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Synthesis and anticholinesterase activity of fumaramide derivatives

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Abstract A series of fumaramide derivatives were synthesized from substituted benzanilines and their cholinesterase inhibitory activity was assayed according to Ellman's method using galanthamine-HBr as the reference compound. Most of the fumaramide compounds showed inhibitory activity of both cholinesterase enzymes. Compounds **29** ($IC_{50} = 0.14$ μM) and **30** ($IC_{50} = 16.50$ μM) were found to be the most active inhibitors on acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) enzymes, respectively. Molecular docking studies were performed with Surflex-Dock programme to provide the possible interactions between compounds and enzymes. A Lineweaver–Burk plot and molecular modelling studies showed that fumaramide compounds targeted both the catalytic anionic site and the peripheral anionic site of AChE. It was revealed that the nature of α,β-unsaturated 1,4-diketone moiety in fumaramide compounds brought about useful and efficient modification especially on AChE inhibition.

Keywords Fumaramide · Benzanilines · Acetylcholinesterase · Butyrylcholinesterase · Molecular docking

Introduction

Alzheimer's disease (AD), which is the most common form of dementia, is a progressive and degenerative disorder. It involves a decrease in cognitive functions such as altered memory ability and learning and behavioural disturbances. AD primarily affects the elderly section of the population, specifically people aged 65 or older (Terry and Buccafusco, 2003). Due to its debilitating nature, this disease places an enormous social and economic burden on society. The significance of AD is further compounded, as it is estimated that the number of identified cases will quadruple by the year 2050 (Brookmeyer *et al*., 2007). Medicines created to cure AD have received significant attention for many years. Still, the molecular causes of this condition remain unknown, in spite of the existence of several theories regarding the pathogenesis of AD (Castro *et al*., 2002; Doraiswamy, 2002; Johannsen, 2004; Wilkinson *et al*., 2004).

Acetylcholinesterase (AChE) is a hydrolytic enzyme that acts on ACh to terminate its actions in the synaptic cleft by cleaving the neurotransmitter to choline and acetate (Shen *et al*., 2002). Recent studies have shown that AD pathogenesis is characterized by the rapid loss of AChE activity in the early stages of the disease, along with the increasing ratio of AChE as the disease progresses (Darvesh *et al*., 2003; Greig *et al*., 2005). These results support the need to control the activity of the AChE enzyme at different stages of AD. On this basis, this hypothesis has become the leading strategy for the development of AD drugs. Up to now, AChE inhibition has represented a purely palliative treatment for AD. However, the importance of AChE's sister enzyme, butyrylcholinesterase (BuChE), has also risen as a pharmacological target for AD therapy in recent years. BuChE has been found to be capable of compensating for the missing AChE catalytic functions in the synaptic cleft (Li *et al*., 2000; Mesulam *et al*., 2002) and that its activity significantly increases, by 30–60 %, during a time course in AD (Jhee *et al*., 2002; Perry *et al*., 1978). Therefore, another promising approach is the development of dual inhibitors using AChE and BuChE (Fallarero *et al*., 2008), as BuChE activity seems to

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correlate with AChE activity in AD, such that a cognitive improvement could be reached (Decker *et al*., 2008; Kamal *et al*., 2008). Currently, several AChE inhibitors (such as tacrine, galanthamine, donepezil and rivastigmine) are used for the treatment of AD. However, they can only treat mild to moderate levels of the disease (Grossberg, 2003) and some of the drugs approved for therapeutic use show hepatotoxicity (Knapp *et al*., 1994) and cause gastrointestinal disturbances (Schulz, 2003). There is still a need to develop more efficient drugs for AD.

The X-ray crystallographic structure of AChE has also been reported (Harel *et al*., 1996). The active sites of AChE comprise these binding sites: anionic substrate binding site, such as Trp84, Glu199 and Phe330; an esteratic site that contains the catalytic triad Ser200- His440- Glu327 (Giacobini, 2003); acyl binding site, Phe288 and Phe299, which binds to the acetyl group of ACh (Castro and Martinez, 2001). Besides these, AChE also has a peripheral anionic site, such as Trp279, Tyr70, Tyr121, Asp72, Glu199 and Phe290 (Harel *et al*., 1996; Pang *et al*., 1996; Zhou *et al*., 2008).

It was shown that certain α,β-unsaturated compounds exhibit good inhibition potency towards both AChE and BuChE. These are chalcones (Hasan *et al*., 2005), which show affinities in the micromolar range, as well as *m*- and *p*-aminobenzoic acid maleamides and maleimides, which are potent nanomolar cholinesterase inhibitors (Correa-Basurto *et al*., 2005, 2006, 2007; Trujillo-Ferrara *et al*., 2003). Some of these derivatives act as irreversible inhibitors, probably because of a Michael-type addition of nucleophilic groups present in the active site gorge of AChE (Vitorović-Todorović *et al*., 2010). To date, there have been no reports regarding the cholinesterase inhibitory activity of any fumaramide derivative. In this regard, several new fumaramide compounds bearing α,β-unsaturated 1,4-diketone moiety were constructed and their cholinesterase inhibiton capacities were evaluated using the microplate inhibition assay.

Results and discussion

Synthesis of targeting compounds

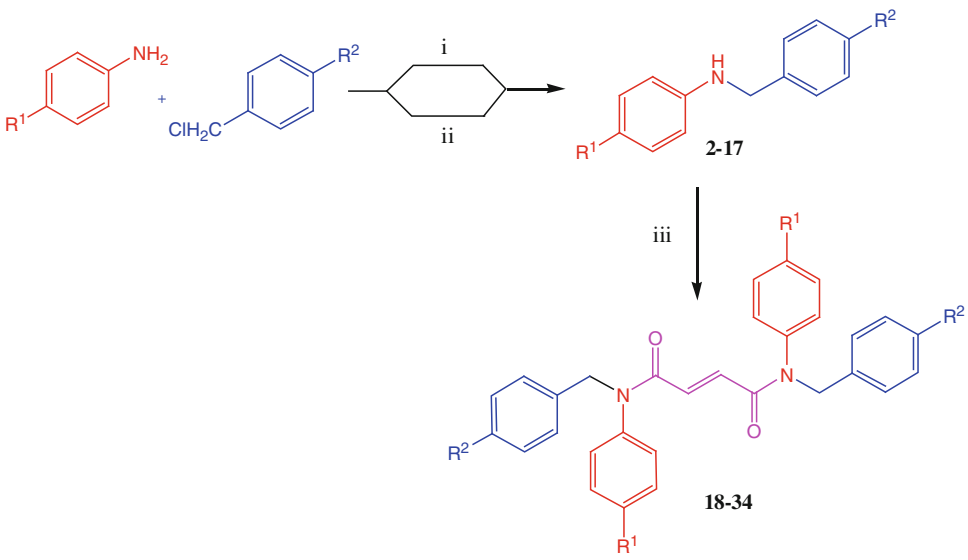
The target fumaramide compounds were synthesized in two steps using the adequate benzanilines **1**–**17** as key intermediates. In the first step, benzanilines were synthesized using microwave heating method under different conditions such as phase-transfer catalysis and the solid supported solvent-free. In the next step, *N*-Alkylation of benzaniline intermediates **1**–**17** with fumaryl chloride in dry ethylacetate in the presence of triethylamine gave targeting fumaramide compounds **18**–**34** in moderate yields (41–72 %). All new fumaramides were fully characterized by ^{1}H and ^{13}C NMR, as well as by high resolution mass spectra and reported for the first time in this study. Amongst the fumaramide series, compounds **23** ($R^1 = OC_2H_5$; $R^2 = CH_3$) and **24** ($R^1 = OC_2H_5$; $R^2 = H$), containing the *p*-ethoxy substituted aniline group, were obtained with the best yields in series (72–71 %). The synthetic pathway is shown in Scheme 1.

In vitro inhibition study

The potency and selectivity of the synthesized compounds **1**–**34** were evaluated by their in vitro inhibitory effects on AChE and BuChE. The inhibitory activities of the compounds against freshly prepared AChE and BuChE were investigated by determining the rate of hydrolysis of acetylthiocholine and butyrylthiocholine in comparison with reference compound galanthamine-HBr, by a modified method of Ellman et al. (1961) as reported (Fawole *et al*., 2010). Primarily, we have chosen to examine the possible AChE inhibitory activity of compounds using thin-layer choromotography/bioautography (TLC-B) assay which was previously described (Marston *et al*., 2002). All of the compounds especially fumaramide derivatives were visualised as white spots on the TLC plates in comparison with the reference compound. TLC-B assay played an important role in monitoring the active compounds. However, this qualitative evaluation gave a positive aspect in the course of the biological study before applying the microplate assay.

Amongst the benzaniline series, **1** and **4** displayed a certain level of inhibitory activity against AChE (IC_{50} values = 1.33–13.01 μM range). *N*-benzylaniline **(1)** and 4-methoxy-*N*-(4-methylbenzyl)aniline **(4)** exhibited weak (IC_{50} = 13.01 μM) and similar (IC_{50} = 1.33 μM) activities on AChE inhibition, respectively, and no inhibition against BuChE (IC_{50} values >100 μM). Merely, 4-ethoxy-*N*-(4-methylbenzyl)aniline (**6**) exhibited potent BuChE inhibitor activity (IC_{50} = 4.07 μM) in the benzaniline series. The other benzaniline compounds in series exhibited no inhibiton of both AChE and BuChE (IC_{50} values >100 μM).

Amongst the fumaramide series, N^1,N^4-bis(4-bromophenyl)-N^1,N^4-bis(4-methylbenzyl) fumaramide (**29**) was found to be the most potent inhibitor on AChE and presented an IC_{50} value of 0.14 μM and superior (169-fold) AChE selectivity. Additionally, N^1,N^4-dibenzyl-N^1,N^4-bis(4-bromophenyl) fumaramide (**30**), which has a similar chemical structure to **29**, was found to be the most potent compound for BuChE inhibition and presented an IC_{50} value of 16.50 μM. Furthermore, N^1,N^4-dibenzyl-N^1,N^4-bis(4-iodophenyl) fumaramide (**34**) was found to be the second most effective inhibitor (IC_{50} = 0.97 μM) and exhibited 24.07-fold selectivity towards AChE. Compounds **23** (IC_{50} = 2.23 μM), **27** (IC_{50} = 2.42 μM), **28** (IC_{50} = 2.53 μM) and **32** (IC_{50} = 4.50 μM) showed moderate inhibition activity toward AChE. Generally,



Scheme 1 Synthesis of the compounds **2–17** and **18–34**. Reagents and conditions: (i) TBAB, K_2CO_3, toluene, microwave (90–360 Watt); (ii) Al_2O_3 (basic), microwave (90–360 Watt); (iii) Triethylamine, fumaryl chloride, 0–5 °C

fumaramide derivatives (except **23**) exhibited slight inhibition activity against BuChE (IC_{50} values = 16.50–89.50 μM range). The IC_{50} values and selectivities of the compounds investigated are summarized in Table 1.

According to the selectivity results, fumaramide compounds have been found to be more selective against AChE than BuChE. Any correlation between both cholinesterase inhibitory activities and the log P values of the compounds or Hammett (σ), Hansch (π) and molar refractivity (MR) values of the aryl substituents were investigated and no correlations were noted ($p \leq 0.05$). Also, further studies are needed to determine the structure activity relationships.

However, the activity results clearly show that almost all of the fumaramides exhibited increased AChE and BuChE inhibitory potency compared to their precursor benzanilines. As an exception, compounds **21** (AChE, IC_{50} = 11.17 μM) and **23** (BuChE, $IC_{50} > 100$ μM) led the decrease of cholinesterase inhibition compared with their starting compounds **4** and **6**, respectively. Although there are these two exceptions, it can be easily said that α,β-unsaturated 1,4-diketone moiety plays a significant role on both cholinesterase inhibition.

Kinetic study of AChE and BuChE

The most potent cholinesterase inhibitors, compounds **29** and **30**, were chosen for kinetic studies with AChE and BuChE to study the inhibitory mechanism of the synthesized fumaramide compounds, respectively. Kinetic analysis of Lineweaver–Burk plots of AChE and BuChE inhibitory activity exhibited that lines crossing the x axis in the same point (unchanged K_m) and decreased V_{max} with increasing inhibitor concentrations. The results indicate non-competitive inhibition and the inhibition data of the compounds are shown in Fig. 1.

Docking study

To understand the binding interactions between compounds **29** and **30**, the most potent inhibitors, molecular docking simulations were performed using Surflex-Dock software. Docking studies indicated that the complex of AChE (PDB code: 1ACJ) and **29**, compound **29** occupied the catalytic anionic site (CAS) and peripheric anionic site (PAS). **29** was bound to CAS with a classic π–π stacking interaction between Phe330 (3.0 Å) and Trp84 (3.3 Å). In the PAS, two hydrogen bond interactions were observed between the carbonyl group of the α,β-unsaturated moiety and the backbone hydroxyl groups of Tyr121 (2.3 Å) and Ser122 (1.6 Å). Similar interactions were found in compound **30**, which was the most potent BuChE inhibitor in complex with HuBuChE (PDB code: 1P0I). Because the crystal structure of BuChE from equine serum had not been reported and the sequences of BuChE from equine showed high homology with human BuChE, the crystal structure of HuBuChE was used in the docking study. A hydrogen bond was found between compound **30** and hydroxyl group of Tyr332 (2.5 Å) and π–π stacking interaction with Trp82 (3.0 Å). Docking models of the compounds are presented in Fig. 2.

Experimental section

Chemistry

The ^1H and ^{13}C Nuclear Magnetic Resonance (NMR) spectra were recorded with tetramethylsilane (TMS) as the internal standard on a Bruker FT-400(100) MHz spectrometer using $CDCl_3$ as the solvent. Coupling constants were given in Hertz (Hz). TOF-Mass (TOF–MS) spectra

Table 1 In vitro inhibition IC_{50} (μM) and selectivity of compounds **1–34** on AChE and BuChE

Compound	R^1	R^2	IC50 (μM)		Selectivity for AChE[c]
			AChE[a]	BuChE[b]	
1	H	H	13.01 ± 5.568	>100	–
2	C_2H_5	CH_3	>100	>100	–
3	C_2H_5	H	>100	>100	–
4	OCH_3	CH_3	1.33 ± 0.577	>100	–
5	OCH_3	H	>100	>100	–
6	OC_2H_5	CH_3	>100	4.07 ± 1.443	–
7	OC_2H_5	H	>100	>100	–
8	CH_3	CH_3	>100	>100	–
9	CH_3	H	>100	>100	–
10	Cl	CH_3	>100	>100	–
11	Cl	H	>100	>100	–
12	Br	CH_3	>100	>100	–
13	Br	H	>100	>100	–
14	F	CH_3	>100	>100	–
15	F	H	>100	>100	–
16	I	CH_3	>100	>100	–
17	I	H	>100	>100	–
18	H	H	11.81 ± 0.850	52.37 ± 0.289	4.43
19	C_2H_5	CH_3	29.38 ± 1.214	44.73 ± 4.571	1.52
20	C_2H_5	H	32.16 ± 1.285	49.27 ± 7.211	1.53
21	OCH_3	CH_3	11.17 ± 0.870	40.97 ± 1.780	3.67
22	OCH_3	H	13.95 ± 0.221	43.60 ± 9.781	3.13
23	OC_2H_5	CH_3	2.23 ± 0.197	>100	–
24	OC_2H_5	H	35.90 ± 0.865	44.03 ± 9.258	1.23
25	CH_3	CH_3	43.08 ± 0.981	54.77 ± 7.948	1.27
26	CH_3	H	12.54 ± 0.758	26.27 ± 1.096	2.09
27	Cl	CH_3	2.42 ± 1.027	45.60 ± 2.606	18.87
28	Cl	H	2.53 ± 0.766	57.67 ± 0.354	22.79
29	Br	CH_3	0.14 ± 0.168	23.10 ± 2.787	169.02
30	Br	H	8.06 ± 0.661	16.50 ± 0.738	2.05
31	F	CH_3	23.90 ± 2.886	56.57 ± 2.325	2.37
32	F	H	4.50 ± 0.971	89.50 ± 7.682	19.89
33	I	CH_3	17.72 ± 2.434	24.63 ± 2.539	1.39
34	I	H	0.97 ± 1.312	23.27 ± 1.168	24.07
Galantamine-HBr			1.02 ± 0.275	14.92 ± 0.252	14.62

[a] 50 % inhibitory concentration (mean ± SD of three experiments) of AChE

[b] 50 % inhibitory concentration (mean ± SD of three experiments) of BuChE

[c] Selectivity for AChE = IC_{50} (BuChE)/IC_{50} (AChE)

were recorded on VG Waters Micromass spectrometer at 70 eV. Melting points of the compounds were obtained on Electrothermal 9100 melting-point apparatus. Benzaniline compounds were synthesized with CEM 3100 microwave oven. Reaction progress and product mixtures were routinely checked by Thin-Layer Chromatography (TLC) on Merck SilicaGel F_{254} aluminium plates. Column chromatography was performed with SiO_2 (70–230 mesh) and Al_2O_3 (neutral). The chemical reagents and solvents used in this study were purchased from Merck or Sigma Aldrich.

General procedure for the synthesis of benzaniline derivatives (**2–17**)

A mixture of substituted aniline (10 mmol), benzyl chloride or *p*-methylbenzyl chloride (5 mmol) was mixed with

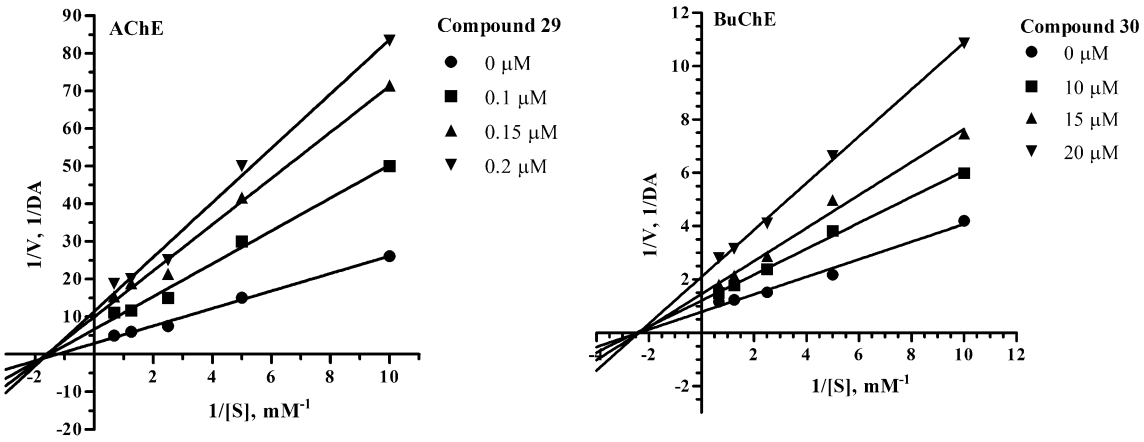


Fig. 1 Lineweaver–Burk plots of the inhibition kinetics of the compounds **29** and **30**

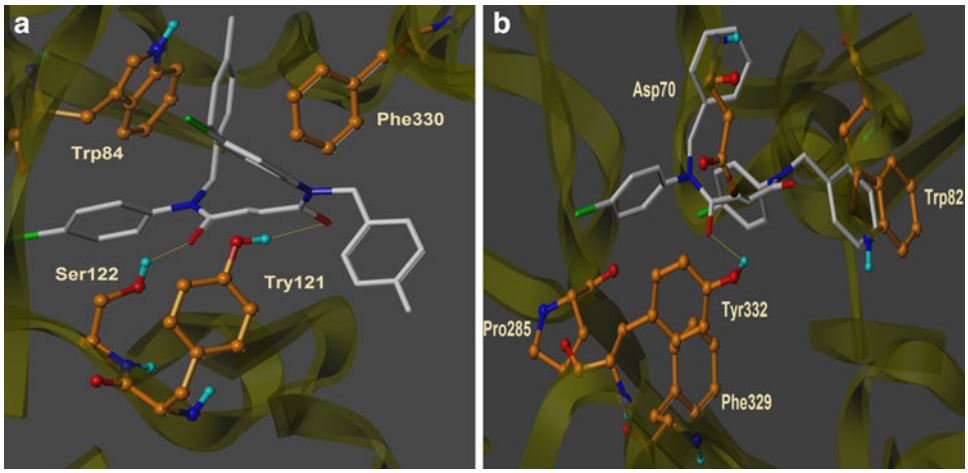


Fig. 2 Docking models (**a**) **29**-AChE complex; (**b**) **30**- HuBuChE complex. Hydrogen bonds are represented as *dots*. The residues are rendered in *orange sticks* (Color figure online)

Al_2O_3 (3 g) under the solvent-free conditions. The resulting fine powder was taken in a 10 ml glass tube with a magnetic stirring bar and subjected to microwave irradiation in microwave oven, in pulses (90–360 W) for a total irradiation time ranging between 2 and 10 min. The reaction was monitored by TLC. After complete conversion, the mass was cooled to room temperature, extracted with dichloromethane (2 × 10 ml) and the combined organic extract was evaporated on rotary evaporator and the crude product was purified by column chromotography on silica gel with hexane:ethyl acetate (8:2) mobile phase. The other synthesis procedure and details were reported in previous study (Yerdelen, 2012).

General procedure for the synthesis of fumaramide derivatives (**18**–**34**)

50 ml two-necked round-bottomed flask, which was equipped with a magnetic stirring bar, was charged with starting benzaniline compound (1.3 mmol), triethylamine (TEA) (1.3 mmol) and dry ethylacetate (5 ml). The mixture was cooled with an ice bath to 0–5 °C and fumaryl chloride (0.65 mmol) in 10 ml dry ethylacetate was added dropwise by syringe over 30 min. The reaction mixture was stirred at room temperature for 12 h. The reaction mixture was quenched with 50 ml of water and the aqueous phase was extracted with two portions of CH_2Cl_2. The combined organic layers were dried over $MgSO_4$, filtered and concentrated by rotary evaporation. The target compounds were purified by SiO_2 or Al_2O_3 (neutral) column chromatography and recrystallized from ethanol.

*N^1,N^4-Dibenzyl-N^1,N^4 diphenylfumaramide (**18**)*

This compound was obtained from *N*-benzylaniline **1** according to general procedure as white solid. Yield 70 %; mp 189–191 °C. The crude compound was purified by column chromatography on SiO_2 eluting with hexane/ethyl acetate (6:4) and recrystallized from ethanol. ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 4.91 (s, 4H, 2× CH_2–N), 6.90 (s, 2H,

fumaryl *CH=CH*), 6.98 (d, $J = 8.1$ Hz, 4H, Ar–H), 7.14-7.16 (dd, $J = 2.6$ and 1.5 Hz, 4H, Ar–H), 7.21–7.24 (m, 4H, Ar–H), 7.31–7.36 (m, 8H, Ar–H). ^{13}C-NMR (100 MHz, $CDCl_3$) 3.76 (CH_2N), 127.72, 128.31, 128.49, 128.67, 128.88, 129.98, 132.37 (*CH=CH*), 137.19, 141.48, 164.61 (*C=O*). TOF–MS ES(+) *m/z* $(M+H)^+$ 447.2031; calcd. for $C_{30}H_{26}N_2O_2 = 447.1994$.

*N^1,N^4-Bis(4-methylbenzyl)-N^1,N^4-bis(4-ethylphenyl)fumaramide (**19**)*

This compound was obtained from 4-ethyl-*N*-(4-methylbenzyl)aniline **2** according to general procedure as white solid. Yield 58 %; mp 174–177 °C. The crude compound was purified by column chromatography on SiO_2 eluting with hexane/ethyl acetate (6:4) and recrystallized from ethanol. ^{1}H-NMR (400 MHz, $CDCl_3$) 1.24–1.26 (t, $J = 3.66$, 6H, 2× CH_2–*CH_3*), 2.28 (s, 6H, 2× *CH_3*-Ph), 2.62–2.65 (q, $J = 3.7$, 4H, 2× *CH_2*–CH_3), 4.83 (s, 4H 2× *CH_2*–N), 6.86–6.90 (m, 4H, Ar–H), 6.91 (s, 2H, fumaryl *CH=CH*), 7.04 (br s, 8H, Ar–H), 7.14 (d, $J = 8.0$ Hz, 4H, Ar–H). ^{13}C-NMR (100 MHz, $CDCl_3$) 15.61 (CH_2–*CH_3*), 22.35 (Ar–*CH_3*), 29.88 (*CH_2*–CH_3), 51.68 (*CH_2*–N), 127.91, 128.34, 128.83, 132.11, 133.40, 134.51 (*CH=CH*), 136.28, 136.41, 143.85, 163.35 (*C=O*). TOF–MS ES(+) *m/z* $(M+H)^+$ 531.2972; calcd. for $C_{36}H_{38}N_2O_2 = 531.2933$.

*N^1,N^4-Dibenzyl-N^1,N^4-bis(4-ethylphenyl)fumaramide (**20**)*

This compound was obtained from *N*-benzyl-4-ethylaniline **3** according to general procedure as white solid. Yield 64 %; mp 151–155 °C. The crude compound was purified by column chromatography on SiO_2 eluting with hexane/ethyl acetate (6:4) and recrystallized from ethanol. ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 1.23-1.26 (t, 6H, 2× *CH_3*, $J = 7.5$ Hz), 2.62–2.67 (q, 4H, 2× *CH_2*, $J = 7.5$ Hz), 4.88 (s, 4H, 2× *CH_2*–N), 6.87–6.89 (d, 4H, $J = 8.4$ Hz, Ar–H), 6.92 (s, 2H, fumaryl *CH=CH*), 7.14–7.17 (m, 8H, Ar–H), 7.21-7.23 (m, 6H, Ar–H). ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 14.96 (CH_2–*CH_3*), 28.22 (*CH_2*–CH_3), 49.82 (*CH_2*–N), 126.84, 126.97, 127.34, 127.93, 128.07, 132.24, 134.47 (*CH=CH*), 136.25, 142.81, 162.48 (*C=O*). TOF–MS ES(+) *m/z* $(M+H)^+$ 503.2601; calcd. for $C_{34}H_{34}N_2O_2 = 503.2620$.

*N^1,N^4-Bis(4-methoxyphenyl)-N^1,N^4-bis(4-methylbenzyl)fumaramide (**21**)*

This compound was obtained from 4-methoxy-*N*-(4-methylbenzyl)aniline **4** according to general procedure as bright yellow solid. Yield 68 %; mp 190–193 °C. The crude compound was purified by column chromatography on SiO_2 eluting with hexane/ethyl acetate (6:4) and recrystallized from ethanol. ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 2.28 (s, 6H, 2× Ar–*CH_3*), 3.80 (s, 6H, 2× O*CH_3*), 4.81 (s, 4H, 2× *CH_2*–N), 6.83–6.87 (m, 10H, Ar–H), 6.89 (s, 2H, fumaryl *CH=CH*), 7.03 (br s, 6H, Ar–H). ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 25.72 (Ar–*CH_3*), 48.40 (*CH_2*–N), 58.49 (O*CH_3*), 118.77, 121.64, 127.81, 128.53, 130.08, 132.74, 135.21 (*CH=CH*), 136.43, 155.81, 165.80 (*C=O*). TOF–MS ES(+) *m/z* $(M+H)^+$ 535.2553; calcd. for $C_{34}H_{34}N_2O_4 = 535.2519$.

*N^1,N^4-Dibenzyl-N^1,N^4-bis(4-methoxyphenyl)fumaramide (**22**)*

This compound was obtained from *N*-benzyl-4-methoxyaniline **5** according to general procedure as white solid. Yield 70 %; mp 199–200 °C. The crude compound was purified by column chromatography on SiO_2 eluting with hexane/ethyl acetate (6:4) and recrystallized from ethanol. ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 3.80 (s, 6H, 2× O*CH_3*), 4.86 (s, 4H, 2× *CH_2*–N), 6.81–6.83 (d, 4H, $J = 7.8$ Hz), 6.85 (s, 2H, fumaryl *CH=CH*), 6.86–6.88 (m, 6H, Ar–H), 7.13–7.15 (dd, 4H, $J = 2.9$ Hz and 1.5 Hz, Ar–H), 7.22 (d, 4H, $J = 6.6$ Hz, Ar–H). ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 46.21 (*CH_2*–N), 55.84 (O*CH_3*), 112.39, 120.19, 125.71, 126.80, 128.56, 132.41, 135.38 (*CH=CH*), 138.29, 159.11, 167.45 (*C=O*). TOF–MS ES(+) *m/z* $(M+H)^+$ 507.2245; calcd. for $C_{32}H_{30}N_2O_4 = 507.2206$.

*N^1,N^4-Bis(4-ethoxyphenyl)-N^1,N^4-bis(4-methylbenzyl)fumaramide (**23**)*

This compound was obtained from 4-ethoxy-*N*-(4-methylbenzyl)aniline **6** according to general procedure as white solid. Yield 71 %; mp 182–184 °C. The crude compound was purified by column chromatography on SiO_2 eluting with hexane/ethyl acetate (6:4) and recrystallized from ethanol. ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 1.40–1.43 (t, 6H, 2× OCH_2–*CH_3*, $J = 6.9$ Hz), 2.28 (s, 6H, 2× *CH_3*), 3.98–4.03 (q, 4H, 2× O*CH_2*CH_3, $J = 6.9$ Hz), 4.80 (s, 4H, 2× *CH_2*–N), 6.78–6.87 (m, 12 H, Ar–H), 7.02 (s, 2H, fumaryl *CH=CH*), 7.05 (d, 4H, $J = 6.8$ Hz, Ar–H). ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 13.25 (OCH_2*CH_3*), 25.63 (Ar–*CH_3*), 41.38 (*CH_2*–N), 63.25 (O*CH_2*CH_3), 108.34, 116.22, 123.49, 125.71, 130.52, 133.25, 135.41 (*CH=CH*), 137.63, 154.39, 165.21 (*C=O*). TOF–MS ES(+) *m/z* $(M+H)^+$ 563.2870; calcd. for $C_{36}H_{38}N_2O_4 = 563.2832$.

*N^1,N^4-Dibenzyl-N^1,N^4-bis(4-ethoxyphenyl)fumaramide (**24**)*

This compound was obtained from *N*-benzyl-4-ethoxyaniline **7** according to general procedure as white solid. Yield 72 %; mp 175–178 °C. The crude compound was purified

by column chromatography on SiO_2 eluting with hexane/ethyl acetate (6:4) and recrystallized from ethanol. ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 1.40–1.43 (t, 6H, 2× OCH_2–CH_3, $J = 6.9$ Hz), 3.98–4.03 (q, 4H, 2× $OCH2$–CH_3, $J = 6.9$ Hz), 4.85 (s, 4H, 2× CH_2–N), 6.88 (s, 2H, fumaryl *CH=CH*), 6.79–6.85 (m, 8H, Ar–H), 7.13–7.15 (dd, 4H, $J = 2.6$ Hz and 1.8 Hz, Ar–H), 7.21–7.24 (m, 6H, Ar–H). ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 14.10 (OCH_2CH_3), 42.49 (CH_2–N), 64.01 (OCH_2CH_3), 110.84, 114.33, 122.21, 125.08, 131.47, 132.14, 135.95 (*CH=CH*), 137.88, 157.60, 163.49 (*C=O*). TOF–MS ES(+) *m/z* $(M+H)^+$ 535.2562; calcd. for $C_{34}H_{34}N_2O_4 = 535.2519$.

*N^1,N^4-Bis(4-methylbenzyl)-N^1,N^4-bis(4-methylphenyl)fumaramide (**25**)*

This compound was obtained from 4-methyl-*N*-(4-methylbenzyl)aniline **8** according to general procedure as white solid. Yield 66 %; mp 205–206 °C. The crude compound was purified by column chromatography on SiO_2 eluting with hexane/ethyl acetate (6:4) and recrystallized from ethanol. δ_H (400 MHz, $CDCl_3$) δ: 2.28 (s, 6H, 2× Benzyl–CH_3), 2.34 (s, 6H, 2× Ar–CH_3), 4.83 (s, 4H, 2× CH_2–N), 6.83 (d, 4H, $J = 8.4$ Hz, Ar–H), 6.85 (d, 4H, $J = 8.4$ Hz, Ar–H), 6.86 (s, 2H, fumaryl *CH=CH*), 7.03 (br s, 4H, Ar–H), 7.11 (d, 4H, $J = 8.0$ Hz, Ar–H). ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 19.64 (Benzyl–CH_3), 21.63 (Ar–CH_3), 45.21 (CH_2–N), 127.65, 128.08, 130.05, 132.28, 134.95, 135.46 (*CH=CH*), 136.25, 137.12, 140.25, 163.91 (*C=O*). TOF–MS ES(+) m/z $(M+H)^+$ 503.2634; calcd. for $C_{34}H_{34}N_2O_2 = 503.2620$.

*N^1,N^4-Dibenzyl-N^1,N^4-bis(4-methylphenyl)fumaramide (**26**)*

This compound was obtained from *N*-benzyl-4-methylaniline **9** according to general procedure as white solid. Yield 60 %; mp 198–200 °C. The crude compound was purified by column chromatography on SiO_2 eluting with hexane/ethyl acetate (6:4) and recrystallized from ethanol. ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 2.34 (s, 6H, 2× CH_3), 4.88 (s, 4H, 2× CH_2–N), 6.83 (s, 2H, fumaryl *CH=CH*), 6.88 (d, 4H, $J = 8.4$ Hz, Ar–H), 7.00 (d, 4H, $J = 8.0$ Hz, Ar–H), 7.04 (d, 4H, $J = 8.0$ Hz, Ar–H), 7.30 (d, 4H, $J = 8.4$ Hz, Ar–H). ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 22.04 (Ar–CH_3), 45.34 (CH_2–N), 125.92, 126.51, 128.54, 129.48, 132.28, 134.98 (*CH=CH*), 135.47, 136.19, 140.11, 159.04 (*C=O*). TOF–MS ES(+) *m/z* $(M+H)^+$ 475.2341; calcd. for $C_{32}H_{30}N_2O_2 = 475.2307$.

*N^1,N^4-Bis(4-chlorophenyl)-N^1,N^4-bis(4-methylbenzyl)fumaramide (**27**)*

This compound was obtained from 4-chloro-*N*-(4-methylbenzyl)aniline **10** according to general procedure as white solid. Yield 52 %; mp 226–229 °C. The crude compound was purified by column chromatography on SiO_2 eluting with hexane/ethyl acetate (6:4) and recrystallized from ethanol. ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 2.29 (s, 6H, 2×CH_3), 4.83 (s, 4H, 2× CH_2–N), 6.83 (s, 2H, fumaryl *CH=CH*), 6.88 (d, 4H, $J = 8.4$ Hz, Ar–H), 7.00 (d, 4H, $J = 8.0$ Hz, Ar–H), 7.04 (d, 4H, $J = 8.0$ Hz, Ar–H), 7.30 (d, 4H, $J = 8.4$ Hz, Ar–H). ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 26.83 (Ar–CH_3), 47.31 (CH_2–N), 122.09, 125.36, 128.98, 130.47, 132.11, 135.28 (*CH=CH*), 136.19, 137.55, 138.01, 165.14 (*C=O*). TOF–MS ES(+) *m/z* $(M+H)^+$ 543.1515; calcd. for $C_{32}H_{28}Cl_2N_2O_2 = 543.1528$.

*N^1,N^4-Dibenzyl-N^1,N^4-bis(4-chlorophenyl)fumaramide (**28**)*

This compound was obtained from *N*-benzyl-4-chloroaniline **11** according to general procedure as white solid. Yield 69 %; mp 229–232 °C. The crude compound was purified by column chromatography on SiO_2 eluting with hexane/ethyl acetate (6:4) and recrystallized from ethanol. δ_H (400 MHz, $CDCl_3$) δ: 4.93 (s, 4H, 2× CH_2–N), 6.90 (s, 2H, fumaryl *CH=CH*), 6.94 (d, 4H, $J = 8.5$ Hz, Ar–H), 7.16–7.18 (dd, 4H, $J = 3.7$ Hz and 2.0 Hz, Ar–H), 7.27-7.28 (m, 6H, Ar–H), 7.34-7.36 (d, 4H, $J = 8.6$ Hz, Ar–H). ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 46.99 (CH_2–N), 123.18, 125.78, 126.93, 127.51, 129.25, 134.35, 135.47 (*CH=CH*), 137.89, 138.46, 164.98 (*C=O*). TOF–MS ES(+) *m/z* $(M+H)^+$ 515.1218; calcd. for $C_{32}H_{24}Cl_2N_2O_2 = 515.1215$.

*N^1,N^4-Bis(4-bromophenyl)-N^1,N^4-bis(4-methylbenzyl)fumaramide (**29**)*

This compound was obtained from 4-bromo-*N*-(4-methylbenzyl)aniline **12** according to general procedure as white solid. Yield 56 %; mp 225–227 °C. The crude compound was purified by column chromatography on SiO_2 eluting with hexane/ethyl acetate (6:4) and recrystallized from ethanol. ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 2.33 (s, 6H, 2× CH_3), 4.88 (s, 4H, 2× CH_2–N), 6.86 (s, 2H, fumaryl *CH=CH*), 6.88 (d, 4H, $J = 7.5$ Hz, Ar–H), 7.05 (d, 4H, $J = 8.1$ Hz, Ar–H), 7.08 (d, 4H, $J = 8.1$ Hz, Ar–H), 7.50 (d, 4H, $J = 8.6$ Hz, Ar–H). ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 25.67 (Ar–CH_3), 44.34 (CH_2–N), 121.39, 125.64, 127.15, 131.01, 132.89, 134.52 (*CH=CH*), 135.91, 136.73, 138.44, 160.01 (*C=O*). TOF–MS ES(+) *m/z* $(M+H)^+$ 631.0515; calcd for $C_{32}H_{28}Br_2N_2O_2 = 631.0518$.

*N^1,N^4-Dibenzyl-N^1,N^4-bis(4-bromophenyl)fumaramide (**30**)*

This compound was obtained from *N*-benzyl-4-bromoaniline **13** according to general procedure as white solid. Yield

52 %; mp 218–220 °C. The crude compound was purified by column chromatography on Al_2O_3 (neutral) eluting with hexane/ethyl acetate (1:1) and recrystallized from ethanol. ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 4.88 (s, 4H, 2× *CH_2*–N), 6.83 (d, 4H, $J = 8.4$ Hz, Ar–H), 6.85 (s, 2H, fumaryl *CH=CH*), 7.12–7.14 (dd, 4H, $J = 3.3$ Hz and 4.0 Hz, Ar–H), 7.23–7.24 (m, 6H, Ar–H), 7.46 (d, 4H, $J = 8.0$ Hz, Ar–H). ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 45.21 (*CH_2*–N), 122.01, 126.14, 126.85, 128.33, 131.45, 133.21 (*CH=CH*), 137.08, 139.43, 165.38 (*C=O*). TOF–MS ES(+) *m/z* $(M+H)^+$ 603.0237; calcd. for $C_{30}H_{24}Br_2N_2O_2 = 603.0205$.

*N^1,N^4-Bis(4-fluorophenyl)-N^1,N^4-bis(4-methylbenzyl)fumaramide (**31**)*

This compound was obtained from 4-fluoro-*N*-(4-methylbenzyl)aniline **14** according to general procedure as white solid. Yield 70 %; mp 189–201 °C. The crude compound was purified by column chromatography on Al_2O_3 (neutral) eluting with hexane/ethyl acetate (1:1) and recrystallized from ethanol. ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 2.29 (s, 6H, 2× *CH_3*), 4.82 (s, 4H, 2× *CH_2*–N), 6.89 (s, 2H, fumaryl *CH=CH*), 6.89–6.93 (dd, 4H, $J = 4.8$ Hz and 4.7 Hz, Ar–H), 7.00 (d, 4H, $J = 6.9$ Hz, Ar–H), 7.03–7.05 (m, 8H, Ar–H). ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 21.06 (Ar–*CH_3*), 48.39 (*CH_2*–N), 113.27, 122.18, 127.81, 133.25, 133.76, 134.58 (*CH=CH*), 136.13, 159.18, 164.26 (*C=O*). TOF–MS ES(+) *m/z* $(M+H)^+$ 511.2142; calcd. for $C_{32}H_{28}F_2N_2O_2 = 511.2119$.

*N^1,N^4-Dibenzyl-N^1,N^4-bis(4-fluorophenyl)fumaramide (**32**)*

This compound was obtained from *N*-benzyl-4-fluoroaniline **15** according to general procedure as white solid. Yield 70 %; mp 201–203 °C. The crude compound was purified by column chromatography on Al_2O_3 (neutral) eluting with hexane/ethyl acetate (1:1) and recrystallized from ethanol. ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 4.87 (s, 4H, 2× *CH_2*–N), 6.84 (s, 2H, fumaryl *CH=CH*), 6.90–6.94 (dd, 4H, $J = 4.7$ Hz and 5.8 Hz, Ar–H), 7.03 (d, 4H, $J = 8.4$ Hz, Ar–H), 7.13 (d, 4H, $J = 6.9$ Hz, Ar–H), 7.22–7.26 (m, 6H, Ar–H). ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 47.23 (*CH_2*–N), 114.33, 123.21, 125.92, 126.78, 128.19, 132.54 (*CH=CH*), 135.98, 134.75, 162.71, 168.18 (*C=O*). TOF–MS ES(+) *m/z* $(M+H)^+$ 483.1853; calcd. for $C_{30}H_{24}F_2N_2O_2 = 483.1806$.

*N^1,N^4-Bis(4-iodophenyl)-N^1,N^4-bis(4-methylbenzyl)fumaramide (**33**)*

This compound was obtained from 4-iodo-*N*-(4-methylbenzyl)aniline **16** according to general procedure as white solid. Yield 45 %; mp 247–250 °C. The crude compound was purified by column chromatography on Al_2O_3 (neutral) eluting with hexane/ethyl acetate (1:1) and recrystallized from ethanol. ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 2.29 (s, 6H, 2× *CH_3*), 4.83 (s, 4H, 2× *CH_2*-N), 6.69 (d, 4H, $J = 8.4$ Hz, Ar–H), 6.84 (s, 2H, fumaryl *CH=CH*), 7.00 (d, 4H, $J = 8.4$ Hz, Ar–H), 7.04 (d, 4H, $J = 8.1$ Hz, Ar–H), 7.65 (d, 4H, $J = 8.4$ Hz, Ar–H). ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 27.34 (Ar–*CH_3*), 45.69 (*CH_2*–N), 105.27, 125.23, 127.65, 128.03, 132.14, 136.28 (*CH=CH*), 136.43, 137.21, 139.64, 162.39 (*C=O*). TOF–MS ES(+) *m/z* $(M+H)^+$ 727.0255; calcd. for $C_{32}H_{28}I_2N_2O_2 = 727.0240$.

*N^1,N^4-Dibenzyl-N^1,N^4-bis(4-iodophenyl)fumaramide (**34**)*

This compound was obtained from *N*-benzyl-4-iodoaniline **17** according to general procedure as white solid. Yield 41 %; mp 259–261 °C. The crude compound was purified by column chromatography on Al_2O_3 (neutral) eluting with hexane/ethyl acetate (1:1) and recrystallized from ethanol. ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 4.88 (s, 4H, 2× *CH_2*–N), 6.70 (d, 4H, $J = 8.1$ Hz, Ar–H), 6.87 (s, 2H, fumaryl *CH=CH*), 7.12–7.14 (m, 4H, Ar–H), 7.23–7.24 (m, 6H, Ar–H), 7.66 (d, 4H, $J = 8.4$ Hz, Ar–H). ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 44.86 (*CH_2*–N), 101.47, 123.55, 125.12, 125.98, 128.51, 133.28 (*CH=CH*), 136.24, 136.98, 138.23, 161.95 (*C=O*). TOF–MS ES(+) *m/z* $(M+H)^+$ 698.9948; calcd. for $C_{30}H_{24}I_2N_2O_2 = 698.9927$.

Pharmacological activity

TLC-B assay for acetylcholinesterase inhibition

Benzaniline and fumaramide compounds (10 µl, 10 mg/ml) and galanthamine-HBr (5 µl, 0.2 mM) were each applied to Silica gel 60 F254 on a aluminium plate (10 cm × 20 cm) (Merck, Germany) and developed with chloroform: methanol (80:20) in a pre-saturated chromatographic chamber. After drying, the TLC plate was sprayed with 5 mM ATCI and 5 mM DTNB dissolved in buffer until the TLC plate was saturated. The plates were allowed to dry slightly and then sprayed with 3 U/ml AChE dissolved in a buffer. After 2–5 min, a yellow background appeared with white spots indicating the presence of AChE inhibiting compounds. The white spots were observed and recorded within 5 min.

Inhibition studies on AChE and BuChE

Acetylcholinesterase (AChE, E.C. 3.1.1.7, from electric eel), butyrylcholinesterase (BuChE, E.C. 3.1.1.8, from equine serum), 5,5′-dithiobis-(2-nitrobenzoic acid) DTNB, acetylthiocholine iodide (ATCI) and butyrylthiocholine iodide (BTCI) were purchased from Sigma Aldrich. Inhibitory activities of AChE and BuChE of the test compounds were evaluated by colorimetric Ellman's method with some

modifications using commercially available galantamine-HBr as the reference compound. The test compounds were dissolved in ethanol/dimethylsulphoxide (1:1) and then diluted in 50 mM KH_2PO_4/K_2HPO_4 phosphate buffer (pH 8.0) to provide a final concentration range. In a 96-well plate, the assay medium in each well consisted of 50 μl of a phosphate buffer, 125 μl of 3 mM DTNB (Ellman's reagent), 25 μl of 0.2 U/ml enzyme (AChE or BuChE) and 15 mM substrate (ATCI or BTCI). The assay mixture containing enzyme, buffer, DTNB and 25 μl of inhibitor compound was preincubated for 15 min at 37 °C, before the substrate was added to begin the reaction. Galantamine-HBr and all compounds were prepared at different concentrations such as 0.195, 0.39, 0.78, 1.56, 3.13, 6.25, 12.5, 25, 50 and 100 μg/ml. The absorbance of the reaction mixture was then measured three times at 412 nm every 45 s using a microplate reader (Bio-Tek ELx800, USA). Results are presented as means ± standard errors of the experiment. The IC_{50} values of the compounds showing percentage inhibition >50 %; the measurements and calculations were evaluated by non-linear regression analysis using GraphPad Prism software programme.

Kinetic characterization of AChE and BuChE inhibition

The mechanism of AChE and BuChE inhibition was investigated in kinetic studies using Ellman's test. Compounds **29** and **30** were selected for kinetic measurements because they were found to have the highest inhibitory activity against AChE and BuChE, respectively. The test was carried out without the inhibitor and in 0.1, 0.15 and 0.2 μM concentrations of the inhibitor for AChE and 10, 15 and 20 μM concentrations for BuChE. Substrate concentrations were varied from 0.1 to 1.5 mM. The obtained data were used to create substrate-velocity curves, which were transformed in GraphPad Prism programme to Lineweaver–Burk plots.

Molecular modelling

The docking study was performed using Surflex-Dock in Sybyl-X 2.0 by Tripos Associates. 3D structures of the compounds **29** and **30** were constructed using the Sybyl sketcher module of Sybyl-X 2.0. The structures were minimized using the Steepest descent conjugated gradient method until the gradient was 0.001 kcal/mol, max iterations: 5000 with the Tripos force field with the Gasteiger Huckel charge. The simulation system was built on the crystal structures of 1ACJ and 1P0I which were obtained from the Protein Data Bank. At the commencement of docking, all the water and ligands were removed and the random hydrogen atoms were added. Docking calculations using Surflex-Dock for 1ACJ and 1P0I were performed through protomol generation by ligand. The parameters used were threshold 0.5 and bloat 0.

Conclusion

In summary, seventeen new fumaramide derivatives (**18**–**34**) were synthesized and tested towards AChE and BuChE. Assuming biological data, all fumaramides have shown moderate to high antiacetylcholinesterase activity in which compounds **29** and **34** were the most potent inhibitors acting in low micromolar concentrations. All synthetic fumaramide compounds (except **23**) had a very good selectivity for AChE. Conversely, most of the fumaramide compounds exhibited more powerful and selective inhibitory activity than the precursor benzanilines against both the cholinesterase enzymes.

Docking studies were performed with the Surflex-Dock programme in order to clarify the cholinesterase inhibitory activities of the compounds (**29**, **30**) and to provide the ideal interaction mode of the compounds in the binding sites of the related enzymes. Accordingly, molecular modelling simulations of AChE and BuChE inhibitor complexes showed that the nature of α,β-unsaturated 1,4-diketone moiety in fumaramide derivatives obviously contributed to the inhibitory activities through interacting with enzymes. In addition, kinetic studies indicated that these inhibitory compounds exhibited non-competitive inhibition against both types of cholinesterase enzymes. To the best of our knowledge, cholinesterase inhibitors based on fumaramide scaffold have not been previously reported, and further investigations of these compounds are in progress.

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