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Searching for the Multi-Target-Directed Ligands against Alzheimer's disease: Discovery of quinoxaline-based hybrid compounds with AChE, H_3R and BACE 1 inhibitory activities

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ABSTRACT

A novel series of quinoxaline derivatives, as Multi-Target-Directed Ligands (MTDLs) for AD treatment, were designed by lending the core structural elements required for H_3R antagonists and hybridizing BACE 1 inhibitor **1** with AChE inhibitor BYYT-25. A virtual database consisting of quinoxaline derivatives was first screened on a pharmacophore model of BACE 1 inhibitors, and then filtered by a molecular docking model of AChE. Seventeen quinoxaline derivatives with high score values were picked out, synthesized and evaluated for their biological activities. Compound **11a**, the most effective MTDL, showed the potent activity to H_3R/AChE/BACE 1 (H_3R antagonism, IC_{50} = 280.0 ± 98.0 nM; H_3R inverse agonism, IC_{50} = 189.3 ± 95.7 nM; AChE, IC_{50} = 483 ± 5 nM; BACE 1, 46.64 ± 2.55% inhibitory rate at 20 μM) and high selectivity over $H_1R/H_2R/H_4R$. Furthermore, the protein binding patterns between **11a** and AChE/BACE 1 showed that it makes several essential interactions with the enzymes.

1. Introduction

Alzheimer's disease (AD), the most common form of dementia, is a complex neurodegenerative disorder.[1,2] The fact that complexity and multiple etiologies of AD make single-target strategy difficult to shed desirable therapeutic effect makes the choice of Multi-Target-Directed Ligand (MTDL) to be a potentially more effective strategy.[3] MTDL, whose goal is to enhance efficacy and improve safety, is rationally designed to hit multiple targets for a particular disease to improve pharmacological profiles.[4–6]

Until now, most drugs approved for AD treatment are AChE inhibitors, which improve the ACh level in the brain by decreasing the hydrolysis of ACh. In contrast, histamine H_3 receptor antagonists can also increase ACh via a distinct mechanism. Since activated presynaptic histamine H_3 receptor decreases the release of ACh from cholinergic neurons and H_3 receptor antagonists increases ACh release, the approach of integrating AChE inhibitors and H_3R antagonists, both contribute to the same result of increasing synaptic levels of ACh although through different mechanisms, may form synergistic effect in the treatment of AD.[7,8]

Amyloid-β (Aβ), formed by the continuously proteolytic processing of β-amyloid precursor protein (APP) by β and γ-secretase,[9,10] plays a central role in the pathogenesis of AD. Recent evidence indicated certain links between Aβ and AChE.[11] On one hand, the presence of Aβ increases AchE activity, and on the other hand, AChE could form a complex with Aβ, which changes the conformation of Aβ and then promotes the aggregation Aβ. Thus, simultaneously inhibition of AChE and β-secretase (BACE 1) not only reduces Aβ generation and hydrolysis of ACh but also weakens the interaction between ACh and Aβ.

Previously, we reported the establishment of a pharmacophore model for BACE 1 inhibitor, Hypo 1, which was validated by Enrichment and ROC method.[12] We also reported the potent dual-site binding AChE inhibitor BYYT-25 (IC_{50} = 0.05 μM) with the interaction between pyrrolidine and center active site.[13]

Considering the above, we focused on Multi-Target-Directed Ligands which have effects on AChE, H_3R and BACE 1 in this study. According to reported studies, H_3R antagonists may contain a basic center, a ring core and a linker between the two centers.[14] Based on previous work, 2-amino-3,4-dihydroquinazoline of BACE 1 inhibitor **1** which has an IC_{50} around 0.011 μM was selected as the core ring moiety of H_3R antagonist, and benzyl pyrrolidine fragment of BYYT-25 was chosen to function as the basic center of H_3R to

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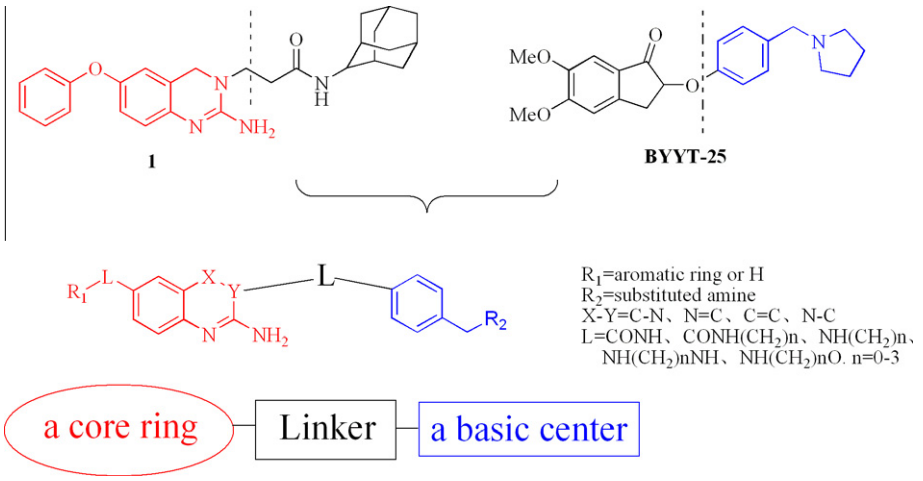


Figure 1. Structure of the parent compounds, compound (**1**) and BBYT-25, and the common structure for compounds included in the virtual database.

design the Multi-Target-Directed Ligands with new scaffold (Fig. 1). A virtual database consisting of new scaffold compounds was built and screened on pharmacophore model of BACE 1 inhibitor which was built in previous work,[12] and then filtered by a molecular docking model of AChE inhibitor. According to the screening result, 17 quinoxaline derivatives were picked out, synthesized, and subsequently evaluated for their AChE/BACE 1 inhibitory activity and H_3R antagonistic action.

2. Materials and methods

2.1. Data preparation

Eighteen AChE inhibitors as the positives for the molecular docking model study were collected from literatures,[13,15] and 1200 negatives were retrieved from Available Chemicals Directory (ACD) database using the Random Percent Filter protocol within PipelinePilot. The test set contains 18 positives and 1200 negatives. All compounds were optimized by Discovery Studio 2.1 software package.

2.2. The pharmacophore model of BACE 1 inhibitor

The pharmacophore model of BACE 1 inhibitor (Hypo 1) was built in a previous work.[12]

2.3. The molecular docking model of AChE inhibitor

Discovery Studio 2.1/Ligandfit was employed to construct the molecular docking model of AChE inhibitor by using the crystal structure of E2020/AChE complex (PDB ID: 1EVE). First, docking and subsequent scoring were performed using default parameters except for the smart Minimization Algorithm process, followed by the best conformation selection based on score values calculated using scoring functions. The parameters for docking and scoring were then changed until the best conformation of docked E2020 was similar with that of E2020 in the crystal structure.

3. Results and discussion

3.1. Molecular docking model validation

The best score function LigScore1 was used to evaluate the result for the docking study. The validation of the molecular docking model was ascertained by the Enrichment Factor (EF) and receiver operating curve (ROC) method.[12] The EF of test set consisting of 1200 and 18 compounds screened by the molecular docking model at 2%, 5% and 10% were 67.6, 18.3 and 8.7, respectively. In addition, the ROC curve of the test set screening by the molecular docking model (Fig. 2) and an AUC of 0.91 meant that a selected randomly active compound has a higher score than a randomly selected inactive compound, 9.1 times out of 10. The results of the EF and ROC metric suggested that the molecular docking model would be a valuable and reliable tool in identifying compounds with AChE inhibitory activity.

3.2. Build in-house database and virtual screening

The virtual database consisting of new scaffold compound was built using the Build 3D database protocol within the Pipeline Pilot software. Various stereoisomers protocol was used to expend the database, which contains 222291 compounds. Followed Lipinski filtration ($A\log P$ was changed as $\geqslant 2$, with the remaining parameters were set as default) led to a reduced set of 118232 compounds. All selected compounds were then optimized by using the Discovery Studio 2.1 software package.

Discovery Studio 2.1/Ligand Pharmacophore Mapping protocol was used to screen the designed database using Hypo 1 as the template. Thousand two seventy three compounds with high Fitvalues (bigger than 3.00, the maximum of the Fitvalue is 5.00) were picked out and screened by the molecular docking model of AChE inhibitor. Finally, 17 compounds were selected based on LigScore1 values and synthesized.

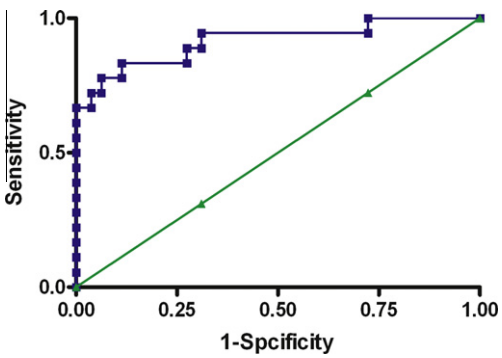


Figure 2. The receiver operating curve of the molecular docking model (Blue curve, test set ROC; green curve, ROC for the random classification of the compounds).

3.3. Chemistry

The synthetic route for **11a–f** is illustrated in Scheme 1. Reaction of methyl 4-(bromomethyl)benzoate with piperidine or pyrrolidine in refluxing CH_2Cl_2 gave **2**, followed by hydrolysis to yield substituted benzoic acid **3**. Condensation of **3** or **4** with 2,3-diaminoquinoxaline in the presence of EDC/HOBt resulted in compounds **11a–c**. Furthermore, compounds **11a,b** were salted using HCl saturated ethylacetate to give target compounds **11d,e**. In addition, further reduction of amide group of compound **11a** afforded compound **11f**.

On the other hand, reaction of 2-chloroacetyl chloride with **5** gave **6** in good yields. Treatment of **7** with 1,2-dibromoethane or 1,3-dibromopropane afforded compounds **8a–d**. Then reaction of **6, 8** with 2,3-diaminoquinoxaline in the presence of NaH in DMF gave the target compounds **11g–m** (Scheme 2).

Condensation of 3-aminoquinoxaline-2-carboxylic acid with **5** in the presence of EDC/HOBt afforded target compounds **11n–p**. In addition, **11o** could be further transformed into **11q** by treatment with aluminum lithium hydride. Reaction of 2,3-diaminoquinoxaline with 4-(piperidin-1-ylmethyl)benzaldehyde **10** in the presence of EDC gave compound **11r** (Scheme 3).

3.4. Biological activity and SAR

H_3R antagonistic action and H_3R inverse agonist action of synthesized compounds were tested by using CRE-luciferace transcription assay with thioperamide as the reference standard. AChE inhibitory activities were evaluated according to a modified Ellman method with donepezil as the reference standard and BACE 1 inhibitory activities were assayed using fluorescence resonance energy transfer (FRET) assay with OM99-2 as the reference standard. The results are summarized in Table 1.

3.4.1. H_3R antagonistic and inverse agonistic action

As shown in Table 1, six compounds showed equivalent or more potent H_3R antagonistic activities in comparison with that of thioperamide. In addition, three compounds (**11a,b,p**) displayed potent H_3R inverse agonistic activity which may be beneficial for the treatment of AD. Compound **11j** exhibited the most potent H_3R antagonistic action with an IC_{50} value of 52.4 ± 23.6 nmol/L, and **11d** showed the most effective inverse agonistic activity with an IC_{50} value of 170.6 ± 98.7 nmol/L. Compound **11a** with a piperidine ring at the end of benzyl exhibited more potent H_3R antagonistic activity over that with an inserted piperidine moiety between quinoxaline and benzyl fragments (**11c**). Similarly, compounds **11n** and **11i**, containing inserted piperidine moiety, showed no H_3R antagonistic activity.

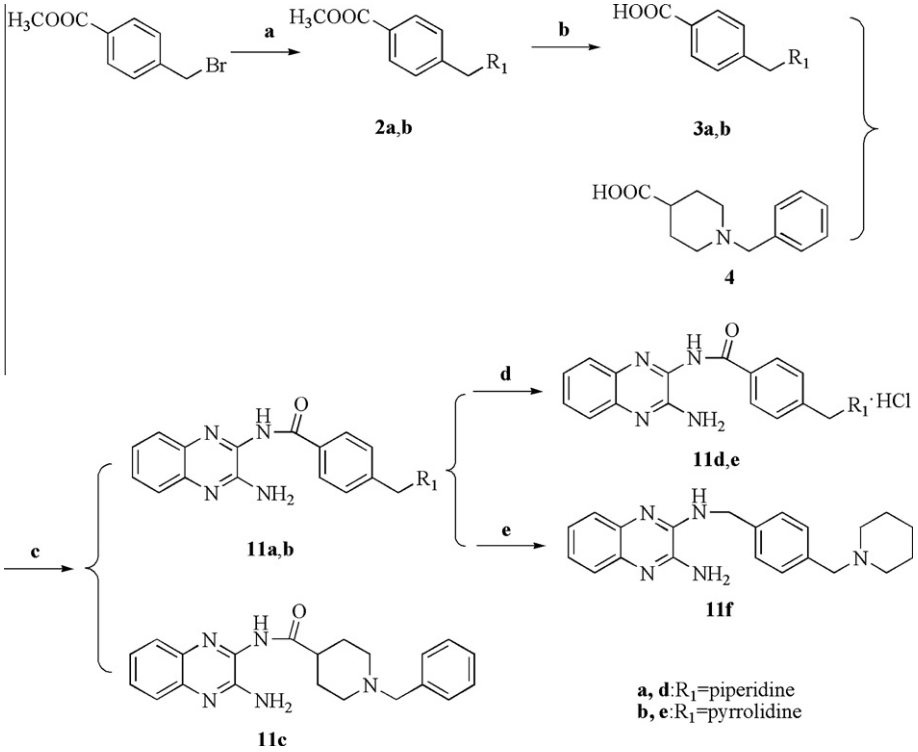
3.4.2. Selectivity for H_1R, H_2R, H_4R

It has been revealed that each of the yet identified four subtypes of histamine GPCRs (namely H_1R, H_2R, H_3R and H_4R) bears distinct biological effects, which makes selectivity an issue of considerable importance in designing H_3R antagonist. Thus, six compounds with potent H_3R antagonistic activities were selected to evaluate their biological activities for H_1R, H_2R and H_4R, using thioperamide as the reference standard. The results are showed in Figs. 3–5 (H: Histamine; T: Thioperamide; F: Forslin; FH: Forslin and Histamine).

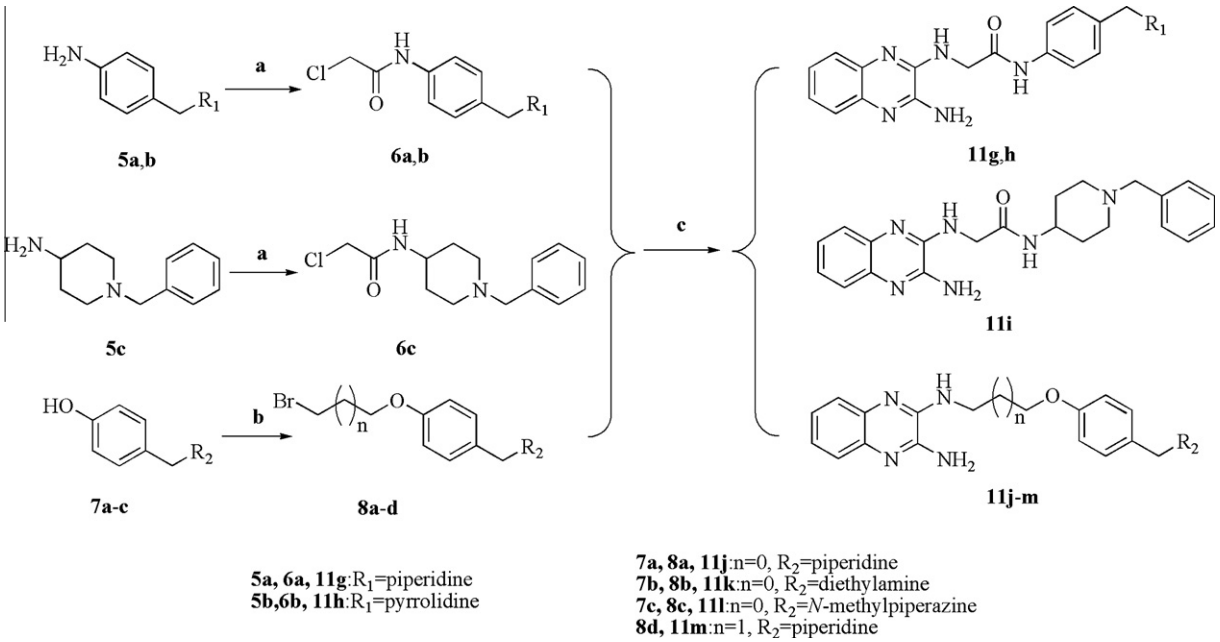
As shown in Figs. 3 and 4, cAMP accumulation induced by 1 μM histamine in HEK-293 cells stably expressing H_1R and H_2R did not been significantly influenced by tested compounds at a dose of 1 μM.

The inhibition of cAMP accumulation induced by 1 μM Histamine (FH) could be partly decreased by co-incubation with 1 μM **11a** and **11d** or thioperamide, respectively (Fig. 5). Further evaluation results are summarized in Table 2.

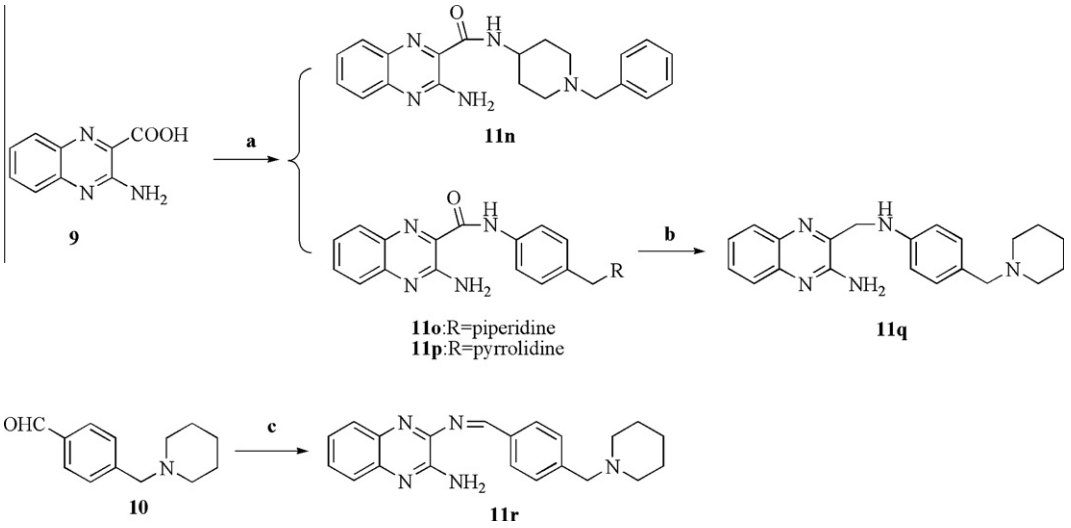
As shown above, all tested compounds, except **11a** and **11d** with the weak function to H_4R, have no effect to H_1R, H_2R or



Scheme 1. Reagents and conditions: (a) amine, Et_3N, acetontrile, reflux; (b) NaOH, MeOH/H_2O, rt; (c) 2,3-diaminoquinoxaline, HOBt, EDC·HCl rt; (d) HCl/AcOEt, rt; (e) $AlLiH_4$, THF, 35 °C.



Scheme 2. Reagents and conditions: (a) $ClCH_2COCl$, CH_2Cl_2, rt; (b) $BrCH_2(CH_2)_nBr$, $NaOH/H_2O$, reflux; (c) 2,3-diaminoquinoxaline, NaH,DMF, rt.



Scheme 3. Reagents and conditions: (a) **5a–c**, HOBt, EDC·HCl. rt; (b) $AlLiH_4$, THF, 35 °C; (c) 3-diaminoquinoxaline, EDC·HCl, acetontrile rt.

H_4R, which indicated that the selectivity of quinoxaline derivatives over H_1R, H_2R, and H_4R is higher than that of thioperamide.

3.4.3. AChE inhibitory activity

The results of AChE inhibitory activity of synthesized compounds showed that most quinoxaline derivatives exhibited moderate to potent AChE inhibitory activities (Table 1). Compound **11d** displayed the most potent AChE inhibitory activity, with an IC_{50} value of 0.313 ± 0.211 μM. Based on these results, some general conclusion can be drawn as follows:

The variation of linker between quinoxaline and benzylamine exhibited obvious effect on AChE inhibitory activity, compounds (**11a**,**d** and **11e**) containing NHCO linkage are more potent than that of containing CONH (**11o,p**), $NHCH_2CONH$ (**11g,h**) or $NHCH_2CH_2O$ (**11j–l**). In addition, reduction of amide to amine (**11a** vs **11f**) has no significantly influence for AChE inhibitory activity, while changing the $NHCH_2$ linkage to N=CH (**11r**) decreases activity. This result indicated that the nitrogen atom in the linkage may form a hydrogen bond interaction with the enzyme. Compounds (**11a**,**o** and **11j**) with piperidine ring at the end of benzyl are more effective than those including pyrrolidine, diethylamine and *N*-methylpiperazine moiety (**11e**, **p**, **q** and **11l**).

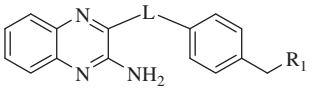
3.4.4. BACE 1 inhibitory activity

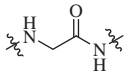
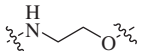
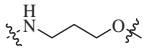
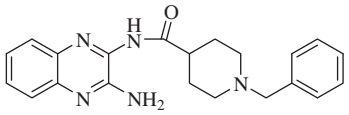
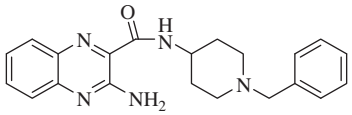
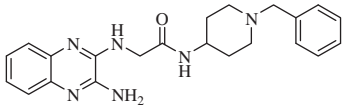
According to the results of BACE 1 inhibitory activities summarized in Table 1, all synthesized compounds displayed moderate to weak activity. The most effective compound **11a** and its hydrochloride (**11d**) showed almost 50% inhibitory rates at 20 μM.

3.4.5. Molecular docking study

Based on results of biological evaluation, compound **11a** showed the most potent activity to H_3R/AChE/BACE 1 and high

Table 1
The biological activity of quinoxaline derivatives



Compd.	L	R_1	H_3R antagonistic action at 1 μM (%) and (IC_{50}, nM)[a,b]	H_3R inverse agonistic action IC_{50}[a] (nM)	AChE inhibitory activity IC_{50}[a] (μM)	BACE 1 inhibitory activity at 20 μg/ml[a] (%)
Thioperamide			(517.5 ± 66.5)	NA[c]	NT[d]	NT
Donepezil			NT	NT	5.0 ± 1.5 nM	NT
OM99-2			NT	NT	NT	0.25 ± 0.03 μM
11a		Piperidine	74.4 ± 2.7 (280.0 ± 98.0)	189.3 ± 95.7	0.483 ± 0.005	46.64 ± 2.55
11d		Piperidine·HCl	65.3 ± 5.4 (511.0 ± 96.0)	170.6 ± 98.7	0.313 ± 0.211	44.79 ± 9.44
11e		Pyrrolidine·HCl	0.8 ± 1.3	NA	NA	10.59 ± 1.39
11f		Piperidine	0.2 ± 2.4	NA	0.476 ± 0.210	1.06 ± 0.23
11g		Piperidine	15.0 ± 2.1	NA	1.155 ± 0.125	0.79 ± 1.27
11h		Pyrrolidine	16.9 ± 4.8	NA	1.173±0.437	36.05 ± 8.13
11j		Piperidine	82.3 ± 8.1 (52.4 ± 23.6)	NA	1.599 ± 0.478	NA
11k		Diethylamine	65.5 ± 3.1 (182.0 ± 52.4)	NA	3.866 ± 2.756	NA
11l		*N*-Methylpiperazine	7.4 ± 1.2	NA	NA	NA
11m		Piperidine	40.5±1.4	NA	1.929 ± 0.544	6.56 ± 2.32
11o		Piperidine	34.6 ± 11.9	NA	0.892 ± 0.132	NA
11p		pyrrolidine	72.8 ± 1.6 (260.0 ± 77.0)	378.5 ± 157.5	1.498 ± 0.058	4.58 ± 5.05
11q		Piperidine	78.2±4.8 (129.7 ± 66.3)	NA	NA	NA
11r		Piperidine	0.5 ± 2.2	NA	0.748 ± 0.014	NA
11c			0.6 ± 0.2	NA	NA	8.96 ± 2.35
11n			1.8±2.3	NA	NA	NA
11i			4.5 ± 1.4	NA	1.329 ± 0.486	30.80 ± 9.49

[a] Data are means ± standard deviation of duplicate independent experiments.
[b] IC_{50} value only determined for compounds attaining over 50% H_3 antagonism at 1 μM and put in brackets.
[c] NA, no activity.
[d] NT, not tested.

selectivity over $H_1R/H_2R/H_4R$. In an attempt to understand the molecular interaction between **11a** and AChE/BACE 1, a molecular docking study was performed using the Discovery Studio 2.1/CDOCKER protocol. The crystal structure of OM99-2/BACE 1 complex (PDB ID: 2ZHR) and E2020/AChE complex (PDB ID:1EVE) were used as the templates. As shown in Fig. 6a, hydrogen bond interaction was observed between amide group and Tyr121 with a distance of 2.14 Å. The charged nitrogen made a cation-π interaction with Trp84, benzene and quinoxaline ring formed π–π stacking with Tyr334 and Trp279, respectively. Besides, the result of molecular docking between **11a** and BACE 1 showed that the amino group formed hydrogen bond with catalytic aspartate Asp32, with a distance of 2.45 Å (Fig. 6b). In addition, hydrogen bonds were also presented between nitrogen atom and Thr72 with a distance of 2.41 and 2.03 Å, and between amide group and Thr72 with a distance of 2.36 Å.

4. Conclusions

Based on SAR analysis of H_3R antagonists, the strategy of hybridizing AChE inhibitor BYYT-25 with BACE 1 inhibitor **1** was utilized to design quinoxaline-based hybrid compounds as Multi-Target-Directed Ligands in this study. The designed database of new scaffold was built and screened against a previous built and validated BACE 1 inhibitor pharmacophore model and filtered using an AChE molecular docking model. Seventeen compounds with high score values were selected, synthesized and evaluated for their biological activities. Most compounds showed moderate to potent inhibitory/antagonistic activities to the AChE, BACE 1 and H_3R. Compound **11a** showed the potent activity to H_3R/AChE/BACE 1 and high selectivity to $H_1R/H_2R/H_4R$. In addition, further receptor-binding studies of **11a** and AChE/BACE 1 showed that it can make several essential interactions to the enzymes. These result suggested that the development

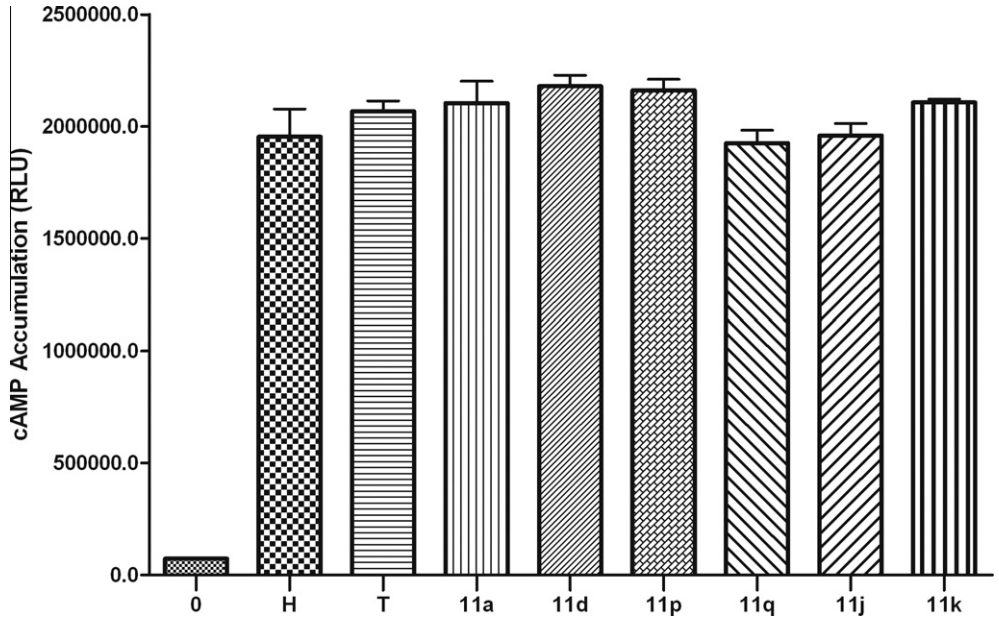


Figure 3. Selectivity of compounds over H_1R in a CRE-luciferace transcription assay.

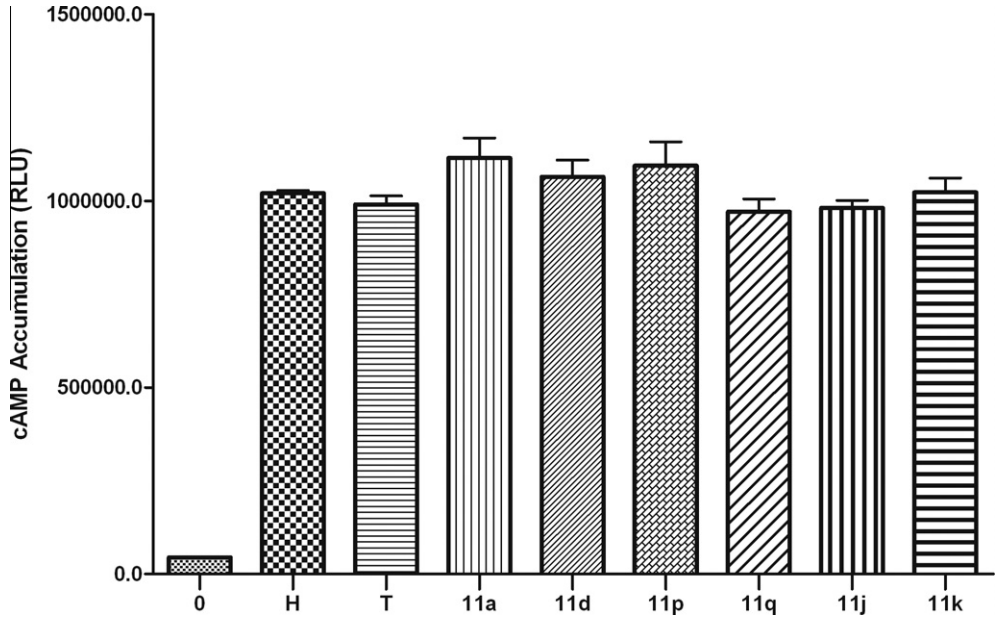


Figure 4. Selectivity of compounds over H_2R in a CRE-luciferace transcription assay

of quinoxaline derivatives may shed instructive light for the treatment of AD in the near future.

5. Experimental

5.1. General

Melting points were recorded on a Buchi apparatus and uncorrected, IR spectra were recorded on a Bruker VECTOR 22 FTIR spectrophotometer. ^{1}H NMR spectra were recorded on a Bruker Avance III 500 M instrument (chemical shifts are expressed as δ values relative to TMS as internal standard). Mass spectra (MS) were recorded on an Esquire-LC-00075 spectrometer. The database of quinoxaline derivatives was built by Pipeline Pilot software. The pharmacophore model (Hypo 1) of BACE 1 inhibitors was built in a previous work.[12] Molecular model studies were performed using the Discovery Studio 2.1.

5.2. Chemistry

Compounds **2**,[14] **4**,[16] **5a–b**,[17] **7**,[13] **9**,[18] and **10**[19] were synthesized according to the known methods.

5.2.1. General procedure for the preparation of compounds 3

Methyl benzoate **2** (7.6 mmol) was dissolved in a mixture of 40 mL methanol and 10 mL water. NaOH (0.33 g, 8.5 mmol) was added, and the mixture was refluxed for 7 h. After methanol evaporation, the remaining aqueous layer pH was adjusted to 7. The solid precipitate was filtered and washed with water (2×10 mL) to obtain white solid **3**.

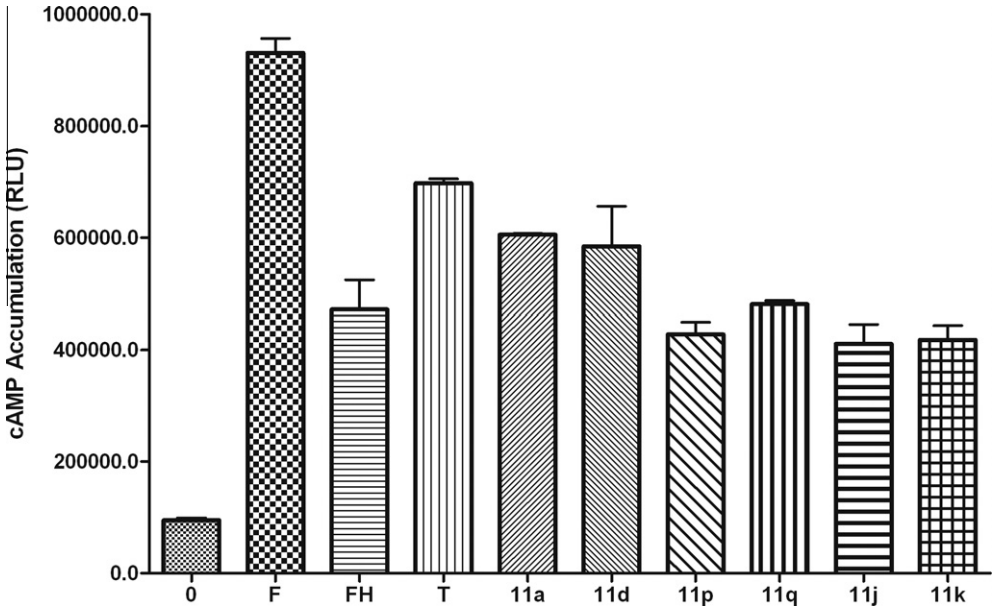


Figure 5. Selectivity of compounds over H_4R in a CRE-luciferace transcription assay.

Table 2
The selectivity of compounds **11a** and **11d** for H_4R

Compd.	**11a**	**11d**	Thioperamide
Function to H_4R IC_{50}	>10 μM	>10 μM	667 ± 114.0 nM

5.2.1.1. 4-(Piperidin-1-ylmethyl)benzoic acid (3a). White solid (50%); mp: 234–236 °C. ^1H NMR (δ, DMSO-d_6): 12.73 (s, 1H, –OH), 7.88 (d, 2H, J = 8.0 Hz, ArH), 7.40 (d, 2H, J = 8.0 Hz, ArH), 3.49 (s, 2H, $ArCH_2N$–), 2.28–2.35 (m, 4H, piperidine), 1.47–1.51 (m, 4H, piperidine), 1.37–1.40 (m, 2H, piperidine).

5.2.1.2. 4-(Pyrrolidin-1-ylmethyl)benzoic acid (3b). White solid (51%); mp: 227–229 °C. ^1H NMR (δ, DMSO-d_6): 12.75 (s, 1H, –OH), 7.89 (d, 2H, J = 7.5 Hz, ArH), 7.40 (d, 2H, J = 7.5 Hz, ArH), 3.51 (s, 2H, $ArCH_2N$–), 2.43–2.49 (m, 4H, pyrroline), 1.73–1.78 (m, 4H, pyrroline).

5.2.2. *N*-(3-aminoquinoxalin-2-yl)-4-(piperidin-1-ylmethyl) benzamide (11a)

Compound **3a** (219 mg, 1 mmol), EDC·HCl (316 mg, 1.65 mmol) and HOBt (223 mg, 1.65 mmol) were dissolved in a solution of 3.5 mL CH_2Cl_2 and 1.5 mL DMF, and the mixture was stirred for 1 h at 0 °C. 2,3-Diaminoquinoxaline (320 mg, 2 mmol) was added to the mixture, and then the mixture was refluxed for 12 h. The solid precipitate was filtered and washed with CH_2Cl_2 (2 × 5 mL) to obtain **11a**. Yellow solid (78%); mp: >250 °C. IR (KBr): 3450, 2932, 2779, 1718, 1633, 1393, 1327 cm^{-1}; ^1H NMR (δ, DMSO-d_6): 7.83 (d, 2H, J = 7.0 Hz, ArH), 7.75–7.78 (m, 2H, ArH), 7.46 (d, 2H, J = 7.0 Hz, ArH), 7.12–7.13 (m, 2H, ArH), 4.76 (s, 2H, $–NH_2$), 2.88–3.05 (m, 4H, piperidine), 2.73 (s, 2H, $–NCH_2$), 1.71–1.75 (m, 4H, piperidine), 1.40–1.60 (m, 2H, piperidine); MS (ESI): m/z = 362 $[M+H]^+$.

5.2.3. 4-Carboxamide-*N*-(3-aminoquinoxalin-2-yl)-1-benzyl piperidine (11c)

Using the previous procedure and starting from 1-benzylpiperidine-4-carboxylic acid **4** (219 mg, 1 mmol) and 2,3-diaminoquinoxaline (320 mg, 2 mmol), **11c** was obtained as yellow solid (79%); mp: 122–125 °C. IR (KBr):3449, 2931, 2851, 1636, 1389 cm^{-1}; ^1H

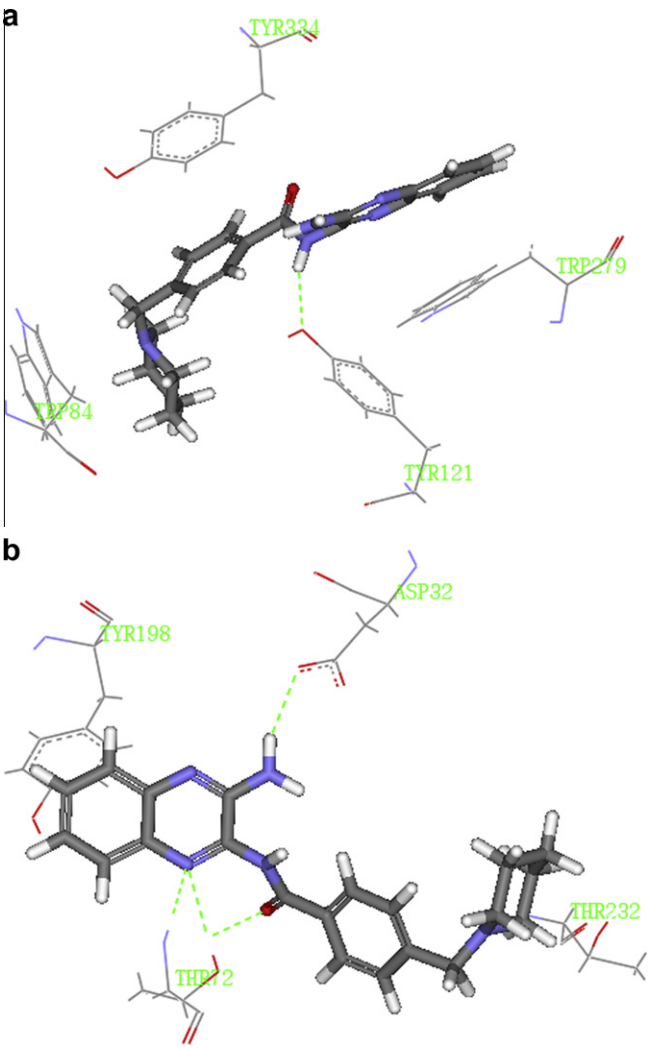


Figure 6. (a) The binding pattern of **11a** into AChE; (b) The binding pattern of **11a** into BACE 1.

NMR (δ, $CDCl_3$): 8.15–8.22 (m, 2H, ArH), 7.71–7.74 (m, 2H, ArH), 7.32–7.37 (m, 4H, ArH), 7.27–7.29 (m, 1H, ArH), 3.62 (s, 2H, –NCH_2), 3.18–3.24 (m, 1H, piperidine), 3.08–3.11 (m, 2H, piperidine), 2.15–2.30 (m, 6H, piperidine); MS (ESI): m/z = 362 $[M+H]^+$.

5.2.4. 2-Carboxamide-3-amino-*N*-(1-benzylpiperidin-4-yl)quinoxaline (11n)

Using the previous procedure and starting from 3-aminoquinoxaline-2-carboxylic acid **9** (189 mg, 1 mmol) and 4-(piperidin-1-ylmethyl)benzenamine (380 mg, 2 mmol), **11n** was obtained as yellow solid (73%); mp: >250 °C. IR (KBr):3452, 2933, 2857, 1635, 1557, 1375 cm^{-1}; ^1H NMR (δ, $CDCl_3$): 8.06 (d, 1H, J = 8.5 Hz, ArH), 7.87 (d, 1H, J = 8.5 Hz, ArH), 7.65–7.69 (m, 2H, ArH), 7.46 (t, 1H, J = 7.0 Hz, ArH), 7.34–7.37 (m, 2H, ArH), 6.99 (t, 1H, J = 7.0 Hz, ArH), 6.92 (t, 1H, J = 7.0 Hz, ArH), 6.24 (s, 1H, –CONH), 3.87 (s, 2H, –NCH_2Ph), 2.75–2.78 (m, 2H, piperidine), 2.56–2.59 (m, 1H, piperidine), 2.46–2.49 (m, 2H, piperidine), 2.00–2.06 (m, 4H, piperidine); MS (ESI): m/z = 362 $[M+H]^+$.

5.2.5. 2-Carboxamide-3-amino-*N*-(4-(piperidin-1-ylmethyl) phenyl)quinoxaline (11o)

Using the previous procedure and starting from 3-aminoquinoxaline-2-carboxylic acid **9** (189 mg, 1 mmol) and 4-(pyrrolidin-1-ylmethyl)benzenamine (380 mg, 2 mmol), **11o** was obtained as yellow solid (83%); mp: >250 °C. IR (KBr):3450, 2931, 2639, 1635, 1525, 1415, 1373 cm^{-1}; ^1H NMR (δ, $CDCl_3$): 10.10 (s, 1H, –CONH), 7.94 (d, 1H, J = 8.0 Hz, ArH), 7.66–7.73 (m, 4H, ArH), 7.48 (t, 1H, J = 6.0 Hz, ArH), 7.39 (d, 2H, J = 8.5 Hz, ArH), 3.53 (s, 2H, –NCH_2Ph), 2.40–2.47 (m, 4H, piperidine), 1.58–1.63 (m, 4H, piperidine), 1.43–1.47 (m, 2H, piperidine); MS (ESI): m/z = 362 $[M+H]^+$.

5.2.6. 2-Carboxamide-3-amino-*N*-(4-(pyrrolidin-1-ylmethyl) phenyl)quinoxaline (11p)

Using the previous procedure and starting from 3-aminoquinoxaline-2-carboxylic acid **9** (189 mg, 1 mmol) and 1-benzylpiperidin-4-amine (380 mg, 2 mmol), **11p** was obtained as yellow solid (79%); mp: >250 °C. IR (KBr):3451, 2932, 2854, 1637, 1533, 1412, 1373 cm^{-1}; ^1H NMR (δ, $CDCl_3$): 10.16 (s, 1H, –CONH), 7.96 (d, 1H, J = 7.0 Hz, ArH), 7.78 (d, 1H, J = 7.0 Hz, ArH), 7.67–7.71 (m, 2H, ArH), 7.50 (d, 2H, J = 8.0 Hz, ArH), 6.98–7.03 (m, 1H, ArH), 6.93 (t, 1H, J = 7.0 Hz, ArH), 3.88 (s, 2H, –NCH_2Ph), 2.75–2.84 (m, 4H, pyrrolidine), 1.91–1.97 (m, 4H, pyrrolidine); MS (ESI): m/z = 348 $[M+H]^+$.

5.2.7. General procedure for the preparation of compounds 11d,e

11a or **11b** (0.1 mmol) was dissolved in 2 mL HCl saturated ethyl acetate, stirred at room temperature for 3 h. The precipitate was filtered to obtain compound **11d** or **11e**.

5.2.7.1. *N*-(3-aminoquinoxalin-2-yl)-4-(piperidin-1-ylmethyl) benzamide hydrochloride (11d). Yellow solid (91%); mp: >250 °C. IR (KBr): 3454, 2775, 2632, 1718, 1633, 1473, 1393 cm^{-1}; ^1H NMR (δ, DMSO-d_6): 10.75 (s, 1H, –CONH), 8.12–8.14 (m, 2H, ArH), 7.91 (d, 2H, J = 7.0 Hz, ArH), 7.76–7.78 (m, 2H, ArH), 7.48 (d, 2H, J = 7.0 Hz, ArH), 4.37 (s, 2H, –NH_2), 2.85–2.95 (m, 4H, piperidine), 2.71 (s, 2H, –NCH_2), 1.70–1.80 (m, 4H, piperidine), 1.32–1.40 (m, 2H, piperidine); MS (ESI): m/z = 398 $[M+H]^+$.

5.2.7.2. *N*-(3-Aminoquinoxalin-2-yl)-4-(pyrrolidin-1-ylmethyl) benzamide hydrochloride (11e). Yellow solid (84%); mp: >250 °C. IR (KBr):3451, 2933, 2857, 1636, 1383, 1109 cm^{-1}; ^1H NMR (δ, CD_3OD): 7.94 (d, 2H, J = 8.0 Hz, ArH), 7.81 (d, 2H, J = 9.0 Hz, ArH), 7.60–7.64 (m, 2H, ArH), 7.49–7.51 (m, 2H, ArH), 3.45 (s, 2H, –NCH_2Ph), 2.95 (m, 4H, pyrrolidine), 1.94 (m, 4H, pyrrolidine); MS (ESI): m/z = 385 $[M+H]^+$.

5.2.8. General procedure for the preparation of compounds 11f,q

To a solution of **11a** or **11o** (0.14 mmol) in 5 mL THF, $AlLiH_4$ (10.5 mg) was added. After the mixture was stirred for 3 h at 50 °C, the reaction was stopped by addition of 5 mL water. The THF was evaporated, and the remaining aqueous layer was extracted with AcOEt (2 × 15 mL). The combined AcOEt layers were dried over Na_2SO_4. The solvent was removed under vacuum to give crude product, which was purified by silica gel column chromatography (PE:AcOEt:TEA = 2:1:0.1) to afford target compounds **11f,q**.

5.2.8.1. 2,3-Diamine-N^2-(4-(piperidin-1-ylmethyl)benzyl)quinoxaline (11f). Yellow solid (34%); mp: >250 °C. IR (KBr):3456, 2929, 2856, 1636, 1474, 1382 cm^{-1}; ^1H NMR (δ, $CDCl_3$): 8.41 (d, 1H, J = 8.0 Hz, ArH), 8.15–8.17 (m, 2H, ArH), 7.79–7.81 (m, 2H, ArH), 7.64 (d, 2H, J = 8.0 Hz, ArH), 3.65 (m, 1H, –$NHCH_2$), 3.55 (s, 2H, –NCH_2Ph), 3.07–3.10 (m, 2H, –NHCH_2), 2.40–2.49 (m, 4H, piperidine), 1.55–1.62 (m, 4H, piperidine), 1.43–1.47 (m, 2H, piperidine); MS (ESI): m/z = 348 $[M+H]^+$.

5.2.8.2. 2-Amine-3-((4-(piperidin-1-ylmethyl)phenylamino)methyl)quinoxalin (11q). Yellow solid (26%); mp: >250 °C. IR (KBr):3451, 2932, 1635, 1513, 1371 cm^{-1}; ^1H NMR (δ $CDCl_3$): 7.35 (d, 2H, J = 8.5 Hz, ArH), 7.14 (d, 2H, J = 8.0 Hz, ArH), 6.99 (t, 1H, J = 7.0 Hz, ArH), 6.93 (t, 1H, J = 7.0 Hz, ArH), 6.62–6.64 (m, 4H, ArH), 6.54–6.56 (m, 2H, –NH_2), 3.70–3.74 (m, 1H, –$NHCH_2$), 3.42–3.44 (m, 4H, –NHCH_2, –NCH_2), 2.34–2.42 (m, 4H, piperidine), 1.55–1.61 (m, 4H, piperidine), 1.41–1.44 (m, 2H, piperidine); MS (ESI): m/z = 348 $[M+H]^+$.

5.2.9. General procedure for the preparation of compounds 11g–i

To a solution of **5** (1 mmol) in 3 mL CH_2Cl_2, 2-chloroacetyl chloride (0.08 mL, 1.1 mmol) was added. After the mixture was stirred for 0.5 h, the solid was filtered and purified by silica gel column chromatography (PE:AcOEt:TEA = 3:1:0.1) to afford **6**. NaH (29 mg, 1.24 mmol) was added to 2,3-diaminoquinoxaline (125 mg, 0.79 mmol)[20] in 3 mL DMF. After the mixture was stirred for 0.5 h, **6** (238 mg, 0.79 mmol) was added, and the mixture was stirred for 0.5 h at 35 °C. Water (10 mL) was added to the mixture, and the crude product was extracted with AcOEt (2 × 15 mL), the organic layer was washed with water (2 × 20 mL) and dried over Na_2SO_4. The solvent was removed under vacuum to give crude product, which was purified by silica gel column chromatography (PE:AcOEt:TEA = 3:1:0.1) to afford target compounds **11g–i**.

5.2.9.1. 2-(3-Aminoquinoxalin-2-ylamino)-*N*-(4-(piperidin-1-ylmethyl)phenyl)acetamide (11g). Yellow oil (11%); IR (KBr): 3457, 2929, 1637, 1381, 1118 cm^{-1}; ^1H NMR (δ, DMSO-d_6): 10.17 (s, 1H, –CONH), 7.59 (d, 2H, J = 8.0 Hz, ArH), 7.39 (d, 2H, J = 7.5 Hz, ArH), 7.24 (d, 2H, J = 8.0 Hz, ArH), 7.20 (t, 2H, J = 7.5 Hz, ArH), 6.80 (s, 2H, –NH_2), 4.50 (m, 1H, –$NHCH_2$), 4.30 (d, 2H, J = 5.5 Hz, –NCH_2CO), 3.43 (s, 2H, –NCH_2Ph), 2.33 (m, 4H, piperidine), 1.49–1.52 (m, 4H, piperidine), 1.40–1.42 (m, 2H, piperidine); MS (ESI): m/z = 391 $[M+H]^+$.

5.2.9.2. 2-(3-Aminoquinoxalin-2-ylamino)-*N*-(4-(pyrrolidin-1-ylmethyl)phenyl)acetamide (11h). Yellow oil (12%); IR (KBr): 3455, 2931, 2855, 1636, 1572, 1494, 1381 cm^{-1}; ^1H NMR1H NMR (500 MHz, CD_3OD) δ: 7.67–7.69 (m, 1H, ArH), 7.61–7.63 (m, 1H, ArH), 7.54–7.58 (m, 2H, ArH), 7.45–7.49 (m, 2H, ArH), 7.26–7.31 (m, 2H, ArH), 4.78 (s, 2H, –NH_2), 4.21–4.23 (m, 2H, –NHCH_2), 3.67 (s, 2H, –NCH_2Ph), 3.45–3.55 (m, 4H, pyrrolidine), 2.12–2.20 (m, 4H, pyrrolidine); MS (ESI): m/z = 377 $[M+H]^+$.

5.2.9.3. 2-(3-Aminoquinoxalin-2-ylamino)-*N*-(1-benzylpiperidin-4-yl)acetamide (11i). Yellow oil (10%); IR (KBr): 3457, 2933, 2857, 1637, 1375, 1120 cm^{-1}; ^1H NMR (δ, $CDCl_3$): 7.37–7.39 (m, 2H, ArH), 7.28–7.34 (m, 5H, ArH), 7.20–7.22 (m, 2H, ArH), 3.59 (s, 2H, $-NCH_2Ph$), 2.51–2.53 (m, 5H, piperidine, $-NHCH_2CONH$), 2.00–2.03 (m, 6H, piperidine); MS (ESI): *m/z* = 391 $[M+H]^+$.

5.2.10. General procedure for the preparation of compounds 11j–m

To a solution of **7** (5 mmol) in 5 mL 1,2-dibromoethane or 1,3-dibromopropane, 40% NaOH solution 5 mL and tetrabutyl ammonium bromide (0.05 g) were added. After the mixture was refluxed for 4 h, the organic layer was separated, and the aqueous layer was extracted with CH_2Cl_2 (2×15 mL). The combined organic layer was washed with brine, dried over Na_2SO_4. The solvent was removed under vacuum to give crude product, which was purified by silica gel column chromatography (PE:AcOEt:TEA = 10:1:0.1) to afford compound **8** .

Compounds **11j–m** were obtained by the procedure of **11g–i** using compound **8** as the starting material instead of compound **6**.

5.2.10.1. 2,3-Diamine-N^2-(2-(4-(piperidin-1-ylmethyl)phenoxy)ethyl)quinoxaline (11j). Yellow oil (18%); IR (KBr):3445, 2857, 1628, 1511, 1459, 1404, 1307 cm^{-1}; ^1H NMR (δ, CD_3OD): 7.53–7.55 (m, 1H, ArH), 7.38–7.42 (m, 3H, ArH), 7.22–7.25 (m, 2H, ArH), 7.09 (d, 2H, *J* = 9.0 Hz, ArH), 4.33 (t, 2H, *J* = 5.5 Hz, $-OCH_2CH_2$), 4.09 (s, 2H, $-NCH_2$), 3.98 (t, 2H, *J* = 5.5 Hz, $-OCH_2CH_2$), 3.02–3.06 (m, 4H, piperidine), 1.76–1.81 (m, 4H, piperidine), 1.62 (m, 2H, piperidine); MS (ESI): *m/z* = 378 $[M+H]^+$.

5.2.10.2. 2,3-Diamine-N^2-(2-(4-((diethylamino)methyl)phenoxy)ethyl)quinoxaline (11k). Yellow oil (12%). IR (KBr):3447, 2929, 2861, 1731, 1636, 1512, 1463, 1381 cm^{-1}; ^1H NMR (δ, CD_3OD): 7.69–7.74 (m, 2H, ArH), 7.52–7.55 (m, 2H, ArH), 7.41 (d, 2H, *J* = 8.5 Hz, ArH), 6.94 (d, 2H, *J* = 8.5 Hz, ArH), 5.92 (m, 1H, $-NHCH_2CH_2O$), 5.37 (s, 2H, $-NH_2$), 4.08 (d, 2H, *J* = 6.5 Hz, $-NHCH_2$), 3.95 (s, 2H, $-NCH_2Ph$), 2.94 (q, 4H, *J* = 7.5 Hz, $-N(CH_2CH_3)_2$), 2.06 (d, 2H, *J* = 6.5 Hz, $-NHCH_2CH_2O$), 0.90 (t, 6H, *J* = 7.5 Hz, $-N(CH_2CH_3)_2$); MS (ESI): *m/z* = 366 $[M+H]^+$.

5.2.10.3. 2,3-Diamine-N^2-(2-(4-((4-methylpiperazin-1-yl)methyl)phenoxy)ethyl)quinoxaline (11l). Yellow solid (15%); mp: 114–117 °C. IR (KBr):3447, 2977, 2734, 1639, 1570, 1464, 1398 cm^{-1}; ^1H NMR (δ, CD_3OD): 7.67 (d, 1H, *J* = 7.0 Hz, ArH), 7.54 (d, 1H, *J* = 7.0 Hz, ArH), 7.37–7.40 (m, 4H, ArH), 7.12 (d, 2H, *J* = 8.0 Hz, ArH), 5.06 (s, 2H, $-NH_2$), 4.42 (t, 2H, *J* = 5.5 Hz, $-NHCH_2CH_2O$), 4.09 (t, 2H, *J* = 5.5 Hz, $-NHCH_2CH_2O$), 3.75 (s, 2H, $-NCH_2Ph$), 3.10–3.16 (m, 4H, piperazine), 2.81–2.87 (m, 4H, piperazine), 2.77 (s, 3H, $-NCH_3$); MS (ESI): *m/z* = 393 $[M+H]^+$.

5.2.10.4. 2,3-Diamine-N^2-(3-(4-(piperidin-1-ylmethyl)phenoxy)propyl)quinoxaline (11m). Yellow solid (8%); mp: 117–121 °C. IR (KBr):3452, 2930, 1636, 1486, 1414, 1372 cm^{-1}; ^1H NMR (δ, CD_3OD): 7.84–7.87 (m, 1H, ArH), 7.75–7.77 (m, 1H, ArH), 7.62–7.63 (m, 1H, ArH), 7.51–7.53 (m, 1H, ArH), 7.42 (d, 2H, *J* = 8.5 Hz, ArH), 7.09 (d, 2H, *J* = 8.5 Hz, ArH), 4.76 (s, 1H, $-NHCH_2CH_2CH_2O$), 4.29 (t, 2H, *J* = 5.5 Hz, $-NHCH_2CH_2CH_2O$), 3.91 (m, 4H, $-NCH_2Ph$, $-NHCH_2CH_2CH_2O$), 2.82–2.90 (m, 4H, piperidine), 2.32–2.36 (m, 2H, $-NHCH_2CH_2CH_2O$), 1.81–1.84 (m, 4H, piperidine), 1.65–1.70 (m, 2H, piperidine); MS (ESI): *m/z* = 392 $[M+H]^+$.

5.2.11. 2,3-Diamine-N^2-(4-(piperidin-1-ylmethyl)benzylidene) quinoxaline (11r)

2,3-Diaminoquinoxaline (40 mg, 0.25 mmol), 4-(piperidin-1-ylmethyl)benzaldehyde **10**(20 mg, 0.1 mmol) and 100 mg EDC·HCl were dissolved in 3 mL acetontrile, and the mixture was refluxed for 3 h. After cooled to room temperature, 10 mL water was added to the mixture, and extracted with ethyl acetate (2×20 mL). The organic layer was dried over Na_2SO_4. The solvent was evaporated. The residue was purified by silica gel column chromatography (PE:AcOEt:TEA = 4:1:0.1) to afford **11r**. Yellow oil (35%); IR (KBr): 3456, 2927, 2858, 1637, 1469, 1384, 611 cm^{-1}; ^1H NMR (δ, $CDCl_3$): 8.35 (d, 1H, *J* = 8.0 Hz, ArH), 8.10–8.12 (m, 2H, ArH), 7.74–7.75 (m, 2H, ArH), 7.58 (d, 2H, *J* = 8.0 Hz, ArH), 6.61 (s, 1H, –NCH–), 3.56 (s, 2H, $-NCH_2Ph$), 2.31–2.40 (m, 4H, piperidine), 1.50–1.53 (m, 4H, piperidine), 1.37–1.42 (m, 2H, piperidine); MS (ESI): *m/z* = 346 $[M+H]^+$

5.3. In vitro BACE 1 inhibit activity screening

All synthesized compounds were tested for their BACE 1 inhibitor activities, using fluorescence resonance energy transfer (FRET) assay, which uses purified insect-expressed BACE 1 and a specific substrate. An excitation wavelength of 355 nm and an emission wavelength of 460 nm were used to monitor the hydrolysis of substrate. Compounds with inhibitory rates at 20 μg/mL above 50% were tested for IC_{50} values.

5.4. In vitro AChE inhibitory activity screening

AChE activities were measured by the spectrophotometric method with slight modification with rat cortex homogenate was used as the resource of AChE. The brain homogenate was preincubated for 5 min with tetraisopropyl pyrophosphoramide (iso-OMPA) (0.04 mmol/L), a selective inhibitor of BuChE. For assay of AChE activity, a reaction mixture of 200 ml containing acetylthiocholine iodide 0.3 mmol/L, sodium phosphate buffer (0.1 mmol/L, pH 7.4) 100 mL, homogenate or serum 20 mL and different concentrations of test compounds 20 mL were incubated at 37 °C for 15 min. The reaction was terminated by adding 50 mL 3% sodium lauryl sulfate, then, 50 mL 0.2% 5,5-dithio-bis(2-nitrobenzoic acid) was added to produce the yellow anion of 5-thio-2-nitrobenzoic acid. The rate of color production was measured spectrophotometrically at 440 nm. Assays were performed with at least seven concentrations of compounds and IC_{50} (drug concentration that inhibits 50% AChE activity) was calculated according to the inhibition curve. Donepezil was applied as the positive control. All samples were assayed in duplicate.

5.5. In vitro H_1R, H_2R, H_3R, H_4R antagonistic action screening

5.5.1. Cell culture and generation of stable cell lines

HEK-293 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C. For transfection, H_1R, H_2R, H_3R or H_4R cDNA plasmid constructs were transfected or co-transfected into HEK-293 cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Stable transfectants were selected in the presence of 800 μg/mL G418.

5.5.2. cAMP accumulation

Stable HEK-293 cells co-transfected with H_3R and pCRE-Luc were seeded in a 48-well plate overnight and were grown to 90–95% confluence. Next, the cells were stimulated with 10 μM forskolin or 10 μM forskolin plus different concentrations of histamine or compound in serum-free DMEM, and the cells were incubated for 5 h at 37 °C. Luciferase activity was detected using a firefly luciferase kit (Promega, Madison, WI). When required, cells were treated overnight with or without PTX (100 ng/mL) in serum-free DMEM before the experiment.

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