dementias is a loss of cholinergic neurons and a resultant decline in acetylcholine (ACh) in brain regions which regulate behavioral and emotional responses.[4-6] This cholinergic deficit can be partly corrected by inhibiting ChEs. Well known AChE inhibitors such as tacrine (THA, Cognex), E2020 (Donepezil, Aricept), rivastigmine (Exelon), and galantamine (Raminyl) are approved by FDA as drugs against AD.[7-9] Initial research has focused on AChE-selective agents, but it is now thought that dual inhibitors of AChE and BuChE may provide more sustained efficacy over the course of AD and may help to slow disease progression.[10] In the healthy brain, AChE predominates (80%) and BuChE is considered to play a minor role in regulating brain ACh levels. In the AD brain, BuChE activity rises while AChE activity remains unchanged or declines. Therefore, both enzymes are likely to have involvement in regulating ACh levels and represent legitimate therapeutic targets to ameliorate the cholinergic deficit.

Abeles *et al*. showed that fluoromethylketones are potent AChE inhibitors with the inhibition constant in nano molar range. Most of them are more potent toward AChE over BuChE.[11] It is believed that BuChE also plays important roles and thus ChE inhibitors should have potency selectivity.[12,13] It is known that *m*-(*N*,*N*,*N*-trimethylammonio)-phenol, *m*-(*N*,*N*,*N*-trimethylammonio)trifluoroacetophenone are very potent reversible AChE inhibitors having the inhibition constant of submicro and femto molar range, respectively.[14,15] It is also known that the trimethylammonio group binds to the hydrophobic region of AChE known as an anionic locus previously and the serine residue in the active site binds to the carbonyl group having the trifluoromethyl group.[14,15] Thus, if a compound has *N*,*N*,*N*-trimethylammoniophenyl group and an electropositive element, it is expected to be a good inhibitor of AChE. It is also known that boronic acid and borinic acid bind to the AChE active site. Koehler *et al*. synthesized a new, specific and reversible bifunctional alkylborinic acid inhibitor of AChE.[16] Herz *et al*. also showed that erythrocyte membrane AChE is inactivated by tetraphenylboron.[17]

Powers *et al*. showed that serine protease such as elastase and chymotrypsin is inactivated by isobenzofuranones and benzopyrandiones.[18] If a compound has a functionality similar to the thioester of acetylthiocholine and a hydrophobic region, it is expected that the compound would bind to the AChE active site. Most insecticides are irreversible inhibitors of AChE and thus specific irreversible inhibitors are also required to develop. The benzothiophenone compounds have hydrophobic group and the carbonyl group and expected to inhibit ChEs. Since AChE and BuChE belong to the same serine hydrolases, we designed and synthesized compounds **1-4** as anticholinesterases and measured their inhibition potencies on ChEs.

Experimental Section

Materials. Electric eel AChE, type V-S lyophilized powder and horse serum BuChE were purchased from Sigma Chemical

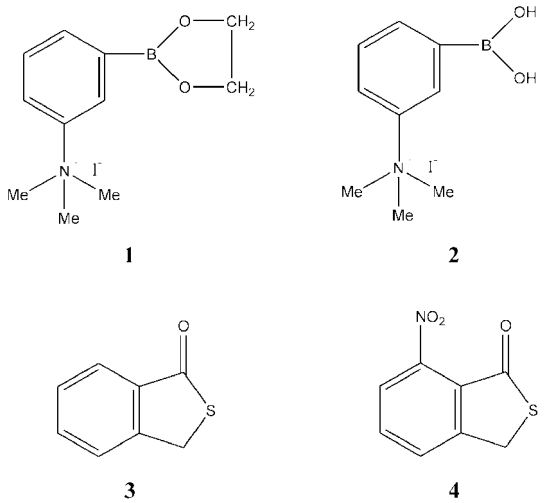

Figure 1.

Co and was used as received. Prior to use they were dissolved in 0.1 M, pH 7.3 sodium phosphate buffer, containing 0.1 M NaCl. Acetylthiocholine (ATCh), butyrylthiocholine (BuTCh), 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB), and buffer components were also purchased from Sigma Chemical Co. Water used in experiments was distilled and deionized by passage through a mixed bead ion-exchange column. NMR spectra were obtained with a Bruker AC200 spectrometer (200 MHz) using tetramethylsilane or D_2O as internal standards.

Synthesis of inhibitors. The boronic acid derivatives **1** and **2** were synthesized from 3-aminophenyl boronic acid through 3-aminophenyl-1-boro-2,5-dioxolane.[19] The benzothiophenone **3** and **4** were synthesized using the known methods from ethyl *o*-methylbenzoate and ethyl 2-methyl-3-nitrobenzoate, respectively.[20,21]

Synthesis of 3-aminophenyl-1-boro-2,5-dioxolane. Vacuum dried 3-aminophenylboronic acid monohydrate (0.5 g, 3.2 mmol) was added to a 50 mL one-necked flask and then 5 mL ethylene glycol was added to the flask under nitrogen. The mixture was heated to 100 °C slowly and kept the temperature for 3 hr. Then, the mixture was cooled to room temperature and 20 mL of methanol was added to the flask and stirred. The unreacted ethylene glycol was removed with a separatory funnel and MeOH was removed with a rotary evaporator. The product was vacuum dried to give 3-aminophenyl-1-boro-2,5-dioxolane (4.84 g, 93%): IR (neat) ν_{max} (cm^{-1}) 3640, 3405, 3030, 2925; ^1H NMR (200 MHz, D_2O) δ 4.31 (s, 4H), 4.35 (s, 2H), 6.75-7.31 (m, 4H); Mass (m/z, CI) 163 (M^+), 132, 119, 106, 92, 65.

Synthesis of *m*-[(*N,N,N*-trimethylammnio)phenyl]-1-boro-2,5-dioxolane (1). Methyl iodide (3 mL, 3.5 mmol) was added to a flask containing 3-aminophenyl-1-boro-2,5-dioxolane (0.5 g, 0.003 mol) with methanol (10 mL) and K_2CO_3 (3 g). The reaction mixture was slowly warmed to reflux for 12 hr. After completion of the reaction the reaction mixture was cooled to room temperature. After filtration of K_2CO_3, methyl iodide was removed with a rotary evaporator and dried in a vacuum to give a white solid (0.66 g, 90%): IR (KBr) ν_{max} (cm^{-1}) 3030, 2960, 2925; ^1H NMR (200 MHz, D_2O) δ 3.36 (s, 4H), 3.64 (s, 9H), 7.49-7.88 (m, 4H); Anal. calcd for $C_{11}H_{17}BNO_2I$: C, 39.70; H, 5.15; N, 4.21. Found: C, 39.72; H, 5.13; N, 4.19%.

Synthesis of *m*-[(*N,N,N*-trimethylammnio)phenyl]boronic acid (2). Compound **1** (0.5 g, 0.0024 mol) was added to a one-necked round bottomed flask having 20 mL of 15% HCl solution. The reaction mixture was warmed to 80 °C. After 1 hr the reaction mixture was cooled to room temperature. The reaction mixture was neutralized with 10% NaOH solution and extracted the product with dichloromethane. Dichloromethane was removed with a rotary evaporator and the product was vacuum dried to give a brownish solid (0.61 g, 83%): IR (KBr) ν_{max} (cm^{-1}) 3630, 3030, 2978; ^1H NMR (200 MHz, D_2O) δ 3.41 (s, 9H), 3.73 (s, 2H), 7.58 (m, 4H); Anal. calcd for $C_9H_{15}BNO_2I$: C, 35.22; H, 4.93; N, 4.56. Found: C, 35.16; H, 4.93; N, 4.55%.

Synthesis of 3*H*-benzo[*C*]thiophen-1-one (3). To a solution of ethyl *o*-methylbenzoate (8.21 g, 50 mmol) and benzoylperoxide (10 mg, 41.3 mmol) in carbon tetrachloride (125 mL) was added *N*-bromosuccinimide (8.90 g, 50 mmol) and refluxed for 6 hr. After completion of the reaction the mixture was filtered and evaporated. The residue in acetone (50 mL) was mixed with thiourea (4.19 g, 55 mmol) and refluxed for 5.5 hr. After removal of acetone by evaporation, the obtained thiouronium salt was warmed to 80-90 °C in 20 % $NaHCO_3$ solution (50 mL) under nitrogen for 2 hr. Then, the solution was acidified with 20% HCl solution. The oily residue was subjected to silica gel column chromatography (eluent; hexane : ethyl acetate = 10 : 1, v/v) to give **3** (4.81 g, 64%): m.p. 58-60 °C (Ref.[17] m.p. 58-60 °C); IR (KBr) ν_{max} (cm^{-1}) 3030, 1686, 1595, 1237; ^1H NMR (200 MHz, $CDCl_3$) δ 4.45 (s, 2H), 7.38-7.97 (m, 4H); Anal. calcd for C_8H_6OS: C, 63.97; H, 4.03; S, 21.35. Found: C, 63.84; H, 4.02; S, 21.30%.

Synthesis of 4-nitro-3*H*-benzo[*C*]thiophen-1-one (4). To a solution of ethyl 2-methyl-3-nitrobenzoate (10.45 g, 50 mmol) and benzoylperoxide (10 mg, 41.3 mmol) in carbon tetrachloride (125 mL) was added *N*-bromosuccinimide (8.90 g, 50 mol) and refluxed for 7 hr. After completion of the reaction the mixture was filtered and evaporated. The residue in acetone (50 mL) was mixed with thiourea (4.19 g, 55 mmol) and refluxed for 5.5 hr. After removal of acetone by evaporation, the obtained thiouronium salt was warmed to 80-90 °C in 20% $NaHCO_3$ solution (50 mL) under nitrogen for 2 hr. Then, the solution was acidified with 20% HCl solution. The oily residue was subjected to silica gel column chromatography (eluent; hexane : ethyl acetate = 10 : 1, v/v) to give **4** (4.39 g, 45%): m.p. 105 °C; IR (KBr) ν_{max} (cm^{-1}) 3030, 1685, 1595, 1530; ^1H NMR (200 MHz, $CDCl_3$): δ 4.40 (s, 2H), 7.04 (d, 1H), 7.29 (d, 1H), 7.72 (t, 1H); Anal. calcd for $C_8H_5NO_3S$: C, 49.23; H, 2.58; N, 7.18; S, 16.43. Found: C, 49.28; H, 2.53; N, 7.19; S, 16.32%.

Cholinesterase assay. AChE-catalyzed hydrolysis of the thiocholine esters was monitored by following production of the thioanion of nitrobenzoic acid at 412 nm by the Ellmans coupled assay.[22] Assays were conducted on HP8452A or HP8453A diode array UV-visible spectrophotometers and the cell compartments were thermostatted by circulating water or Peltier temperature controller, respectively. In BuChE assay, BuTCh was used as a substrate.

Results and Discussion

The designed compounds were synthesized and tested as anticholinesterases. They turned out to be ChE inhibitors with the inhibition constant shown in Table 1. The inhibition constant obtained by a replot of K_m/V_{max} *vs.* inhibitor concentration ranges from μM to nM. The boronic acid and its protected form are effective inhibitors of AChE and BuChE. The inhibition constant for AChE, 78 nM is close to that of the most potent AChE inhibitors such as tacrine and huperzine A. Since the inhibitors increased the Michaelis constant while they have little effect on the maximal velocities, the inhibition mechanism is mixed (data not shown)

Table 1. The anticholinesterase activities of the compounds

Compound	K_i for AChE (μM)	K_i for BuChE (μM)	K_i(BuChE)/ K_i(AChE)
1	0.137 ± 0.005	74.2 ± 6.6	541
2	0.078 ± 0.003	23.5 ± 1.0	301
3	59.8 ± 3.1	4894 ± 358	82
4	11.8 ± 1.2	341 ± 30	29

though it is close to competitive inhibition. Compounds **1** and **2** did not show any slow binding inhibition. AChE and compound **2** were incubated and the enzyme activity was measured at different times. There is no change in the inhibition potency as time goes for 1 hr. The protection of the two hydroxyl group in the boronic acid does not have a significant effect on the inhibition constant. The protection decreased the inhibition potency only by 2-fold. The trimethylammoniophenyl group of **1** and **2** is believed to bind to the hydrophobic site of ChE active site. The active site of AChE and BuChE composed of hydrophobic amino acid residues such as phenylalanine, tryptophan, and tyrosine.[23,24] This kind of π-charge interaction plays an important role in ligand binding to protein active site.[25] The positive charge on the nitrogen atom of trimethylammonio group interacts with the hydrophobic amino acid residues in ChE active site. This π-charge interaction is much stronger than normal hydrogen bond and the binding of ACh to the AChE active site is due to this interaction. The active site serine residue bind to boron atom to enhance the binding affinity of **1** and **2**.

The benzothiophenone (**3**) and the nitro substituted one (**4**) inhibit ChE with much less potency. The nitro group in **4** increased the inhibition potency by 5 fold toward AChE compared to **3**. These compounds are not good inhibitors for BuChE, either. Compound **3** is the least potent BuChE inhibitor with the inhibition constant of 4.9 mM among the tested compounds. This may due to the increased hydrophobic site of BuChE and the absence of any electronic effect by the nitro group. As the inhibition potency decrease, the K_i (BuChE)/K_i(AChE) also decreases, meaning the selectivity decreases. The more potent inhibitor toward AChE, **1** and **2** are more selective than benzothiophenones. The irreversible inhibition of ChEs with benzothiophenones is underway.

Recent evidence suggests that BuChE may also have a role in the etiology and progression of AD beyond regulation of synaptic ACh levels. Experimental evidence from the use of agents with enhanced selectivity for BuChE (cymserine, MF-8622) and ChE inhibitors such as rivastigmine, which have a dual inhibitory action on both AChE and BuChE, indicate potential therapeutic benefits of inhibiting both AChE and BuChE in AD and related dementias.[26,27] In this respect, the boronic acid inhibitors has the potential for the further refinement as ChEs inhibitors.

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