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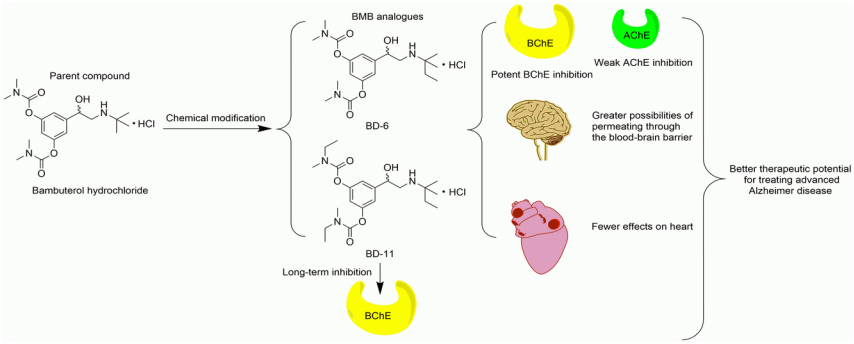

Design, Synthesis and Biological Evaluation of Bambuterol Analogues as Novel Inhibitors of Butyrylcholinesterase

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Abstract:

An increase activity of butyrylcholinesterase is believed to contribute to Alzheimer's disease. Bambuterol is a known potent inhibitor of butyrylcholinesterase, but it has undesired cardiac effects and less lipophilicity. Thirteen bambuterol analogues were synthesized using 1-(3, 5-dihydroxyphenyl) ethanone as a starting material. *In-vitro* cholinesterase assay established that the majority of the compounds are specific butyrylcholinesterase inhibitors. Out of the 13 compounds, two bambuterol derivatives, BD-6 and BD-11 exhibited similar efficacies in inhibiting butyrylcholinesterase with fewer effects on heart and enhanced possibilities of permeating through the blood-brain barrier as compared to bambuterol. These bambuterol analogues may provide better alternatives for treatments of Alzheimer's disease.

Keywords: Bambuterol; Butyrylcholinesterase; Alzheimer's disease; Specific butyrylcholinesterase inhibitor.

1. Introduction

Alzheimer`s disease (AD) is a type of progressive neurodegenerative disorder commonly associated with dementia, which gradually worsens over a period of time leading to severe behavioral and psychiatric symptoms in the long run. According to the cholinergic hypothesis of AD, the progressive loss of cognitive and behavioral functions was related to a significant reduction in cholinergic neurotransmission in brain [1-3]. Presently there is no cure for AD. Recent progresses in the knowledge and interpretation of AD pathogenesis have led to the development of several compounds that may ameliorate disease progression by amending the loss of presynaptic cholinergic functions. Few of these compounds have established their effectiveness by deferring the deterioration of the symptoms of AD, a valued treatment approach considering the progressive nature of the disease. Acetylcholinesterase (EC 3.1.1.7; AChE) inhibitors have been widely reported to relieve dementia and improve reasoning competences and behavior in patients with mild to moderate AD [4]. Advanced AD

is characterized by the declining levels of AChE in the brain with a constant or progressive increase in the levels of butyrylcholinesterase (EC 3.1.1.8; BChE). In this stage, BChE is considered to play a prominent role in regulating brain acetylcholine (ACh) levels as BChE too can hydrolyze ACh, though at a lower rate as compared to AChE. Therefore BChE inhibitors may provide therapeutic advantages in patients with advanced AD. AChE inhibitors not only augment ACh levels in brain but may also trigger increased expressions of AChE jeopardizing their sole purpose, but no such consequences have been reported in case of BChE inhibitors [3, 5-8]. Recent studies have demonstrated that BChE inhibition results in improved cognitive potential with elevated levels of ACh in brain and hence, it may act as an effective therapeutic strategy for AD. [3, 9-12].

Currently available cholinesterase inhibitors (ChEIs) for AD in clinic are donepezil, rivastigmine and galantamine. Huperzine A is becoming increasingly considered as a potential strategy for AD as it can easily traverse the blood-brain barrier and has shown to improve cognitive functions in clinical set up. But poor size and quality of the clinical trials demands more and larger studies to weight the potential benefits and long term risks of huperzine A as a treatment for Alzheimer's disease[13]. All the ChEIs mentioned above are thought to work primarily by inhibiting AChE whereas rivastigmine exhibits dual AChE and BChE inhibitory effect. No single specific BChE inhibitors have been approved for AD to date [4, 7, 14]. Therefore the discovery of safe, effective and specific BChE inhibitors, able to ease out the symptoms of AD without any adverse physiological effects would represent a significant advancement.

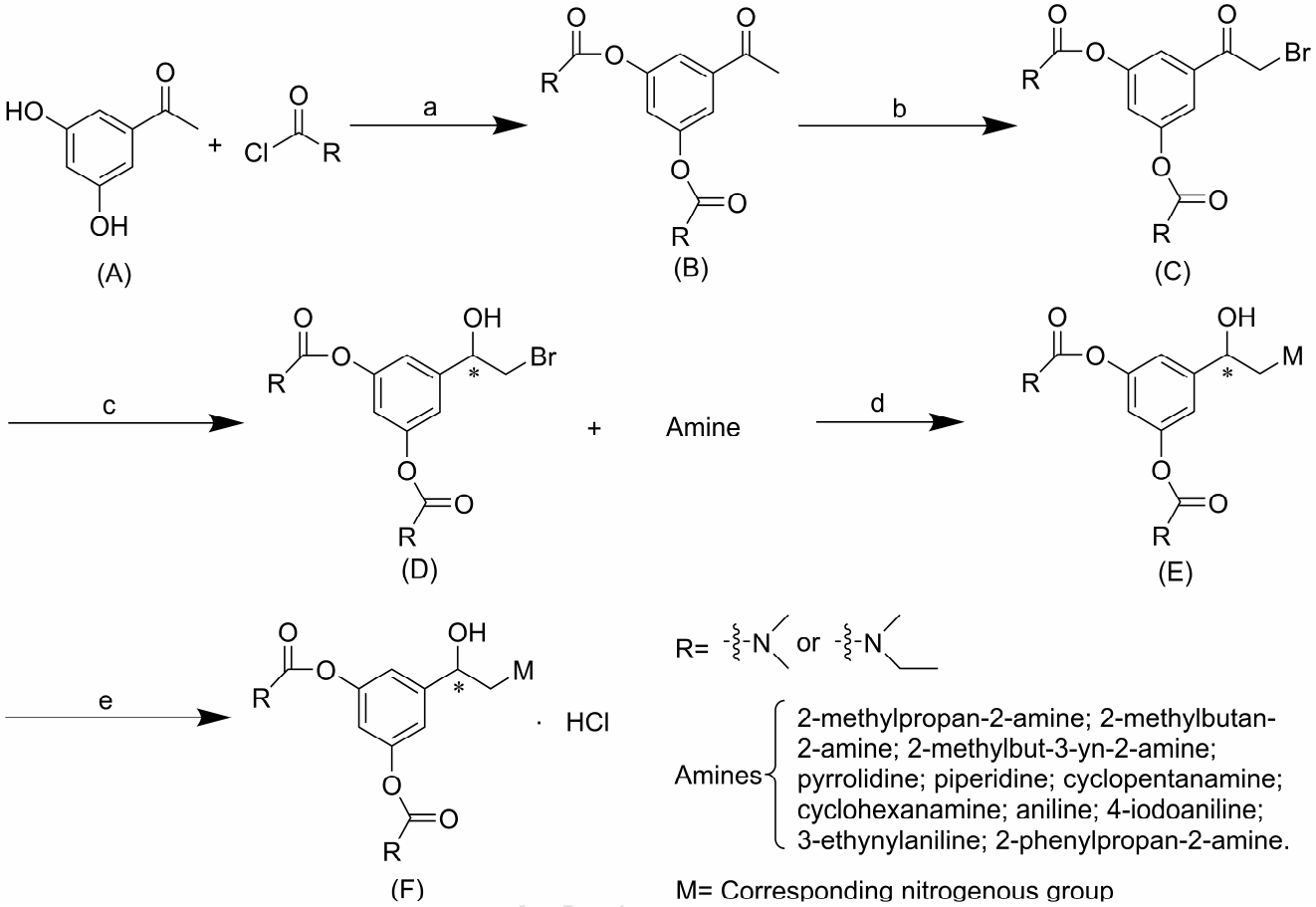
Bambuterol (BMB) is a biscarbamate ester prodrug of terbutaline, a β_2-adrenergic agonist used to treat asthma. This prodrug is hydrolyzed by BChE to produce the active metabolite terbutaline [15]. During metabolism studies, BMB displayed high inhibitory potential against BChE with IC_{50} value of 3×10^{-9} M. BMB also inhibited AChE with a 10000-fold lower efficacy ($IC_{50}=3 \times 10^{-5}$ M) as compared to BChE, indicating it to be more capable in inhibiting BChE than AChE [16, 17]. Moreover, no notable deleterious physiological effects have been reported for BMB, making it a suitable drug candidate. Few roadblocks in this study were the inability of BMB to penetrate the blood-brain barrier [18, 19] and the possibility of terbutaline (active metabolite of BMB) to cause cardiac arrythmogenecity or heart palpitations [20]. However, the modification of the branched alkyl group of terbutaline could alter its affinity for β_2-adrenoceptor thereby reducing its side effects [20, 21]. This approach of structure modification is somehow similar to that used to design rivastigmine. On this basis, thirteen BMB analogues were designed, synthesized and evaluated as ChEIs. In addition, the biological activity of BD-6 and BD-11 was further explored using electric eel AChE, equine serum BChE, human plasma, human erythrocyte and their effect on mice heart rate was measured and compared with BMB. Log P estimation and structure activity relationship analysis were also performed to demonstrate the improved ability of the analogues over BMB.

2. Results and discussion

2.1. Chemistry

The synthesis of BMB analogues are systematically represented in Scheme 1. Sodium borohydride was used to produce racemic products in place of chiral catalytic agent, as mentioned in

previous studies [22]. BD-1, BD-6, BD-11 and BD-12 formed white solid salts and precipitated when their free bases were treated with hydrochloric acid in ethanol. The chemical purity of all compounds was higher than 90%. Moreover, BD-6 and BD-11, used for further studies, were more than 99% pure.



Scheme 1: Synthesis of bambuterol derivatives. Reagents and conditions: (a) K_2CO_3, $K_2CO_3 \cdot 3/2H_2O$, pyridine, ethyl acetate, 70℃; (b) $CuBr_2$, ethyl acetate:$CHCl_3$=1:1, 60℃; (c) $NaBH_4$, CH_2Cl_2, methanol; (d) Isopropanol, 80℃; (e) HCl in ethanol, 0℃. *: Chiral center.

2.2. Measurement of IC_{50}

The cholinesterase inhibitory activities of the synthesized BMB derivatives were firstly compared to that of BMB utilizing electric eel AChE and equine serum BChE due to the economic viability of these enzymes. The IC_{50} values are included in Table 1. A comparative study of the IC_{50} values indicated that all the compounds were more potent in inhibiting BChE as compared to AChE. We observed that the IC_{50} values for most of the compounds were higher than 100μM for electric eel AChE, except BD-4 and BD-10 which presented IC_{50} values less than 100 μM, whereas the IC_{50} values of all the compounds for equine serum BChE were between 100 nM and 4700 nM. The n-butyl amine modified derivatives BD-9, BD-11, BD-12 and BD-13, with IC_{50} values of 100.3±3.5 nM, 116.0±9 nM, 113.0±3 nM and 108.3±4 nM were equally effective in inhibiting equine serum BChE as compared to BMB, which under the same experimental conditions displayed an IC_{50} of 119.3±3.5 nM. The IC_{50} values of other analogues, BD-1, BD-2 and BD-8 were found to be higher than 1000 nM, leading to the conclusion that tailoring the n-butyl amine groups of BMB will have a greater influence on their efficacy. On the contrary, using ethyl methylcarbamate instead of dimethylcarbamate will

have less impact on the inhibitory potential. However, previous studies have already reported some differences between the cholinesterase activities of humans and other organisms[23]. Among the analogues prepared, BD-6 and BD-11 are those with the structure most similar to BMB. We predicted that they would have less unexpected side effects and thus more chances to become drugs than the other analogues. Hence, human erythrocytes and plasma were used to confirm the ability of BMB, BD-6 and BD-11 to repress the activity of human AChE and BChE (Table 1). By comparing the IC_{50} values of these compounds for human AChE and human BChE, we found that, consistently with BMB, BD-6 and BD-11 were specific BChE inhibitors. Although the IC_{50} of BD-6 and BD-11 were higher than BMB, they were very potent and specific BChE inhibitors. IC_{50} values of both the compounds were in the nano molar range and the ratios of their AChE/BChE IC_{50} were higher than 1000 folds.

Table 1: Measured kinetic parameters for the hydrolysis of BMB and its analogues by AChE/BChE.

	Electric eel AChE IC_{50} mean±SD (nM)	Human erythrocyte IC_{50} mean±SD (nM)	Equine serum BChE IC_{50} mean±SD (nM)	Human plasma IC_{50} mean±SD (nM)	Equine serum BChE k_{I1} mean±SD ($\times 10^5$ $M^{-1}min^{-1}$)	Human plasma k_{I1} mean±SD ($\times 10^5$ $M^{-1}min^{-1}$)	Equine serum BChE k_3 mean±SD (h^{-1})	Human plasma k_3 mean±SD (h^{-1})
BMB	>100000	>10000	119.33±3.51	4.29±0.41	1.44 ± 0.02	77.7± 3.5	0.0839±0.0028	0.409±0.025
BD-1	>100000		1133.33±120.51					
BD-2	>100000		2402.33±118.15					
BD-3	>100000		686.00±63.59					
BD-4	65950±5027		130.33±24.79					
BD-5	>100000		476.33±43.73					
BD-6	>100000	>10000	125.67±30.02	7.28±0.20	1.42 ± 0.05	60.0±2.5	0.0851±0.0023	0.402±0.035
BD-7	>100000		224.00±16.70					
BD-8	>100000		4699.00±428.15					
BD-9	>100000		100.33±3.51					
BD-10	41330±23740		253.33±10.12					
BD-11	>100000	>10000	116.00±9.00	7.12±1.30	1.49 ± 0.04	73.3±6.7	0.0092±0.0003	0.116±0.007
BD-12	>100000		113.00±3.00					
BD-13	>100000		108.33±4.04					

2.3. Kinetic study of the inhibition of BChE by BMB, BD-6 and BD-11

The inhibition constants (k_{I1}) for BMB and its derivatives were calculated based on the equations reported earlier[17].

$$R_t = v_i / v_0 \quad (1)$$

$$R_t = R_0 \cdot \exp(-k_{obs} \cdot t) + R_\infty \quad (2)$$

$$k_{obs} = k_I \cdot [I] \quad (3)$$

Where v_i is the enzyme activity measured after pre-incubation with inhibitors for a determined time period, while v_0 is the enzyme activity measured after pre-incubation with water for the same given time of pre-incubation, and represents the maximum enzyme activity. R_t, R_0 and R_∞ are the

residual fractional activities at time t, 0 and infinite, respectively. Pseudo-first order rate constant values (k_{obs}) of BMB, BD-6 and BD-11 were obtained by fitting the residual activity measured after different pre-incubation times to a one-phase exponential decay equation (eq. 2). All data were analyzed as for a simple irreversible inhibition and the second-order carbamylation rate constant (k_{I1}) was calculated using eq. (3). As observed for BMB (Figure 1A), the inhibition of BChE (either equine or human) by BD-6 and BD-11 was time-dependent (i.e. the extent of the inhibition increased while increasing the pre-incubation time) and concentration-dependent (i.e. the extent of the inhibition increased while increasing the inhibitor concentration) (Figure 1B-C).The results obtained with both equine and human BChE depicted a linear relationship between the concentration of the inhibitors and k_{obs} with the line passing through the origin (Figure 1D). The linearity of this relationship indicates that the range of concentration employed is below the affinity (K_D) of the enzyme-inhibitor Michaelis complexes of all the three compounds, therefore the fraction of the observed inhibition that can be attributed to the enzyme-inhibitor Michaelis complex is negligible. In addition, an intercept with the y-axis close to the origin indicates a slow recovery of the enzyme (Figure 1D). The k_{I1} of BMB, BD-6 and BD-11 were in the order of 1.5×10^5 $M^{-1}min^{-1}$ for equine BChE and about 50-fold higher for human BChE (Table 1), leading to the conclusion that all the three compounds displayed similar effectiveness in inhibiting BChE, with a higher potency over the human enzyme, consistently with the pattern of IC_{50} values (Table 1).

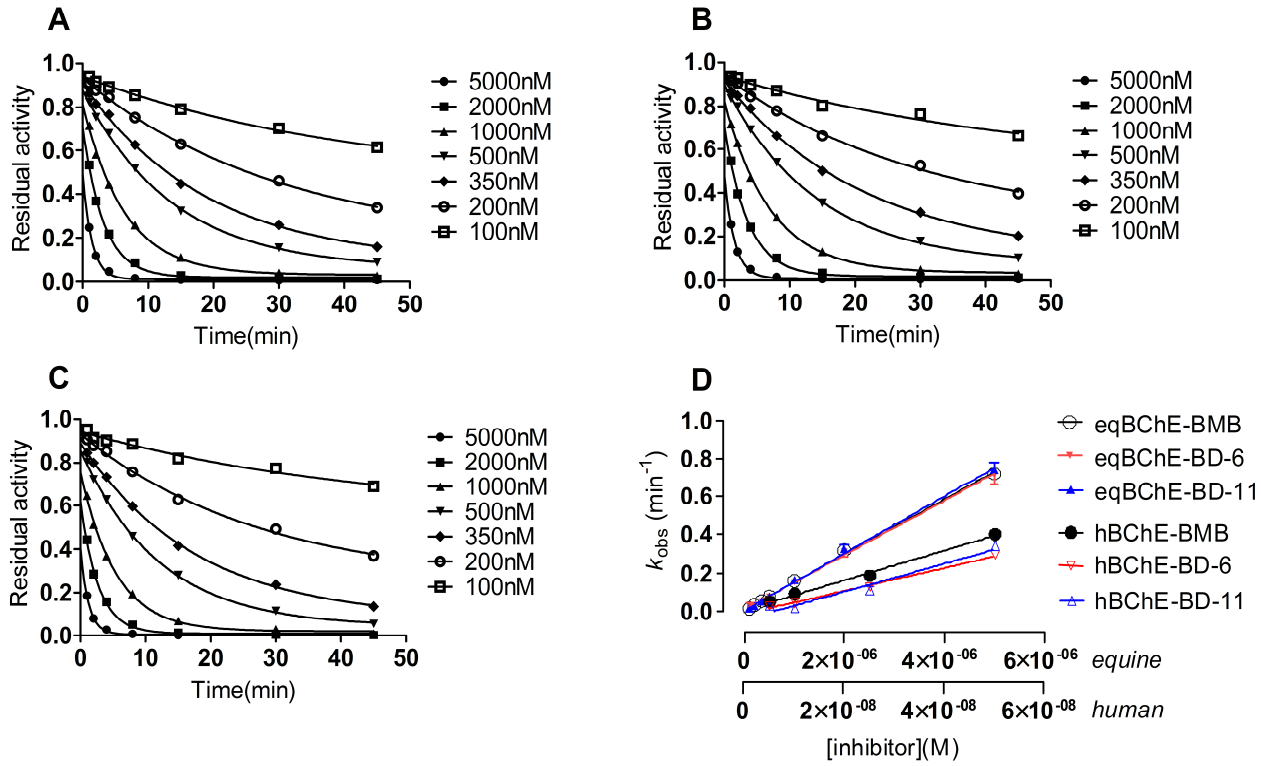


Figure 1. Time and concentration dependent inhibition of equine BChE after pre-incubation with various concentrations of BMB (A), BD-6 (B) and BD-11 (C). Linear correlations of the k_{obs} values obtained for BMB, BD-6 and BD-11 versus their respective concentrations (D).

2.4. Determination of the decarbamylation rate constants of N, N-dimethyl-BChE and N-methyl-N-ehyl-BChE, k_3

The decarbamylation rate constant (k_3) was calculated according to eq. 4[17].

$$\ln[1-(v_i / v_0)] = -k_3 \cdot t \qquad (4)$$

Where v_i and v_0 are the measured activities of the enzyme pre-incubated with the inhibitors or water at a predetermined time points (t) after dilution. $-k_3$ is the slope of linear data regression (Figure 2). The decarbamylation rate constants were obtained by using a single concentration of each inhibitor. This concentration has been selected to be sufficiently high to provide a high initial fractional concentration of carbamylated enzyme, but sufficiently low to avoid that the excess of inhibitor could carbamylate the enzyme while monitoring the decarbamylation process. The inhibition of both equine and human BChE by all three compounds was found to be pseudo irreversible in nature. As expected, the k_3 of N, N-dimethyl-BChE produced by BD-6 and BMB were similar, whereas BD-11 displayed a nearly 10-fold (equine BChE) and 4-fold (human BChE) lower k_3 as compared to others under similar conditions, indicating that BD-11 acted as a longer-term BChE inhibitor with respect to BD-6 and BMB (Table 1). The k_3 values obtained with human BChE were 5-fold (BD-6 and BMB) and 10-fold (BD-11) higher than equine BChE, indicating that the human enzyme recovered more easily than the equine enzyme from the inhibition (Table 1).

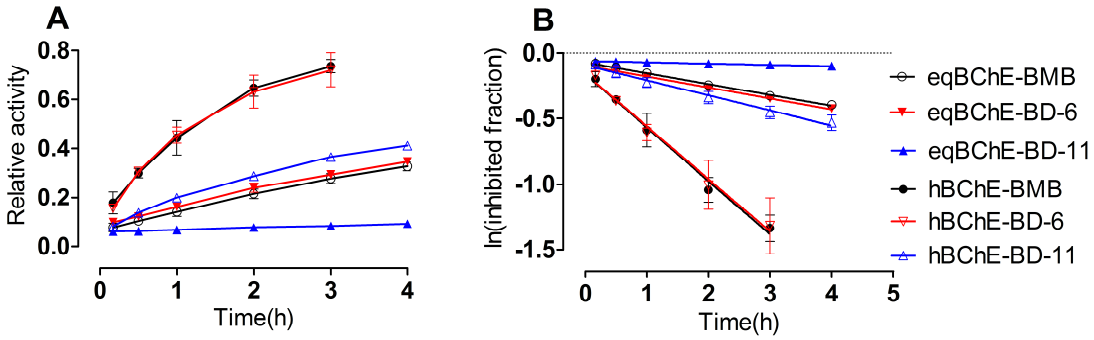


Figure 2: (A) Recovered activities of equine and human BChE after inhibition with BMB, BD-6 and BD-11 respectively. (B) Decarbamylation kinetics of N, N'- dimethylcarbamyl-BChE (BMB and BD-6) and N-methyl-N-ehyl-BChE (BD-11) determined using Eq.4. Data are represented in the form of mean ± SD (n=2-3). The slope of the linear fitting corresponds to -k_3.

2.5. Measurement of Heart rate in anesthetized mice

Heart rates of mice were recorded for every 5 minutes from the 40th minute to 220th minute after anesthesia to procure 36 periods per group (n=6), which were then subtracted from the average heart rate of the anesthetized mice between the 35th to 40th minute to determine the change in heart rates under the influence of the test compounds[24]. We observed a slight decrease in the heart rates of mice of the blank group whereas mice of the group treated with BMB displayed elevated heart rates. Pentobarbital sodium was used as the anesthetic. In spite of the adverse effects of pentobarbital on cardiac haemodynamics and contractile functions, this drug has been widely used as an injectable general anesthetic agent in cardiovascular research[25]. Pentobarbital causes respiratory depression and lowers heart rate, thus explaining the slightly reduced heart rates of mice of the control group. Despite of its effects on cardiac rhythmicity, the mechanism of action of pentobarbital is very different

from BMB and its analogues, thereby enabling us to precisely detect the effects of BMB and its derivatives on heart. In fact, the heart rates of anaesthetized mice increased appreciably after BMB administration. Unpaired t-test revealed that the heart rates (Δ Heart rate) between treatment groups differed substantially from 40 min to 150min after drug administration (Figure 3A). We did not detect any significant difference between the heart rates of mice treated with BD-6 and control (Figure 3B), whereas unpaired t-test between BD-11 and blank groups indicated that heart rates were significantly different from each other (Figure 3C). When the treated groups (BMB, BD-6 and BD-11) were compared with each other we observed a significant difference between the heart rates of mice treated with BMB and BD-6 from 60 min to 125 min (period 5-17) after the intraperitoneal administration (Figure 3D). A noticeable deviation between heart rates of BMB and BD-11 groups was detected between 100-105 minutes (period 13), 115-120 minutes (period 16) and 130-135 minutes (period 19) (Figure 3E). Comparing the heart rates of BD-6 group with BD-11 group, we could find that BD-11 was more potent in triggering heart rate than BD-6 (Figure 3F).

Although there were substantial differences in heart rates between BD-11 and blank groups, the changes were comparatively weaker and more moderate compared with BMB. No major differences were detected between BD-6 and blank groups. The synthesized BMB derivatives BD-6 and BD-11 demonstrated reduced side effects on heart presenting significant advantage over BMB to be used as therapeutic agents.

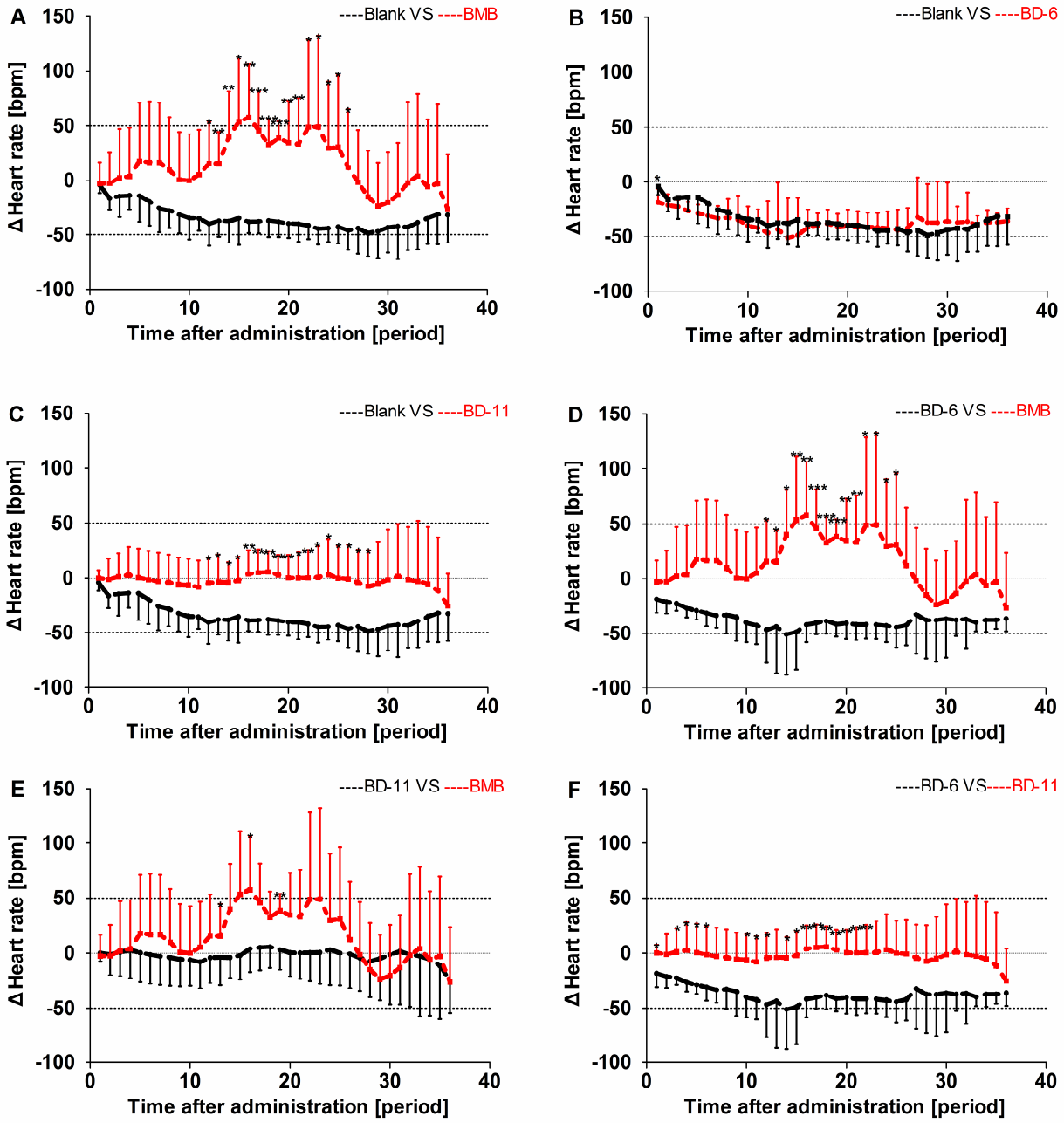
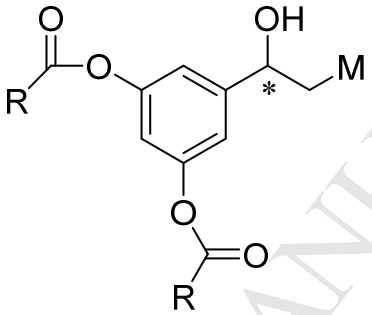


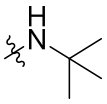
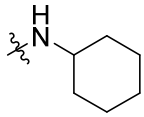
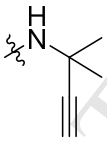
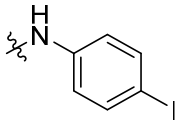
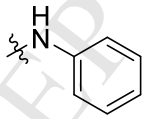
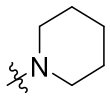
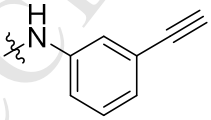
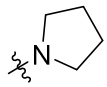
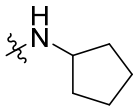
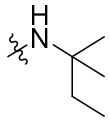
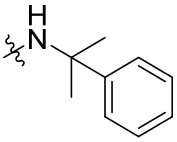
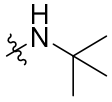
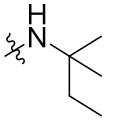
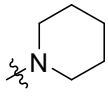
Figure 3: Effects of BMB, BD-6 and BD-11 on heart rates of anesthetized mice. Δ Heart rate is the measure of the balance of average heart rate calculated at each period (after drug administration) from the average heart rate measured in between 35-40 minutes under anesthesia. Each period in the graphs corresponds to a mean HR of five minutes. The data sets obtained for each treatment are depicted in different panels to help visual comparison. (A) Blank *versus* BMB. (B) Blank *versus* BD-6. (C) Blank *versus* BD-11. (D) BMB *versus* BD-6 (E) BMB *versus* BD-11 (F) BD-6 *versus* BD-11. BD-6 had no effects on hearts and followed the same trend as the control group. BMB produced considerable hyper arrhythmia and was more potent in activating cardiac contractions as compared to BD-11. *: $p < 0.05$, **: $p < 0.01$, ***$p<0.001$ w.r.t. the group which was marked in black (n =6, unpaired t-test).

2.6. In-silico chemical analysis

The ability of BMB and its derivatives to penetrate the blood-brain barrier was estimated by calculating their lipophilicity (LogP) using ChemDraw Ultra 14.0. (Table 2). The acid group was ignored for salts. The LogP values of the derivatives were found to be higher than BMB. BD-8 has the highest LogP value, but its inhibitory potential towards BChE was not satisfactory (Table 1). BMB analogue BD-11 displayed a high LogP value of 2.56, second only to BD-8 among the studied compounds, indicating that BD-11 is more likely to cross the blood-brain barrier when compared with BMB and BD-6 [26, 27].

Table 2: *In-silico* analysis of BMB and its analogues.



Compound	R	M	Log P	Compound	R	M	Log P
BMB	-N(Me, Me)		1.4	BD-7	-N(Me, Me)		2.07
BD-1	-N(Me, Me)		1.27	BD-8	-N(Me, Me)		3.54
BD-2	-N(Me, Me)		2.19	BD-9	-N(Me, Me)		1.63
BD-3	-N(Me, Me)		2.34	BD-10	-N(Me, Me)		1.21
BD-4	-N(Me, Me)		1.65	BD-11	-N(Me, Et)		2.56
BD-5	-N(Me, Me)		2.34	BD-12	-N(Me, Et)		2.07
BD-6	-N(Me, Me)		1.88	BD-13	-N(Me, Et)		2.31

*: Chiral center.

2.7. Structure-activity relationship of BMB, BD-6 and BD-11

BMB and all thirteen analogues were metabolized by BChE into monocarbamates. In this section we discuss only about BD-6, BD-11 and BMB. The two-step hydrolysis is illustrated by second-order kinetic constants k_{I1} and k_{I2}, as shown in figure 4. The hydrolysis mechanism in each step consists of two stages. In the first stage, one carbamate group of BD-6, BD-11, BMB or the corresponding monocarbamates is transferred to the active site of the enzyme inhibiting it temporarily and the second stage is characterized by restoration of enzyme activity by removal of the carbamyl group from the active site with a rate constant k_3. The carbamyl-enzyme for BD-6 and BMB are the same species and the final products of the two-step hydrolysis are identical for BD-6 and BD-11.

In our study, we have only measured k_{I1} of various BMB analogues. The k_{I1} of BD-11 was similar to that of BD-6, which led us to infer that the use of the N-methyl-N-ethyl carbamyl group does not decrease the inhibitory potential of BChE compared to the N, N-dimethyl carbamyl group of BMB. Substitution of the tert-butyl group of BMB with a tert-pentyl group (BD-6) led only to a slight decrease in the inhibition potency of BChE, thus also this modification preserved most of the inhibitory properties. The major difference between BMB, BD-6 and BD-11 was in their decarbamylation rate constants k_3. In fact, k_3 of BD-11 was significantly lower than that of BD-6 and BMB, thus suggesting that the modification of the N, N-dimethyl group of BMB can have a significant influence on the recovery time of BChE after inhibition. The 10-fold (equine BChE) and 4-fold (human BChE) lower k_3 measured for BD-11 could also be the result of reinhibition of the enzyme by BD-11 monocarbamate during the recovery experiment. Based on this hypothesis the k_{I2} of the monocarbamate should be higher than the k_{I2} of BMB and BD-6, and perhaps higher than the k_{I1} of BD-11, thus it could be a better inhibitor than any of the biscarbamates studied in this work. This possibility highlights the future necessity to study also the properties of the relative monocarbamate before proposing an optimal drug candidate belonging to this class of molecules.

Theoretically BD-6 and BD-11 should have similar influence on heart as they are metabolized to produce the same final active product. But we observed that BD-11 was more effective in increase the heart rate compared to BD-6. The explanation behind the enhanced activity of BD-11 may lie in the pharmacokinetic profile of these compounds which need to be properly studied to pinpoint the differences.

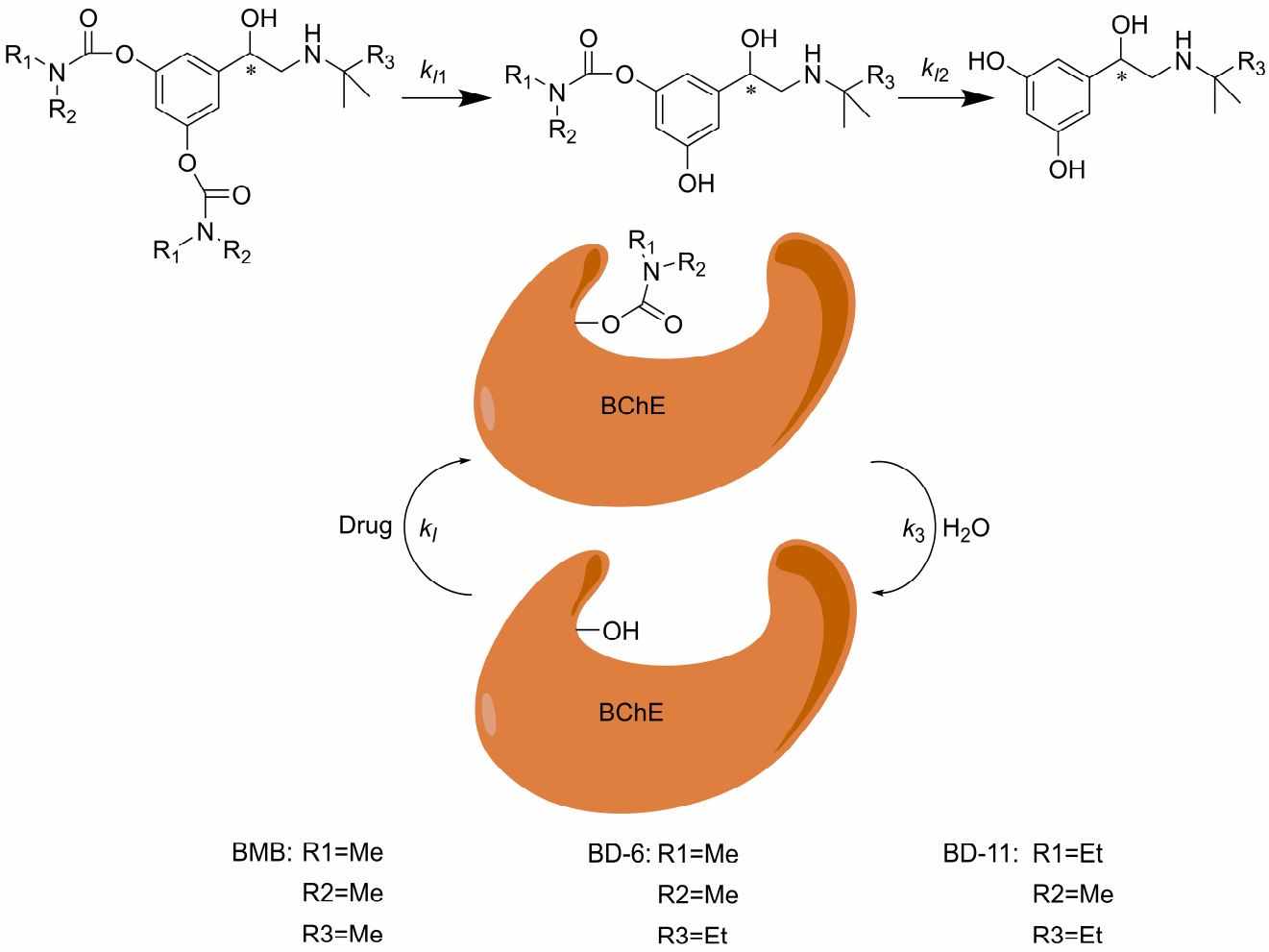


Figure 4: Model of the hydrolysis of BMB, BD-6 and BD-11 by BChE. *: Chiral center.

3. Conclusion

BMB has been widely used in the clinics for over 30 years with no serious side effects. We designed and synthesized 13 BMB analogues and determined their ability to inhibit BChE. We report all BMB derivatives to be specific BChE inhibitors. *In-vivo* mice experiment demonstrated reduced cardiac stress in mice treated with BMB analogues BD-6 and BD-11 as compared to BMB. Moreover, structural modification also reduces their hydrophilicity making them more competent to cross the blood-brain barrier.

BChE is inhibited by BMB during the hydrolytic process. Recent evidences suggest that BChE could be a potential target for treating AD. Adverse drug event (ADE) or drug toxicity remains the foremost reason behind the restricted use of drugs in clinics. One risk free approach of discovering novel drug candidates is to structurally modify established therapeutic agents to increase their efficacies. The most noticeable fallout of BMB has been its influence on heart, which resulted from the pharmacological activity of terbutaline rather than from cholinesterase inhibition. In our study BMB analogues were found to be potent inhibitors of BChE with reduced effect on heart and greater potential ability to permeate the blood-brain barrier. Hence, BMB could be an excellent lead compound to discover novel specific BChE inhibitors for AD treatment.

4. Experimental Section

4.1. Chemistry

IR spectra were recorded using a PerkinElmer FT-IR C93810. ^{1}H-NMR spectra were captured with a Bruker AvanceIII 400MHz or 600MHz spectrometers. ^{13}C-NMR was performed with a Bruker AvanceIII 151MHz or 101MHz spectrometers. All NMR analyses were carried out at room temperature. Chemical shifts were reported as parts per million (ppm) relative to TMS, used as an internal standard for both ^{1}H-NMR and ^{13}C-NMR. Mass spectra were recorded on a Q-TOF mass spectrometer (Synapt G2-S HDMS, Waters) equipped with an ESI source. HPLC was carried out on a SPD-M20A separation system (Shimadzu, Japan), comprising a Shimadzu SPD-M20A detector, a Shimadzu CBM-20A controller, two Shimadzu LC-20AT pumps and a Shimadzu LC Labsolution workstation. TLC was performed on silica F254 and detection done by UV light at 254 nm. Anhydrous solvents were used in all experiments, unless otherwise specificed.

4.2. General procedures for the preparation of BMB analogues

4.2.1. Method 1: preparation of compounds without salt formation.

A mixture of 3, 5-dihydroxyacetophenone (78.9mmol), $K_2CO_3 \cdot 3/2H_2O$ (124.2mmol), K_2CO_3 (34.0mmol) and pyridine (0.5g) in ethyl acetate (40ml) were stirred and heated to 70 ºC. 60 ml solution of acyl chloride (232.5mmol) in ethyl acetate was added drop wise to this mixture and the black suspension was stirred at 70 ºC for 3.5h (dimethylcarbamic chloride) or 24h (ethyl(methyl)carbamic chloride) . 60ml water was added to the mixture and stirred again for 1.5h at 70 ºC. The contents were cooled down to room temperature and the reaction mixture was separated. The organic phase was washed twice with water and twice with dilute sulfuric acid (2% H_2SO_4), dried over $MgSO_4$ and filtered. The filtrate was concentrated to give the product B, which was used in the next step without further purification.

Two equivalents of $CuBr_2$ were mixed with B (20g) in ethyl acetate (100ml) and chloroform (100ml) and stirred at 60 ºC for 5h. The mixture was strained to remove the solid residues and the filtrate was washed with water five times, dried over $MgSO_4$ and filtered again. The filtrate was finally concentrated to yield a yellow solid. The crude product was crystallized using ethyl acetate and petroleum to give pure compound C.

The compound C (5g) was mixed with 2.5 equivalents of $NaBH_4$ and the mixture was dissolved in 80 ml of dichloromethane at room temperature. 20 ml methanol was added to the suspension under ice bath and mixed for 10 minutes. Then the mixture was stirred for 2 hours at 40 ºC. 60 ml of NH_4Cl solution was added to the reaction mixture followed by another round of regulated mixing for 2 hours at room temperature to form distinct aqueous and organic layers. The resulting solution was separated and the water phase was further extracted with dichloromethane twice. The combined organic phase was dried over $MgSO_4$, filtered and concentrated to produce a yellow oil D. The product D was used in the next step without further purification.

D (1g) was mixed with 3 equivalents of amines in 20ml isopropanol and was constantly stirred for 10.5h at 80 ºC. After cooling down to room temperature, the resulting solution was concentrated to generate yellow oil. The oil was treated with water and saturated K_2CO_3 solution to adjust its pH to 9. The residual compound in the water phase was subsequently extracted by washing thrice with dichloromethane (50ml, 50ml and 30ml). The combined organic phase was dried over $MgSO_4$, filtered and concentrated to give yellow oil E. The crude product E was purified by flash column chromatography using ethyl acetate and petroleum ether or dichloromethane and methanol.

4.2.2. Method 2: preparation of compounds with salt formation.

The oil E formed during the process was dissolved in ethanol and the pH of the solution was adjusted to 1 using hydrochloric acid in ethanol. The mixture was stirred continuously at 0 ºC until it precipitated to produce white solid. The precipitate was recrystallized with methanol, ethanol and hexane whereas the fraction which could not form solid was purified by flash column chromatography using dichloromethane and methanol.

4.2.3. 5-(1-hydroxy-2-((2-methylbut-3-yn-2-yl)amino)ethyl)-1,3-phenylene bis(dimethylcarbamate) hydrochloride (BD-1)

The desired product **BD-1** was prepared based on Method 2. Yield: 38%; Mp:232.6-234.0 ºC ; Purity of compound was 99.3%(determined by RP HPLC at 220nm, t_R=16.14min); IR(cm^{-1}): 3243.49, 2933.72, 2702.45, 1711.5, 1619.15, 1562.95, 1451.54, 1385.12, 1314.96, 1298.18, 1267.14, 1158.21, 1096.16, 1069.82, 1026.28, 955.52, 922.16, 888.64, 835.59, 813.86, 754.44, 692.34, 655.93, 639.25, 609.71, 524.29, 464.47 cm^{-1}; ^{1}H NMR (DMSO, 400 MHz) δ= 1.63 (d, J=8.8Hz, 6H, $(CH_3)_2C$), 2.92 (s, 6H, $(CH_3)_2N$), 3.04 (s, 6H, $(CH_3)_2N$), 3.08 (m, 1H, CH_2), 3.19(m, 1H, CH_2), 3.83 (s, 1H, HC≡C), 5.01 (d, J=9.3Hz, 1H, CH) , 6.38(s, 1H, OH), 6.91(t, J=2.1 Hz, 1H, H_{arom}), 7.06(d, J=2.0 Hz, 2H, H_{arom}), 9.18(s, 1H, NH), 10.00(s, 1H, HCl) ppm; ^{13}C NMR (DMSO, 151 MHz) δ=26.11, 36.59, 36.80, 50.26, 53.72, 68.37, 78.49, 82.31, 115.71, 116.55, 144.23, 152.10, 154.10 ppm; TOF MS ES(+)(m/z): calculated for $C_{19}H_{28}N_3O_5$ ($[M+H]^+$) 378.2023; found 378.2028.

4.2.4. 5-(1-hydroxy-2-(phenylamino)ethyl)-1,3-phenylene bis(dimethylcarbamate) (BD-2)

BD-2 was synthesized based on Method 1. Yield: 43%; Purity of compound was 99.6%(determined by RP HPLC at 220nm, t_R=16.97min); IR(cm^{-1}): 3456.04, 3364.91, 2923.5, 1700.98, 1600.28, 1535.57, 1500.42, 1459.61, 1439.42, 1384.37, 1338.27, 1261, 1167.24, 1125.55, 1052.94, 1024.7, 999.19, 987.45, 950.94, 898.01, 863.72, 839.55, 822.75, 747.59, 718.88, 694.87, 669.35, 624.33, 607.85, 563.22, 507, 453.81 cm^{-1}; ^{1}H NMR (DMSO, 600 MHz) δ= 2.91 (s, 6H, $(CH_3)_2NCO$), 3.03 (s, 6H, $(CH_3)_2NCO$), 3.06 (dd, J = 8.3, 4.4 Hz, 1H, CH_2), 3.25 – 3.20 (m, 1H, CH_2), 4.73 (dt, J = 8.1, 4.1 Hz, 1H, CH), 5.50 (s, 1H, NH), 5.60 (d, J=4.5Hz, 1H, OH) , 6.53(t, J=7.2Hz, 1H, H_{arom}), 6.63(d, J=7.7 Hz, 2H, H_{arom}), 6.82(t, J=2.2 Hz, 1H, H_{arom}), 7.02 (d, J = 2.0 Hz, 2H, H_{arom}), 7.07 (dd, J = 8.5, 7.3 Hz, 2H, H_{arom}) ppm; ^{13}CNMR (DMSO, 151MHz) δ=36.58, 36.78, 51.54, 70.52, 112.80, 114.75, 116.32, 116.52, 129.35, 146.82, 149.02, 151.84, 154.21 ppm; TOF MS ES(+)(m/z): calculated for $C_{20}H_{26}N_3O_5$ ($[M+H]^+$) 388.1867; found 388.1877.

4.2.5. 5-(2-((3-ethynylphenyl)amino)-1-hydroxyethyl)-1,3-phenylene bis(dimethylcarbamate) (BD-3)

BD-3 was formulated based on Method 1. Yield: 69%; Purity of compound was 99.3%(determined by RP HPLC at 220nm, t_R=25.60min); IR(cm^{-1}): 3386.47, 3287.18, 2935.41, 1705.87, 1599.12, 1580.39, 1485.08, 1440.07, 1381.08, 1323.42, 1293.35, 1263.27, 1157.7, 1130.55, 1038.52, 1002.11, 955.43, 887.8, 840.32, 819.89, 779.93, 754.77, 688.86, 633.29, 607.86, 563.12, 460.11 cm^{-1}; ^{1}H NMR (DMSO, 600 MHz) δ= 2.91 (s, 6H, $(CH_3)_2NCO$), 3.03 (s, 6H, $(CH_3)_2NCO$), 3.10 – 3.06 (m, 1H, CH_2), 3.27 – 3.19 (m, 1H, CH_2), 3.99(s, 1H, HC≡C), 4.73 – 4.69 (m, 1H, CH),

5.63 (d, J=4.6 Hz, 1H, NH) , 5.79 – 5.75 (m, 1H, OH), 6.63(d, J=7.5 Hz, 1H, H_{arom}), 6.69(dd, J=8.2 and 1.8Hz, 1H, H_{arom}), 6.73 (d, J = 1.8 Hz, 1H, H_{arom}), 6.82(t, J=2.1 Hz, 1H, H_{arom}), 7.01(d, J=2.1 Hz, 2H, H_{arom}), 7.06(t, J=7.9 Hz, 1H, H_{arom})ppm; ^{13}CNMR (DMSO, 151MHz) δ=36.58, 36.78, 51.19, 70.54, 79.68, 84.95, 113.55, 114.78, 115.53, 116.55, 119.64, 122.53, 129.62, 146.66, 149.12, 151.83, 154.20ppm; TOF MS ES(+)(m/z): calculated for $C_{22}H_{26}N_3O_5$ ($[M+H]^+$) 412.1867; found 412.1873.

4.2.6. 5-(2-(cyclopentylamino)-1-hydroxyethyl)-1,3-phenylene bis(dimethylcarbamate) (BD-4)

BD-4 was prepared by Method 1. Yield: 80%; Purity of compound was 96.3%(determined by RP HPLC at 220nm, t_R=3.00min); IR(cm^{-1}): 2946.63, 2867.88, 1716.14, 1615.4, 1439.62, 1378.94, 1292, 1264.36, 1153.56, 1129.3, 1064.09, 1033.3, 1001.98, 953.44, 886.2, 837.38, 818.01, 755.02, 694.19, 608.48, 563.6, 456.45 cm^{-1}; ^{1}H NMR (DMSO, 600 MHz) δ=1.32 – 1.24 (m, 2H, CH_{2ring}), 1.50 – 1.41 (m, 2H, CH_{2ring}) , 1.60 (dd, J = 9.6, 5.2 Hz, 2H, CH_{2ring}), 1.70 (ddd, J = 20.3, 13.0, 6.4 Hz, 2H, CH_{2ring}), 2.55(dd, J=11.8 and 8.8 Hz, 1H, CH_2), 2.65(dd, J=11.9 and 3.7 Hz, 1H, CH_2) , 2.90 (s, 6H, $(CH_3)_2NCO$), 3.03 (s, 7H, $(CH_3)_2NCO$ and CH_{ring}), 4.59(dd, J=8.4 and 3.6 Hz, 1H, CH), 5.41 (s, 1H, OH) , 6.79(t, J=2.1 Hz, 1H, H_{arom}), 6.94(d, J=2.1 Hz, 2H, H_{arom}) ppm; ^{13}CNMR (DMSO, 151 MHz) δ=24.03, 32.96, 33.04, 36.57, 56.45, 59.35, 71.35, 114.52, 116.36, 147.25, 151.77, 154.21ppm;TOF MS ES(+)(m/z): calculated for $C_{19}H_{30}N_3O_5$ ($[M+H]^+$) 380.2180; found 380.2185.

4.2.7. 5-(1-hydroxy-2-((2-phenylpropan-2-yl)amino)ethyl)-1,3-phenylene bis(dimethylcarbamate) (BD-5)

BD-5 was derived using Method 1. Yield: 83%; Purity of compound was 96.1%(determined by RP HPLC at 220nm, t_R=6.73min); IR(cm^{-1}): 3443.86, 2933.3, 1715.9, 1615.69, 1600.09, 1492.77, 1440.99, 1379.42, 1291.92, 1264.09, 1154.32, 1129.81, 1030.71, 1002.02, 954.83, 886.61, 837.17, 818.51, 755.45, 699.84, 608.36, 563.82, 456.64 cm^{-1}; ^{1}H NMR (DMSO, 600 MHz) δ= 1.35(s, 6H, $(CH_3)_2C$), 2.34 (ddd, J = 19.4, 11.4, 6.3 Hz, 2H, CH_2), 2.89 (s, 6H, $(CH_3)_2NCO$), 3.01 (s, 6H, $(CH_3)_2NCO$), 4.52(s, 1H, CH), 5.38 (s, 1H, OH) , 6.77(t, J=2.2 Hz, 1H, H_{arom}), 6.86(d, J=2.1Hz, 2H, H_{arom}), 7.16(t, J=7.3Hz, 1H, H_{arom}), 7.28(t, J=7.7Hz, 2H, H_{arom}), 7.42 – 7.39 (m, 2H, H_{arom}) ppm; ^{13}CNMR (DMSO, 151MHz) δ=29.77, 30.10, 36.56, 36.76, 51.37, 55.64, 72.34, 114.52, 116.42, 126.20, 126.31, 128.42, 147.20, 151.69, 154.19 ppm; TOF MS ES(+)(m/z): calculated for $C_{23}H_{32}N_3O_5$ ($[M+H]^+$) 430.2336; found 430.2343.

4.2.8. 5-(1-hydroxy-2-(tert-pentylamino)ethyl)-1,3-phenylene bis(dimethylcarbamate) hydrochloride (BD-6)

BD-6 was synthesized by Method 2. Yield: 73 %; Mp:195.8-197.2 ºC ; Purity of compound was 99.1%(determined by RP HPLC at 220nm, t_R=3.21min); IR(cm^{-1}): 3362.49, 2928.15, 1709.43, 1618.31, 1464.35, 1385.9, 1294.4, 1261.51, 1159.56, 1095.95, 1070.75, 1033.12, 953.05, 887.31, 836.04, 814.05, 754.25, 694.35, 607.46, 506.91, 474.15 cm^{-1}; ^{1}H NMR (DMSO, 400 MHz) δ= 0.88 (t, J=7.5Hz, 3H, CH_3CH_2), 1.26(s, 6H, $(CH_3)_2C$), 1.67 (q, J = 7.5 Hz, 2H, CH_3CH_2), 2.92 (s, 7H, $(CH_3)_2NCO$ and CH_2), 3.05 (s, 7H, $(CH_3)_2NCO$ and CH_2), 4.96 (d, J = 10.2 Hz, 1H, CH), 6.32 (d, J=3.9 Hz, 1H, OH) , 6.91(t, J=2.1 Hz, 1H, H_{arom}), 7.08(d, J=2.1 Hz, 2H, H_{arom}), 8.34 (s, 1H, HCl), 9.02 (s, 1H, NH) ppm; ^{13}CNMR (DMSO, 151 MHz) δ=8.36, 22.35, 22.47, 30.27, 36.58, 36.79, 48.44,

59.96, 68.45, 115.62, 116.63, 144.28, 152.03, 154.11 ppm; TOF MS ES(+)(m/z): calculated for $C_{19}H_{32}N_3O_5$ ($[M+H]^+$) 382.2336; found 382.2343.

4.2.9. 5-(2-(cyclohexylamino)-1-hydroxyethyl)-1,3-phenylene bis(dimethylcarbamate) (BD-7)

BD-7 was formulated using Method 1. Yield: 81%; Purity of compound was 95.1% (determined by RP HPLC at 220nm, t_R=9.63min); IR(cm^{-1}): 3443.56, 2926.9, 2853.07, 1715.8, 1615.53, 1440.81, 1378.92, 1291.99, 1264.12, 1154.09, 1128.48, 1064.2, 1032.52, 1002.2, 954.36, 886.94, 837.63, 817.83, 754.97, 694.16, 608.81, 563.29, 456.29 cm^{-1}; ^{1}H NMR (DMSO, 600 MHz) δ=1.04 – 0.95 (m, 2H, CH_{2ring}), 1.15 – 1.06 (m, 1H, CH_{2ring}), 1.25 – 1.19 (m, 2H, CH_{2ring},), 1.54 (dd, J = 8.6, 3.7 Hz, 1H, CH_{2ring}), 1.68 – 1.62 (m, 2H, CH_{2ring}) 1.83 – 1.74 (m, 2H, CH_{2ring}), 2.37(m, 1H, CH_{ring}), 2.55(dd, J=11.9 and 8.7Hz,1H, CH_2), 2.69(dd, J=11.9 and 3.9Hz,1H, CH_2) , 2.90 (s, 6H, $(CH_3)_2NCO$), 3.03 (s, 6H, $(CH_3)_2NCO$), 4.56(dd, J=8.5 and 3.7Hz, 1H, CH), 5.38 (s, 1H, OH), 6.78(t, J=2.2Hz, 1H, H_{arom}), 6.94(d, J=2.2Hz, 2H, H_{arom}) ppm; ^{13}CNMR (DMSO, 151MHz) δ=24.87, 26.33, 33.43, 33.66, 36.56, 54.91, 56.16, 71.53, 114.48, 116.36, 147.35, 151.76, 154.21 ppm; TOF MS ES(+)(m/z): calculated for $C_{20}H_{32}N_3O_5$ ($[M+H]^+$) 394.2336; found 394.2342.

4.2.10. 5-(1-hydroxy-2-((4-iodophenyl)amino)ethyl)-1,3-phenylene bis(dimethylcarbamate) (BD-8)

BD-8 was devised based on Method 1.Yield: 69%; Purity of compound was 95.1% (determined by RP HPLC at 220nm, t_R=5.76min); IR(cm^{-1}): 3385.04, 2931.78, 1706.1, 1615.43, 1591.18, 1486.61, 1439.32, 1380.26, 1317.06, 1292.63, 1260.56, 1244.08, 1155.72, 1129.2, 1061.37, 1037.58, 1001.43, 954.93, 887.92, 814.28, 754.25, 694.28, 607.62, 563.56, 504.11, 456.97 cm-1; ^{1}H NMR ($DCCl_3$, 400 MHz) δ= 2.91 (s, 6H, $(CH_3)_2NCO$), 2.99 (s, 6H, $(CH_3)_2NCO$), 3.11 (dd, J=12 and 8Hz,1H, CH_2), 3.23 (dd,J=12 and 4Hz, 1H, CH_2),4.70(dd,J=8 and 4Hz, 1H, CH), 6.39(dd, J = 9.3, 2.3 Hz, 2H, H_{arom}),6.79(t, J=2.1 Hz, 1H, H_{arom}), 6.92(d, J=2.1 Hz, 2H, H_{arom}), 7.36 – 7.31 (m, 2H, H_{arom})ppm; ^{13}CNMR (DMSO, 151MHz) δ= 36.61, 36.81, 51.27, 70.44, 76.59, 114.85, 115.51, 116.58, 137.57, 146.68, 148.78, 151.87, 154.22(Ethyl acetate:14.58, 21.25, 60.25, 170.82); TOF MS ES(+)(m/z): calculated for $C_{20}H_{25}IN_3O_5$ ($[M+H]^+$) 514.0833; found 514.0833.

4.2.11. 5-(1-hydroxy-2-(piperidin-1-yl)ethyl)-1,3-phenylene bis(dimethylcarbamate) (BD-9)

BD-9 was prepared based on Method 1. Yield: 68%; Purity of compound was 92.1% (determined by RP HPLC at 220nm, t_R=18.76min); IR(cm^{-1}): 3443.26, 2933.87, 2855.85, 1718.65, 1615.95, 1599.09, 1489.19, 1440.23, 1377.9, 1292.08, 1264.83, 1242.3, 1152.01, 1129.22, 1033.63, 1001.67, 954.29, 886, 836.63, 817.96, 794.7, 755.18, 732.03, 693.46, 607.72, 563.04, 456.71 cm^{-1}; ^{1}H NMR (DMSO,400 MHz) δ=1.37 (d, J = 5.4 Hz, 2H, piperidine), 1.48 (dd, J = 10.2, 5.0 Hz, 4H, piperidine), 2.37 (ddd, J = 16.9, 12.7, 6.3 Hz, 6H, piperidine and CH_2), 2.90 (s, 6H, $(CH_3)_2NCO$), 3.03 (s, 6H, $(CH_3)_2NCO$), 4.68(dd, J=8.1, 4.4 Hz, 1H, CH), 5.08 (s, 1H, OH), 6.78(t, J=2.2 Hz, 1H, H_{arom}), 6.94(d, J=2.1 Hz, 2H, H_{arom}) ppm. ^{13}CNMR (DMSO, 151MHz) δ=24.43, 26.02, 36.56, 36.76, 54.83, 67.26, 69.39, 114.49, 116.45, 147.38, 151.74, 154.21 ppm; TOF MS ES(+)(m/z): calculated for $C_{19}H_{28}N_3O_5$ ($[M+H]^+$) 380.2180; found 380.2190.

4.2.12. 5-(1-hydroxy-2-(pyrrolidin-1-yl)ethyl)-1,3-phenylene bis(dimethylcarbamate) (BD-10)

BD-10 was synthesized by Method 1. Yield: 71%; Purity of compound was 97.3% (determined by RP HPLC at 220nm, t_R=7.79min); IR(cm^{-1}): 3443.06, 2933.2, 2803.33, 1716.65, 1616.38, 1599.29, 1488.11, 1439.53, 1378.63, 1291.46, 1264.24, 1152.21, 1065.68, 1033.35, 1001.77, 955.03, 887.39, 836.94, 817.88, 754.79, 695.82, 606.83, 563.51, 455.81 cm^{-1}. ^{1}H NMR (DMSO, 400 MHz) δ=1.23 (s, 4H, pyrrolidine), 1.88 (s, 4H, pyrrolidine), 2.91 (s, 6H, $(CH_3)_2NCO$), 3.03 (s, 6H, $(CH_3)_2NCO$), 3.09 (d, J = 9.4 Hz, 2H, CH_2), 4.97(d, J=7.8, 1H, CH) , 6.18 (s, 1H,OH), 6.86(t, J=2.1 Hz, 1H, H_{arom}), 7.03(d, J=2.1 Hz, 2H, H_{arom}) ppm; ^{13}CNMR (DMSO, 101MHz) δ=23.21, 36.58, 36.79, 49.04, 54.18, 115.28, 116.56, 151.96, 154.14ppm; TOF MS ES(+)(m/z): calculated for $C_{19}H_{28}N_3O_5$ ($[M+H]^+$) 366.2023; found 366.2032.

4.2.13. 5-(1-hydroxy-2-(tert-pentylamino)ethyl)-1,3-phenylene bis(ethyl(methyl)carbamate) hydrochloride (BD-11)

BD-11 was derived using Method 2. Yield: 71%; Mp:151.5-153.2 ºC; Purity of compound was 99.8%(determined by RP HPLC at 220nm, t_R=7.25min)); IR (cm^{-1}): 3362.00, 2970.60, 1708.47, 1464.45, 1394.59, 1281.96, 1158.57, 1134.55, 1089.73, 1040.89, 880.44, 754.61cm^{-1}; ^{1}H NMR (DMSO, 400 MHz) δ= 0.88 (t, J=7.5Hz, 3H, CH_3CH_2), 1.14 (dt, J = 31.8, 7.0 Hz, 6H, $(CH_3CH_2)(CH_3)NCO$), 1.26(s, 6H, $(CH_3)_2C$), 1.68(q, J=7.5Hz, 2H, CH_3CH_2), 2.96 – 2.88 (m, 4H, $(CH_3CH_2)(CH_3)NCO$ and CH_2), 3.11 – 3.00 (m, 4H, $(CH_3CH_2)(CH_3)NCO$ and CH_2), 3.33 – 3.28 (m, 2H, $(CH_3CH_2)(CH_3)NCO$), 3.42 (dd, J = 14.0, 6.9 Hz, 2H, $(CH_3CH_2)(CH_3)NCO$), 4.99(d, J = 10.2 Hz, 1H, CH), 6.32 (d, J=4.1Hz, 1H, OH) , 6.90(t, J=2.1Hz, 1H, H_{arom}), 7.06(d, J=1.9Hz, 2H, H_{arom}), 8.38(t, J = 9.6 Hz, 1H, HCl), 9.22(t, J = 9.2 Hz, 1H, NH) ppm. ^{13}CNMR (DMSO, 101MHz) δ=8.36, 12.73, 13.55, 22.40, 22.51, 30.27, 34.06, 34.41, 43.99, 48.25, 60.00, 68.62, 115.69, 116.65, 144.28, 152.03, 153.75ppm; TOF MS ES(+)(m/z): calculated for $C_{19}H_{28}N_3O_5$ ($[M+H]^+$) 410.2649; found 410.2649.

4.2.14. 5-(2-(tert-butylamino)-1-hydroxyethyl)-1,3-phenylene bis(ethyl(methyl)carbamate) hydrochloride (BD-12)

The derivative, **BD-12** was prepared by Method 2. Yield: 61%; Mp: 158.9-160.2 ºC; Purity of compound was 95.9%(determined by RP HPLC at 220nm, t_R=5.44min); IR(cm^{-1}):3346.40, 2978.40, 2786.70, 1711.86, 1396.34, 1281.49, 1159.73, 1135.69, 1090.53, 1074.48, 879.73, 755.57, 691.55 cm^{-1}; ^{1}H NMR (DMSO, 400 MHz) δ= 1.14 (dt, J = 31.6, 6.8 Hz, 6H, $(CH_3CH_2)(CH_3)NCO$), 1.31(s, 9H, $(CH_3)_3C$), 2.87 – 2.94 (m, 4H, $(CH_3CH_2)(CH_3)NCO$ and CH_2), 3.00 – 3.11 (m, 4H, $(CH_3CH_2)(CH_3)NCO$ and CH_2), 3.28 – 3.33 (m, 2H, $(CH_3CH_2)(CH_3)NCO$), 3.42 (dd, J = 13.8, 6.8 Hz, 2H, $(CH_3CH_2)(CH_3)NCO$), 5.00(d, J = 10.1 Hz, 1H, CH), 6.33 (d, J=4.0Hz, 1H, OH) , 6.90(t, J=2.0Hz, 1H, H_{arom}), 7.06(d, J=1.9Hz, 2H, H_{arom}), 8.47(t, J = 9.7 Hz, 1H, HCl), 9.33(t, J = 9.4 Hz, 1H, NH) ppm.; ^{13}CNMR (DMSO, 600MHz) δ=12.73, 13.55, 25.51, 34.07, 34.41, 43.99, 48.42, 56.87, 68.65, 115.68, 116.63, 144.35, 152.03, 153.63ppm; TOF MS ES(+)(m/z): calculated for $C_{19}H_{28}N_3O_5$ ($[M+H]^+$) 396.2493; found 396.2507.

4.2.15. 5-(1-hydroxy-2-(piperidin-1-yl)ethyl)-1,3-phenylene bis(ethyl(methyl)carbamate) hydrochloride (BD-13)

BD-13 was synthesized by Method 2. Yield: 33%; Purity of compound was 96.4%(determined by RP HPLC at 220nm, t_R=4.34min); IR(cm^{-1}): 3232.90, 2935.40, 2536.20, 1712.32, 1394.45, 1283.54,

1251.01, 1155.18, 1129.18, 1080.98, 1035.85, 969.37, 754.50cm^{-1}; ^{1}H NMR (DMSO, 600 MHz) δ= 1.14 (dt, J = 47.2, 6.8 Hz, 6H, (CH_3CH_2)(CH_3)NCO), 1.48 (s, 2H, piperidine), 1.77 (s, 4H, piperidine), 2.90-3.15 (m, 12H, (CH_3CH_2)(CH_3)NCO, piperidine and CH2),3.31 (dd, J = 14.1, 7.1 Hz, 2H, (CH_3CH_2)(CH_3)NCO), 3.41 (dd, J = 13.3, 6.4 Hz, 2H, (CH_3CH_2)(CH_3)NCO), 5.17 (s, 1H,CH), 6.31 (s, 1H,OH), 6.88 (s, 1H, H_{arom}), 7.05 (s, 2H, H_{arom}), 10.11 (s, 1H,HCl)ppm.; ^{13}CNMR (DMSO, 151MHz) δ=12.72, 13.54, 22.11, 22.86, 34.05, 34.42, 43.99, 62.95, 66.79, 115.49, 116.58, 144.68, 151.99, 153.63, 153.75ppm; TOF MS ES(+)(m/z): calculated for $C_{21}H_{34}N_3O_5$ ([M +H]$^+$) 408.2493; found 408.2543.

4.3. Measurement of cholinesterase activity

Modified Ellman`s assay was performed to determine the cholinesterase inhibition ability of the bambuterol derivatives [17, 28]. All experiments were conducted in 96-well plates and the readings were obtained using an EnSpire Multimode 2300 plate reader (Perkin Elmer, MA). AChE (from electric eel), BChE (from equine serum), S-acetylthiocholine iodide (ATCh), S-butyrylthiocholine iodide (BTCh) and 5-5'-dithiobis (2-nitrobenzoic) acid (DTNB) were purchased from Sigma-Aldrich (Shanghai, PRC). Stock solutions of substrates (ATCh or BTCh) were made fresh in water. DTNB (Ellman's reagent) stock solution was freshly prepared in sodium phosphate buffer (100 mM, pH 7, 0.15% $NaHCO_3$ w/v). For IC_{50} measurements with electric eel AChE and equine serum BChE, stock solutions of the test compounds were prepared in a mixture of acetonitrile and water (1:9) and for other assays, the test compounds were dissolved in double distilled water. All the measurements were performed at pH=8.

4.3.1. Modified Ellman`s assay/Cholinesterase assay

200 μL of purified AChE (0.04U/ml) or BChE (0.1 U/mL) in phosphate buffer (sodium phosphate buffer 100 mM, pH=8) were pre-incubated at 37°C with 25 μL solutions of each inhibitor at seven different concentrations. At predetermined time intervals the reactions were terminated by adding 25 μL solution containing the substrate (2.5 mM) and DTNB (3.4 mM) in phosphate buffer (pH 8). The enzyme activity (v_i) was determined by measuring absorbance at 412 nm for 5 min at 37°C. A sample incubated with blank in place of the inhibitor was analyzed in parallel to measure the maximum enzyme activity (v_0) at each incubation time. A blank sample containing phosphate buffer (pH 8) in place of the enzyme was measured for each inhibitor concentration to account for the spontaneous conversion of the substrate into product and the values obtained were subtracted from each measured activity.

4.3.2. Measurement of IC_{50} with electric eel AChE or equine serum BChE

This approach was based on the general method for measuring cholinesterase activity. The residual cholineasterase activity was measured after 60 minutes of pre-incubation of the enzyme with seven different concentrations of the synthesized compounds selected in order to achieve an inhibition between 5% and 95%. The highest concentrations of inhibitors used were 1×10^{-4} M for AChE and 1×10^{-5} M for BChE. Each concentration was analyzed in triplicate.

4.3.3. Measurement of IC_{50} of BMB, BD-6 and BD-11 with human plasma or human erythrocytes

Blood from an apparently healthy individual (25 years, male) was collected and prepared as previously described[29]. Briefly, blood was collected in a heparinized tube and centrifuged at 1000 × g for 20 minutes at 25 °C to separate plasma from the erythrocytes. Plasma served as the source of BChE whereas the sedimented erythrocytes were the source of AChE. The erythrocytes were washed with 0.9 % sodium chloride solution four times prior to storing. Both plasma and erythrocytes were stored at –80 °C until analysis. Cholinesterase assay was performed using diluted plasma (150X) or erythrocytes (600X) to determine the effect of inhibitors on BChE and AChE. The set time was 60min. Inhibitors were used at concentrations of 5×10^{-8} M, 2.5×10^{-8} M, 1×10^{-8} M, 5×10^{-9} M, 2.5×10^{-9} M, 1×10^{-9} M and 5×10^{-10}M for human plasma. For erythrocytes, only one concentration of 1×10^{-5} M, set as therapeutically relevant[30], was used to measure their ability to inhibit human AChE. Since none of the compounds investigated showed to inhibit 50% of the enzyme at this concentration, other concentrations were not tested. Each concentration was analyzed in triplicate. The experiment was performed in accordance to the declaration of Helsinki.

4.3.4. Kinetic analysis of the inhibition of equine serum BChE and human plasma BChE by BMB, BD-6 and BD-11: determination of k_{I1}

The inhibitory enzyme kinetic analysis of BMB and its analogues were determined by measuring the BChE activities at predetermined time intervals of 1min, 2min, 4min, 8min, 15min, 30min and 45min. The concentrations of the inhibitors used were 5×10^{-6} M, 2×10^{-6} M, 1×10^{-6} M, 5×10^{-7} M, 3.5×10^{-7} M, 2×10^{-7} M and 1×10^{-7}M for the experiments with equine BChE and 5×10^{-8} M, 2.5×10^{-8} M, 1×10^{-8} M, 5×10^{-9} M for the experiments with human plasma. The origin and preparation of plasma is described in section 4.3.3.

4.3.5. Determination of the decarbamylation rate constants of N, N-dimethyl-BChE and N-methyl-N-ehyl-BChE by BMB, BD-6 and BD-11: determination of k_3

Decarbamylation rate constants of equine BChE were measured by pre-incubating the enzyme with the inhibitors such that the final concentration of the reaction mixtures was BChE 30U/ml, inhibitors 800nM in 5μL solution. The samples were pre-incubated at 37℃ for 30 minutes followed by the addition of 1.495mL of phosphate buffer (pH 8, 37℃) to each reaction mixture (dilution 1:300). Decarbamylation rate constants of human plasma BChE were measured by pre-incubating 5 μL of plasma with 5 μL of the inhibitor solution in water at room temperature for 30 minutes (final inhibitor concentration: 100nM). An aliquot of 1.463mL of phosphate buffer (pH 8, 37℃) was then added to an aliquot of 7μL of each reaction mixture (dilution 1:210). At set time points 225 μL of each sample was added onto a well of a 96-well plate containing 25 μL substrate (BTCh, 2.5 mM) and DTNB (3.4 mM) mix in phosphate buffer (pH 8). The enzyme activities (v_i) were determined as mentioned above. For each incubation time, a sample pre-incubated with water was analyzed to measure the maximum enzyme activity (v_0). Phosphate buffer (pH 8) was used as a blank to account for the spontaneous conversion of the substrate into product and the values obtained were subtracted from each measured activity.

4.4. Effects of BMB, BD-6 and BD-11 on mice heart rate

All animal experiments were performed in compliance with the guidelines issued by the Ethical Committee for Animal Welfare at the South China University of Technology and Nutrition Guidelines for Care and Use of Laboratory Animals. Male Kunming mice (n=24) purchased from the Experimental Animal Center of Guangzhou University of Chinese Medicine were anesthetized using an intraperitoneal injection of pentobarbital sodium (Anhui Shuanghe Zhiyao Co., Ltd., Anhui, China) 80mg/kg and randomly distributed into four experimental groups. Lead I electrocardiograms (ECG) were recorded and heart rates were determined by counting the R waves in ECG. Test compounds were administered intraperitoneally (20mg/kg body weight). Heart rates (HR) of mice were recorded from 40 minutes prior to drug administration to 180 minutes after the intraperitoneal dose of the test compounds (0-220 minutes). Data were calculated for averaged HR of periods ranging from 35 to 40 min before the administration (baseline) and 36, 5-min long epochs of averaged HR after intraperitoneal injection [24].

4.5. Statistical analysis

Data were presented as mean±SD unless otherwise specified. The results of cholinesterase activities were analyzed by Graph Pad Prism Software. Primary analysis of the parametric data on HR used repeated-measures analysis of variance (ANOVA) via SPSS Statistics 19.0.

4.6. Computational methods

Log P values were calculated using ChemDraw Ultra 14.0. professional drawing tool.

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- The analogues displayed reduced cardiac effects and improved lipophilicity
- Two analogues are promising drugs for treatment of Alzheimer's disease