Review

Development of an Efficient Therapeutic Agent for Alzheimer's Disease: Design and Synthesis of Dual Inhibitors of Acetylcholinesterase and Serotonin Transporter

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To date, acetylcholinesterase (AChE) inhibitors have been clinically effective drugs for the palliative treatment of Alzheimer's disease, but their clinical efficacy is limited, mainly due to their adverse effects on peripheral organs. Since patients of Alzheimer's disease often exhibit depression as well as memory impairment, dual inhibitors of AChE and serotonin transporter (SERT) would be a better therapeutic method. Anti-depressive effects based on SERT inhibition would reduce the dose-related side effects of AChE inhibitors. Such dual inhibitors were designed by the hybridization of rivastigmine and fluoxetine based on a hypothetical model of the AChE active site. Various derivatives were synthesized and evaluated for their *in vitro* inhibition, and then (*S*)-5j (RS-1259), which possessed balanced inhibitory activities of AChE (IC_{50}=101 nM) and SERT (IC_{50}=42 nM), was successfully obtained. An *ex vivo* experiment in mice indicated that (*S*)-5j (RS-1259) simultaneously inhibited AChE and SERT in the brain following an oral administration. The simultaneous elevation of extracellular levels of acetylcholine and serotonin in the rat hippocampus was actually confirmed by microdialysis.

Key words Alzheimer's disease; acetylcholinesterase; serotonin transporter; dual inhibitor

1. What Is Dementia

Dementia, in which parts of the brain stop working, occurs more frequently as people age. As a result, in societies where life expectancy has been extended, dementia is one of the most severe health problems of the aging populations. Approximately 15 percent of the people who live to the age of 65 will show some symptoms of dementia and there are currently about 18 million people with dementia in the world. The number of people with dementia is expected to increase globally to about 34 million by 2025.[1)]

Symptoms of dementia include:

- loss of memory
- difficulty in finding the right words or understanding what people are saying
- difficulty in performing previously routine jobs
- personality and mood changes

The most common cause of dementia is Alzheimer's disease (AD), which accounts for 55% of all dementia patients worldwide. The second cause of dementia is vascular dementia (VD), which makes up 20% of all cases. VD used to be the most common cause of dementia in Japan. However, the proportion of AD patients has been increasing yearly and AD is now the leading cause of dementia in Japan.[2)] Consequently, it is important to introduce new therapeutic agents for AD in order to alleviate dementia.

2. About Alzheimer's Disease

AD, a neurodegenerative disorder discovered by Alois Alzheimer in 1907, is characterized by a progressive deterioration of memory and cognition.[3)] Neuropathologically, AD is identified by three major signs: amyloid-β plaques (Aβ), neurofibrillary tangles (NFT) and synaptic loss.[4)] In particular, the degeneration of cholinergic neurons in the cortex and hippocampus and a loss of acetylcholine (ACh), a neurotransmitter for cholinergic neurotransmission, in the brain are characteristically observed in AD patients.[5)] So far, no single factor causing AD has been identified, but it is likely the result of a combination of factors, such as age, genetic makeup, or environmental factors.[6)]

3. Treatment of Alzheimer's Disease

A deficiency in cholinergic neurotransmission is considered to play an important role in the learning and memory impairment of AD patients. Recent progress in the understanding of the pathology of Alzheimer disease has made it possible for potential disease-modifying therapies to be tested in clinical trials.[7—11)] However, no such therapy is available at present. Therefore, cholinergic enhancement remains the most effective therapeutic method to treat AD.[12)]

In the course of neurotransmission at cholinergic synapses (Fig. 1), ACh is released from the presynapse into the synap-

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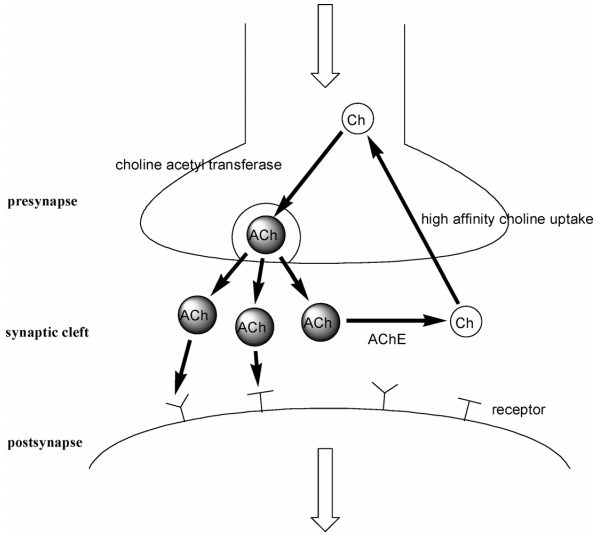

Fig. 1. The Cholinergic Synapse

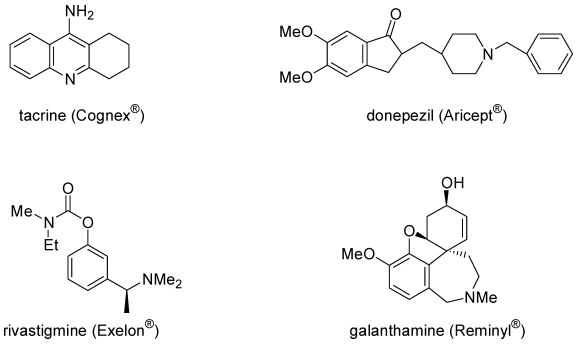

Fig. 2. Structure of Marketed AChE Inhibitors

tic cleft and binds to muscarinic and nicotinic receptors on the postsynapse. This nerve impulse transmission at cholinergic synapses is terminated by acetylcholinesterase (AChE) which catalyzes the hydrolysis of ACh.

To date, several strategies have been explored on the basis of the above neurotransmission mechanism in order to enhance cholinergic functions.

These are:

[1] AChE inhibitors,[13] which increase the level of ACh in the synaptic cleft
[2] ACh precursors,[14] such as choline
[3] ACh releasers,[15] which advance the release of ACh from presynapses
[4] muscarinic agonists,[16] which activate muscarinic receptors on postsynapse
[5] nicotinic agonists,[17] which activate nicotinic receptors on postsynapse

Among these strategies, it is known that the only clinically effective strategy for the palliative treatment of AD is to inhibit AChE.[18] At present, several AChE inhibitors have successfully reached the market, such as tacrine (Cognex®),[19] donepezil (Aricept®),[20] rivastigmine (Exelon®)[21] and galanthamine (Reminyl®)[22] (Fig. 2).

Tacrine (Cognex®), a reversible AChE inhibitor, was the first approved drug for AD from Warner–Lambert. It can improve some of the symptoms of AD patients. However, it also causes a potentially serious side effect, liver damage. Donepezil (Aricept®), a reversible AChE inhibitor, was produced by Eisai. It has now replaced tacrine because of its convenient once-a-day administration (tacrine, four times daily). In addition, it does not cause hepatotoxic effects. Ri-

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vastigmine (Exelon®) was produced by Novartis Pharmaceuticals. From a structural viewpoint, it has a carbamate moiety which is considered to interact with the AChE active center. Recently, Bal-on *et al.* reported that rivastigmine was a pseudo-irreversible inhibitor of AChE based on structure interaction studies.[23] Galanthamine (Reminyl®), a tertiary alkaloid isolated from several plants, was co-developed by Shire Pharmaceuticals and the Janssen Research Foundation. It works as not only an AChE inhibitor but also as a nicotinic agonist.

These drugs do not provide a complete cure, but stabilize some of the symptoms of AD patients for a limited time. However, the usefulness of these AChE inhibitors in a clinical setting is limited mainly due to their adverse effects on peripheral organs, since ACh works as the primary neurotransmitter in the body as well as in the brain.[24]

4. Structure of Acetylcholinesterase

Sussman *et al.* reported on the determination of the crystal structure of AChE from *Torpedo californica* by X-ray analysis to 2.8 angstrom resolution.[25] The AChE enzyme is an α/β protein that contains 537 amino acids forming of 12β-sheets surrounded by 14α helices. A catalytic triad consisting of Ser200, His400 and Glu327, is located at the bottom of a deep and narrow gorge. The gorge is lined with the side chain rings of 14 aromatic residues.

With respect to donepezil, complex structure models and their binding interactions were simulated by molecular modeling methods.[26] Recently, Susmman *et al.* solved the complex crystal structure of donepezil with *Torpedo californica* AChE and reported three major binding interactions: the benzyl moiety to Trp84 at the bottom of the gorge; the charged nitrogen of the piperidine ring to Phe330 in the middle of the gorge; and the indanone ring to Trp279 at the top of the gorge (Fig. 3).[27]

These studies provide some useful information on, for example, inhibitor–enzyme interactions and possible binding sites, for the design of potent AChE inhibitors.

5. Depression in Alzheimer's Disease

Recent studies suggest that cholinergic dysfunction does not completely explain age-related cognitive deficits.[28] The hypofunction of several other neuronal systems such as the glutamatergic, (GABAergic and monoaminergic systems in the brain of AD patients may be partly responsible for this cognitive decline.[29] There are many experimental reports suggesting that several neurotransmitters, including serotonin, norepinephrine, dopamine and γ-aminobutyric acid (GABA), appear to play a role in cognitive processes and interact with the cholinergic systems.[30] Moreover, AD patients often exhibit a number of behavioral abnormalities, such as irritability, anxiety and depression, as well as cognitive impairment.[31] These psychiatric symptoms are sometimes controlled by psychotropic agents.[32] Among these symptoms, it is most often reported that depression is present in about 40% of patients with dementia.[33] Currently, depression in AD patients has been successfully treated with serotonin transporter (SERT) inhibitors,[34] a type of antidepressant which has no anticholinergic side effect, since serotonin is known to be a neurotransmitter associated with many psychiatric symptoms including depression. Currently, the most commonly used treatments for depression in AD patients are SERT inhibitors such as fluoxetine[35] and paroxetine[36] which have no anticholinergic side effects (Fig. 4). At serotonergic synapses, serotonin is released in the brain synaptic cleft and binds to a receptor. Upon its reuptake into the presynaptic neuron by SERT, which terminates the action of serotonin, it is recycled into the neurotransmitter pool (Fig. 5). Therefore, inhibition of SERT results in an increased concentration of serotonin in the brain synaptic cleft, *i.e.* an enhanced serotonergic neurotransmission.

6. Concept of Dual Inhibitors of AChE and SERT as Therapeutic Agents for Alzheimer's Disease

To date, AChE inhibitors are able to enhance memory in AD patients, but their clinical efficacy is limited mainly due

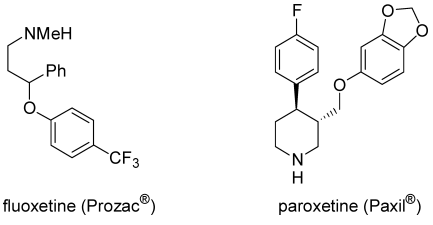


Fig. 4. Examples of SERT Inhibitors

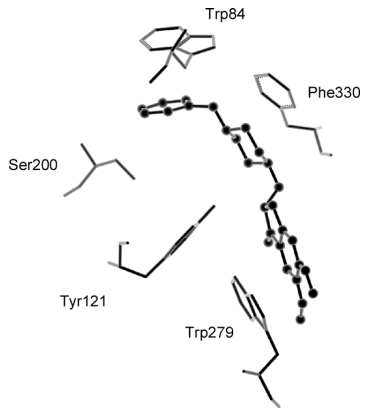


Fig. 3. Major Binding Interactions of Donepezil and *Torpedo californica* AChE

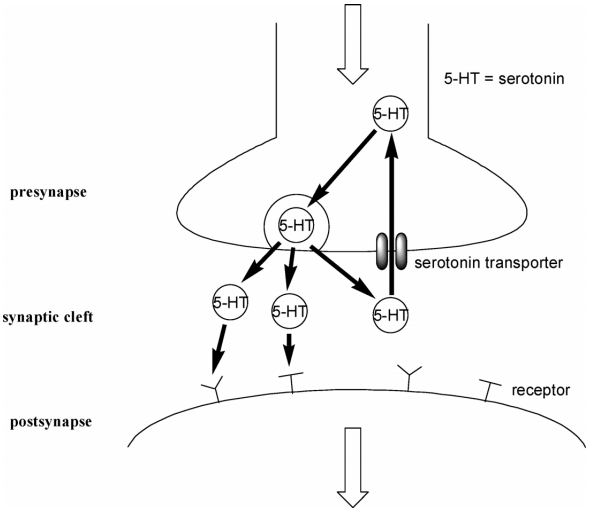


Fig. 5. Serotonin Reuptake Process in the Serotonergic Synapse

to their adverse peripheral effects.[37] SERT inhibitors which have no anticholinergic side effects are also useful for controlling depression in AD patients. However, co-administration of SERT and AChE inhibitors would be undesirable due to the difficulty in managing their different pharmacodynamic and pharmacokinetic properties. Moreover, it may cause harmful drug–drug interactions as well.[38]

In order to overcome these disadvantages, a drug inhibiting both SERT and AChE but in a balanced manner is considered to be a promising method. This combined SERT and AChE inhibition would bring about greater therapeutic benefits than AChE inhibition alone, since further improvement of cognitive deficits could be achieved without the dose-related adverse effects caused by excessive AChE inhibition. Thus, AChE–SERT dual inhibitors would be a novel class of anti-AD drugs that are more effective in alleviating the symptoms of AD than known AChE inhibitors (Fig. 6).

So far, only two groups have studied AChE inhibitors that also possess SERT inhibitory activity. Hirai *et al.* reported that 3-[1-(phenylmethyl)-4-piperidinyl]-1-(2,3,4,5-tetrahydro-1*H*-1-benzazepin-8-yl)-1-propanone fumarate (TAK-147) showed potent AChE inhibition (IC_{50}=51 nM) (Fig. 7). Furthermore, it weakly inhibited noradrenaline transporter and SERT (IC_{50}=4020 and 1350 nM, respectively).[39]

McKenna *et al.* reported that several analogues of tacrine could be useful templates for further investigation of dual AChE and SERT inhibitors (Fig. 7).[40]

The compounds described by these two groups showed potent AChE inhibitory activities and moderate SERT inhibitory activities *in vitro*. However, the extent of inhibition against each was imbalanced when it came to *in vivo* dual inhibition. Consequently, no antidepressive effect resulting from SERT inhibition was observed due to the dominating effect of increased cholinergic transmission. Recently AChE inhibitors such as protopine and S-9977 reportedly have SERT inhibitory activity, although involvement of those activities in clinical efficacy has not been elucidated yet.[41,42]

It is clear that the balance between AChE and SERT inhibition is crucial for the simultaneous activation of the cholinergic and serotoninergic transmissions in the brain *in vivo*. Therefore, the creation of a balanced dual inhibitor of AChE and SERT, one that can achieve the same pharmacological effects as those observed *in vivo*, is challenging but necessary.

7. Design of AChE and SERT Dual Inhibitors

On the basis of the X-ray structure of the complex of AChE and donepezil and molecular modeling studies, we proposed a hypothetical model explaining the inhibition mode at the active site of AChE, which involves four binding sites (Chart 1). In order to design a dual inhibitor, we selected rivastigmine, one of the marketed AChE inhibitors, as a lead compound. Rivastigmine has three binding elements for the hypothetical binding sites of AChE, and they are:

(1) a carbamate group to the catalytic triad site
(2) an aromatic group to hydrophobic binding site A
(3) an amine group to the ionic site

Focusing on the fourth binding site of the hypothetical model, fluoxetine, an antidepressant, was chosen as the pharmacophoric element that would bind to hydrophobic binding site B. Fluoxetine has potent inhibitory activity against SERT

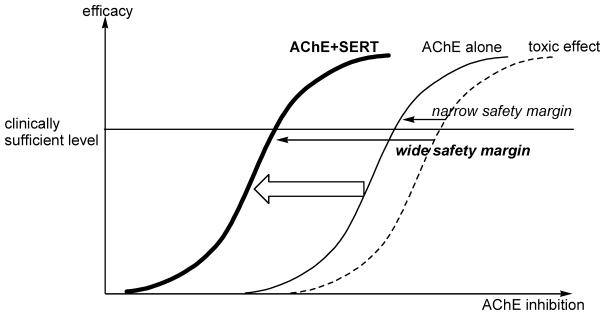


Fig. 6. Wider Safety Margin of AChE–SERT Dual Inhibitors

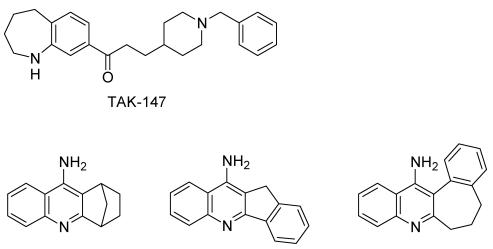


Fig. 7. Reported AChE–SERT Dual Inhibitors: Hirai's TAK-147 and McKenna's Tacrine Analogues

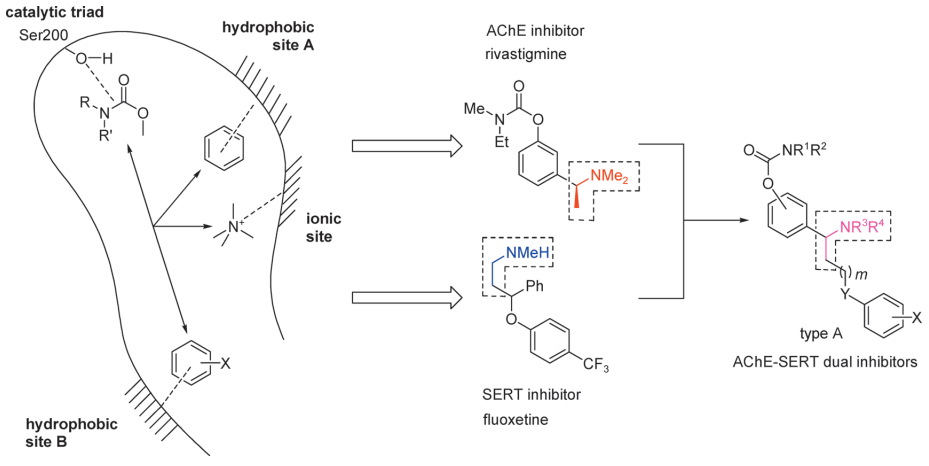


Chart 1. Design of AChE–SERT Dual Inhibitors Based on a Hypothetical Model of the AChE Active Site with Proposed Pharmacophore

and possesses an ethylamine moiety that could be overlapped with that of rivastigmine. Therefore, a type A compound designed by hybridizing of these two compounds at their common ethylamine moiety was expected to be a dual inhibitor of AChE and SERT.

8. Synthesis of Type A Derivatives

Firstly, various type A derivatives were synthesized for optimization of their inhibitory activities as AChE–SERT dual inhibitors.[43]

The preparation of *meta*-carbamate derivatives **4a**—**f** and *para*-carbamate derivatives **5a**—**j** is shown in Chart 2. One-pot condensation[44] of hydroxybenzaldehyde **1**, malonic acid and methylamine, followed by esterification of the carboxylic acid catalyzed by sulfuric acid and the *tert*-butoxycarbonyl (Boc) protection of methylamine provided compound **2**. Compound **2** was treated with dimethylcarbamyl chloride, followed by reduction of the ester group with $LiAlH_4$ to furnish primary alcohol **3**. Primary alcohol **3** was etherified with various phenols, followed by deprotection of the Boc group with 2 N HCl in ethyl acetate to give compounds **4a**—**f** and **5a**—**j**.

The synthesis of both enantiomers of **5j** was described in Chart 3. The primary amine of known (*S*)-**6**[45] was Boc-protected, followed by treatment with dimethylcarbamyl chloride to afford (*S*)-**7**. Reduction of the ester group of (*S*)-**7** with $LiAlH_4$ gave primary alcohol (*S*)-**8**. The alcohol (*S*)-**8** was reacted under Mitsunobu reaction conditions with *p*-nitrophenol, followed by methylation of the Boc-protected amine with iodomethane. Finally, deprotection of the Boc group with 2 N HCl provided (*S*)-**5j**. Enantiomer (*R*)-**5j** was prepared by the same method starting from (*R*)-**6**.

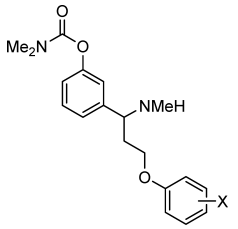
9. Results of Type A Derivatives

All the final compounds were tested for *in vitro* inhibition of AChE from mouse brain and SERT from rat synaptosome. The protocols used here are described in the Experimental section.

Table 1 summarizes the inhibitory activities of compounds **4a**—**f**, which possess the dimethylcarbamate at the *meta* position similar to rivastigmine. For comparison, the assay data of rivastigmine, donepezil and fluoxetine are also included. The effect of the electron-donating (**4a**, **b**) and electron-withdrawing (**4c**—**f**) substituents X at the 4-position of the phenyl ether moiety was examined. Nearly all of the 4-substituted derivatives **4a**—**e** exhibited potential inhibitory activity against SERT ($IC_{50}<50$ nM) but much lower activity against AChE ($IC_{50}>1000$ nM). However, only the 4-nitro-substituted derivative **4f** was found to be a potential dual inhibitor of AChE ($IC_{50}=221$ nM) and SERT ($IC_{50}=52$ nM).

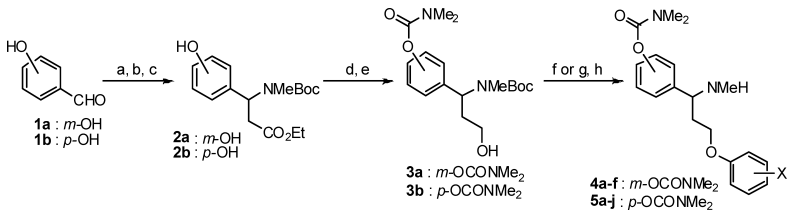
Changing the location of the dimethylcarbamate from the *meta* position to the *para* position mostly increased the potency against AChE, but decreased the potency against SERT

Table 1. *In Vitro* Inhibition of AChE and SERT for *meta*-Carbamate Derivatives



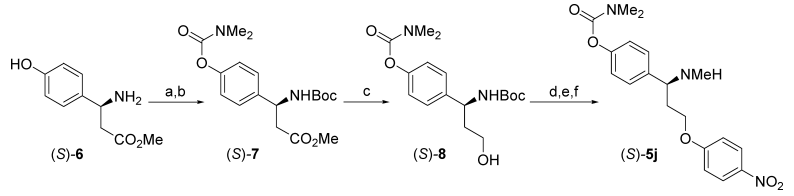
Compound[a]	X	IC_{50} (nM)	
		AChE[b]	SERT[c]
Rivastigmine		11000	>1000
Donepezil		10	>1000
Fluoxetine		>10000	180
4a	4-OMe	>1000	38
4b	4-Me	>1000	49
4c	4-Cl	870	81
4d	4-F	870	16
4e	4-CF_3	>1000	13
4f	4-NO_2	221	52

a) Compounds were tested as their hydrochloride salts. *b*) From mouse brain. *c*) From rat synaptosome.



Reagents and conditions: (a) $CH_2(CO_2H)_2$, MeH_3NOAc, EtOH, reflux; (b) H_2SO_4, EtOH, reflux; (c) Boc_2O, THF, rt; (d) Me_2NCOCl, K_2CO_3, DMF, rt; (e) $LiAlH_4$, THF, −20 °C; (f) (i) MsCl, Et_3N, CH_2Cl_2, 0 °C; (ii) phenol, NaH, DMF, 0 °C to rt; (g) phenol, DEAD, PPh_3, THF, rt; (h) 2 N HCl–AcOEt, rt.

Chart 2



Reagents and conditions: (a) Boc_2O, THF, rt, 72%; (b) Me_2NCOCl, K_2CO_3, DMF, rt, 96%; (c) $LiAlH_4$, −78 to 0 °C, 97%; (d) DEAD, PPh_3, 4-nitrophenol, THF, rt; (e) NaH, MeI, DMF, 0 °C to rt, 77% (2 steps); (f) 2 N HCl–AcOEt, rt, 100%.

Chart 3

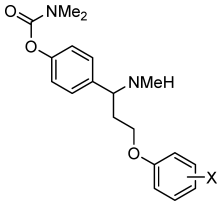
(Table 2). Among the compounds **5a**—**j**, the 4-nitro-substituted derivative **5j** was revealed to possess good potencies against both AChE (IC_{50}=125 nM) and SERT (IC_{50}=44 nM), inhibiting them both to a suitable extent.

The effect of the carbamate moiety in **5j** was examined (Table 3). The carbamate derivatives **9a**, **b** had good anti-SERT activity but moderate anti-AChE activity. The dimethylamide derivative **9d**[43] no longer exhibited an inhibitory effect against AChE, whereas its anti-SERT activity was maintained. The removal of the dimetylcarbamyl moiety from **5j** led to a loss of the inhibitory activities against both AChE and SERT (**7c**). These results suggest that the dimethylcarbamate moiety is essential for anti-AChE activity.

The optimization of the methylamine moiety in **5j** is shown in Table 4. The dimethylamine derivative **10a** was found to be less potent than **5j**. Increasing the size of the amine (**10c**—**g**) resulted in a sharp decline of inhibitory potencies against both AChE and SERT. Ammonium derivative **10b** was found to have lost its inhibitory activities against AChE (IC_{50}>1000 nM), whereas the potency against SERT was maintained (IC_{50}=44 nM). In general, it seems that a small amine like methylamine is particularly favorable for dual inhibition of AChE and SERT.[46]

As shown in Table 5, the effects of the tether length and the replacement of the hetero atom were examined. Compound **11**[43] possessing a shorter tether length exhibited much less activity toward both AChE and SERT than **5j**. However, compound **12**[43] possessing a longer tether length displayed stronger activity against AChE (IC_{50}=77 nM) than **5j** (IC_{50}=125 nM) but lower potency against SERT (IC_{50}>1000 nM). It

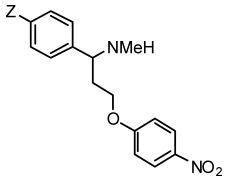
Table 2. *In Vitro* Inhibition of AChE and SERT for *para*-Carbamate Derivatives



Compound[a]	X	IC_{50} (nM)	
		AChE[b]	SERT[c]
5a	4-OMe	220	310
5b	4-Me	726	230
5c	H	341	>1000
5d	4-Cl	493	210
5e	4-F	594	790
5f	4-CF_3	572	130
5g	4-CN	330	500
5h	2-NO_2	52	>1000
5i	3-NO_2	14	175
5j	4-NO_2	125	44

a) Compounds were tested as their hydrochloride salts. *b*) From mouse brain. *c*) From rat synaptosome.

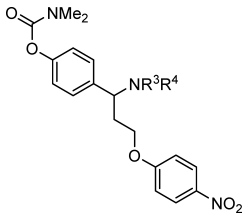
Table 3. *In Vitro* Inhibition of AChE and SERT for Derivatives with Different Carbamates



Compound[a]	Z	IC_{50} (nM)	
		AChE[b]	SERT[c]
5j	–$OCONMe_2$	125	44
9a	–OCONMeH	497	32
9b	–OCONEtH	>1000	46
9d[d]	–CH_2CONMe_2	>1000	60
9c	–OH	>1000	>1000

a) Compounds were tested as their hydrochloride salts. *b*) From mouse brain. *c*) From rat synaptosome. *d*) For the preparation of **9d**, see ref. 43.

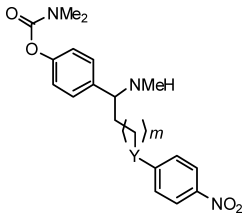
Table 4. *In Vitro* Inhibition of AChE and SERT for Derivatives with Various Amines



Compound[a]	NR^3R^4	IC_{50} (nM)	
		AChE[b]	SERT[c]
5j	NMeH	125	44
10a	NMe_2	319	87
10b	N^+Me_3	>1000	44
10c	NEtH	326	>1000
10d	NMeEt	378	980
10e		852	>1000
10f		>1000	500
10g		>1000	>1000

a) Compounds were tested as their hydrochloride salts. *b*) From mouse brain. *c*) From rat synaptosome.

Table 5. *In Vitro* Inhibition of AChE and SERT for Derivatives with Different Tethers and Replaced Heteroatoms



Compound[a]	*m*	Y	IC_{50} (nM)	
			AChE[b]	SERT[c]
11[d]	0	O	>1000	>1000
5j	1	O	125	44
12[d]	2	O	77	>1000
13	1	NH	36	>1000
14	1	NAc	>1000	>1000
15	1	S	61	680
16	1	None	524	670

a) Compounds were tested as their hydrochloride salts. *b*) From mouse brain. *c*) From rat synaptosome. *d*) For the preparation of **11** and **12**, see ref. 43.

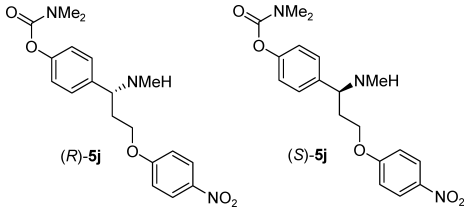
seems that the nitrophenoxy moiety of compound **12** interacted more efficiently with the hydrophobic site B in our hypothetical model of the AChE active site than compounds **5j** and **11**. The replacement of oxygen by nitrogen (**13**, **14**) or sulfur (**15**) resulted in a reduction of inhibitory potency particularly against SERT. Compound **16**, in which the ethereal oxygen was removed from **5j**, was found to be a moderate dual inhibitor of AChE (IC_{50}=524 nM) and SERT (IC_{50}=670 nM). These replacement studies revealed that the ethereal oxygen was crucial for the inhibition of both AChE and SERT.

Since we stated that racemic **5j** was the best dual inhibitor of AChE and SERT, both enantiomers of **5j** were evaluated (Table 6). Compound (*S*)-**5j** showed potent inhibitory potencies against both AChE (IC_{50}=101 nM) and SERT (IC_{50}=42 nM). There was little difference in AChE inhibitory activity between the stereoisomers of **5j**. However, SERT inhibition of (*S*)-**5j** was 3-fold more potent than that of (*R*)-**5j**.

10. Synthesis of Type B Derivatives

For further development of dual AChE and SERT inhibitory activities, conformationally restricted type B derivatives were designed by linking the methylamine moiety and the *ortho*-carbon atom of the phenyl ring of (*S*)-**5j** with a carbon chain of appropriate length (Fig. 8).[47]

Table 6. *In Vitro* Inhibition of AChE and SERT for Enantiomers of **5j**



Compound[a]	IC_{50} (nM)	
	AChE[b]	SERT[c]
rac-**5j**	125	44
(*R*)-**5j**	143	135
(*S*)-**5j**	101	42

a) Compounds were tested as their hydrochloride salts. *b*) From mouse brain. *c*) From rat synaptosome.

Preparation of the 1,2,3,4-tetrahydroisoquinoline derivatives (*n*=0) using a Bischler–Napieralski reaction is shown in Chart 4. Methoxyphenethylamine **17** was treated with ethyl malonyl chloride to give an *N*-acylated compound. The 1,2,3,4-tetrahydroisoquinoline skeleton was constructed by a Bischler–Napieralski reaction using $POCl_3$ or polyphosphate ester (PPE). Following hydrogenation of the exo double bond with Adams' catalyst provided compound **18**. Demethylation of **18** with boron tribromide, followed by *N*-Boc protection and treatment with dimethylcarbamyl chloride, furnished intermediate **19**. The ethyl ester group was reduced with $LiAlH_4$ and the resulting primary alcohol was reacted with various phenols under Mitsunobu reaction conditions. Deprotection of the Boc group afforded the derivatives **21a**—**g** and **22a**, **b**. A methyl group was introduced to the secondary amine moiety by an Eshweiler–Clarke reaction to give the derivatives **24a**—**g** and **25a**, **b**. Derivative **23** was prepared using a similar method *via* compound **20**.

Chart 5 shows the synthesis of the rigid derivatives having a saturated (**35a**, **b** and **36a**, **b**) or unsaturated (**32a**, **b** and **33a**—**h**) seven-membered ring (*n*=1). We formed the seven-membered ring by a ring-closing olefin metathesis reaction,[48] which is a very effective method for making rings of various sizes. Treatment of dihydroxybenzaldehyde **26** with dimethylcarbamyl chloride using sodium hydride or pyridine selectively provided a mono-carbamate product. The remaining phenol was converted to an aryl triflate by treatment with

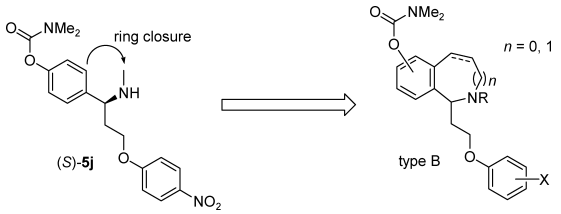
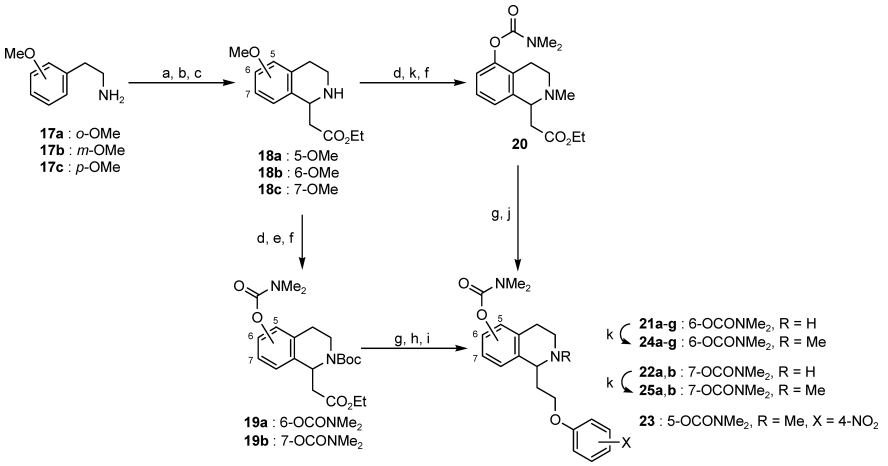
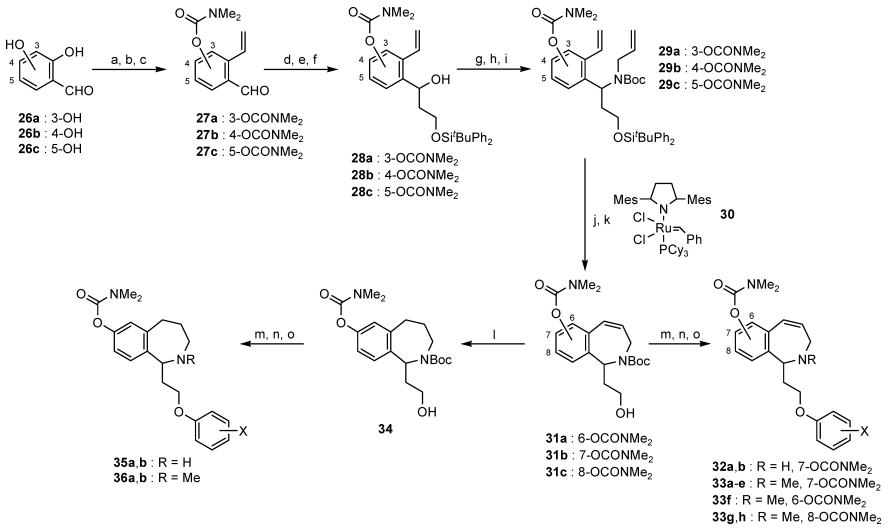


Fig. 8. Development of AChE–SERT Dual Inhibitors Using a Conformational Restriction Approach



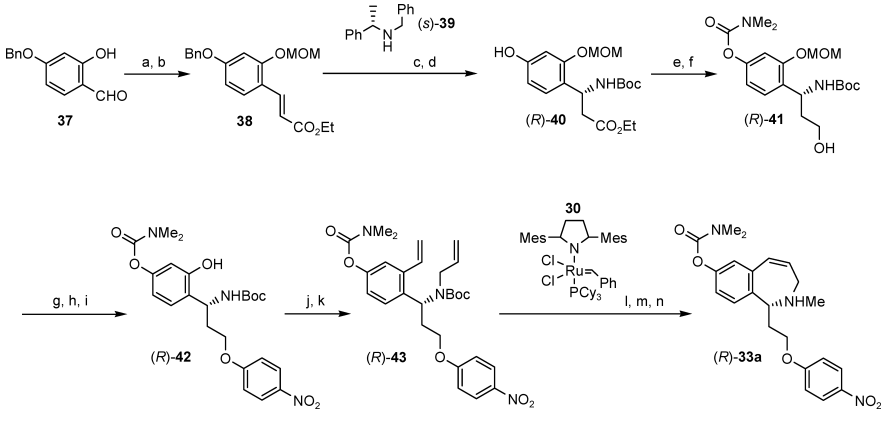
Reagents and conditions: (a) ethyl malonyl chloride, K_2CO_3, CH_2Cl_2, 0 °C; (b) $POCl_3$ or PPE, 80 °C; (c) H_2, PtO_2, AcOH, rt; (d) BBr_3, CH_2Cl_2, −78 °C to rt; (e) Boc_2O, THF, rt; (f) Me_2NCOCl, K_2CO_3, DMF, rt; (g) $LiAlH_4$, THF, −78 to 0 °C; (h) phenol, DEAD, PPh_3, THF, rt; (i) 2 N HCl–AcOEt, rt; (j) 4-fluoronitrobenzene, NaH, DMF; (k) 37% HCHO aq., HCO_2H, 80 °C.

Chart 4



Reagents and conditions: (a) Me_2NCOCl, NaH, DMF, rt or Me_2NCOCl, py, CH_2Cl_2, rt; (b) Tf_2O, py, CH_2Cl_2, 0 °C; (c) $(CH_2{=}CH)(n\text{-}Bu)_3Sn$, $Pd(PPh_3)_4$, LiCl, dioxane, 100 °C; (d) AcOEt, LDA, THF, −78 °C; (e) $LiBH_4$, THF, rt; (f) *t*-$BuPh_2SiCl$, imidazole, DMF, rt; (g) CBr_4, PPh_3, CH_2Cl_2, rt; (h) allylamine, CH_3CN, rt; (i) Boc_2O, Et_3N, THF, 50 °C; (j) **30** (10 mol%), CH_2Cl_2, 45 °C; (k) TBAF, THF, rt; (l) H_2, Pd/C, MeOH, rt; (m) phenol, DEAD, PPh_3, THF, rt; (n) 2 N HCl–EtOAc, rt; (o) 37% HCHO aq., HCO_2H, 80 °C.

Chart 5



Reagents and conditions: (a) MOMCl, *i*-Pr_2NEt, CH_2Cl_2, 0 °C, 79%; (b) $(EtO)_2P(O)CH_2CO_2Et$, NaH, THF, 0 °C, 99%; (c) (i) (*S*)-**39**, *n*-BuLi, THF, −78 °C; (ii) H_2, 20% $Pd(OH)_2$/C, MeOH–H_2O–AcOH (20 : 2 : 1), rt, 67% (2 steps); (d) Boc_2O, Et_3N, MeOH, rt, 97%; (e) Me_2NCOCl, K_2CO_3, DMF, rt, 97%; (f) $LiAlH_4$, THF, −50 to 0 °C, 82%; (g) *p*-nitrophenol, DEAD, PPh_3, THF, rt; (h) concd. HCl, MeOH, rt, 68% (2 steps); (i) Boc_2O, Et_3N, MeOH, rt, 85%; (j) (i) Tf_2O, py, CH_2Cl_2, 0 °C; (ii) $(CH_2{=}CH)(n\text{-}Bu)_3Sn$, $Pd(PPh_3)_4$, LiCl, dioxane, 100 °C, 86% (2 steps); (k) allyl bromide, NaH, DMF, 0 °C to rt, 86%; (l) **30** (10 mol%), CH_2Cl_2 (15 mM), 45 °C, 96%; (m) 2 N HCl–AcOEt, rt, 96%; (n) (i) 37% HCHO aq., HCO_2H, 80 °C, 78%.

Chart 6

trifuluoromethanesulfonic anhydride. The resulting triflate was reacted with tributylvinylstannane by the Stille reaction to give styrene compound **27**. The aldol reaction with ethyl acetate followed by reduction of the ethyl ester with $LiBH_4$ furnished the diol. Selective protection of the primary alcohol with *t*-$BuPh_2SiCl$ and imidazole provided benzyl alcohol **28**. Bromination of **28** using carbon tetrabromide and triphenylphosphine gave benzyl bromide. The benzyl bromide was treated with allylamine in acetonitrile, followed by *N*-Boc protection to afford **29**, the precursor for ring-closing olefin metathesis. Ring-closing olefin metathesis of **29** with Grubbs 2nd generation catalyst **30** successfully gave a seven-membered ring product. Deprotection of the silyl group with tetrabutylammonium fluoride (TBAF) furnished primary alcohol **31**. Treatment of **31** with various phenols under Mitsunobu reaction conditions and deprotection of the Boc group provided derivatives **32a**, **b**. *N*-Methylation by a Eshweiler–Clarke reaction gave the derivatives **33a**—**h**. Derivatives **35a**, **b** and **36a**, **b** were synthesized from **34**, prepared by the hydrogenation of intermediate **31**, in a similar manner to that above.

The asymmetric synthesis of (*R*)-**33a** is shown in Chart 6. Phenol **37**[49] was protected with a methoxymethyl (MOM) group and a Horner–Wadsworth–Emmons reaction provided α,β-unsaturated ester **38**. Compound **38** was subjected to chiral amination by a method reported by Davies and Walters.[50] The resulting amine was protected with a Boc group to furnish (*R*)-**40** (99% ee). Compound (*R*)-**40** was reacted with dimethylcarbamyl chloride and reduced with $LiAlH_4$ to afford primary alcohol (*R*)-**41**. Alcohol (*R*)-**41** was reacted with *p*-nitrophenol under Mitsunobu reaction conditions, and the following treatment with HCl and Boc protection gave (*R*)-**42**. This phenol was converted to a triflate and a Stille reaction with tributylvinylstannane provided a styrene compound. Following allylation of the *N*-Boc-protected amine afforded (*R*)-**43**. Ring-closing olefin metathesis of (*R*)-**43** with

Grubbs 2nd generation catalyst **30** provided a seven-membered ring product. Deprotection of the Boc group and Eshweiler–Clarke methylation furnished (*R*)-**33a**. The enantiomer (*S*)-**33a** was prepared by the same method using chiral amine (*R*)-**39**.

The absolute configuration of (*R*)-**40** was determined as shown in Chart 7. Triflate (*R*)-**44**, which was prepared by triflation of (*R*)-**42**, was converted to (*R*)-**45** by treatment with a catalytic amount of $Pd(OAc)_2$ in *N*,*N*-dimethylformamide (DMF). All the physical properties, including the optical rotation of (*R*)-**45**, were identical to those of the authentic sample, which was prepared from known compound (*R*)-**8**.

11. Results of Type B Derivatives

Table 7 summarizes the inhibitory activities against AChE and SERT of the conformationally restricted derivatives containing a 1,2,3,4-tetrahydroisoquinoline skeleton (**21a**, **b**, **22a**, **b**, **23**, **24a**—**g** and **25a**, **b**). 1,2,3,4-Tetrahydroisoquinoline derivatives were reinvestigated with respect to substituent X using, in particular, an electron-withdrawing group, and the ideal carbamate position. A series of 1,2,3,4-tetrahydroisoquinoline derivatives were observed to have dramatically enhanced inhibitory activity toward AChE, but their inhibitory activity toward SERT had almost disappeared. We supposed that conformational restriction of the flexible amine moiety resulted in the appropriate binding to AChE. The reason for the disappearance of anti-SERT activity could not be determined because we had no information on the structure of SERT. Among the compounds with a 1,2,3,4-tetrahydroisoquinoline structure, **21a** (R=H, X=4-NO_2) showed the most potent inhibitory activity against AChE (IC_{50}=8 nM).

Next, we examined the rigid derivatives **35a**, **b**, **36a**, **b** containing a saturated seven-membered ring and **32a**, **b**, **33a**—**h** containing an unsaturated seven-membered ring (Table 8). Compounds **35a**, **b** (R=H) and **36a**, **b** (R=Me) showed higher inhibitory potencies against AChE but much lower potencies against SERT similarly to 1,2,3,4-tetrahydroquinoline derivatives. Compounds **32a**, **b** (R=H) which possessed a double bond on the seven-membered ring also exhibited good inhibitory activities but only against AChE, whereas compounds **33a**—**e** (R=Me) surprisingly showed potent inhibitory activities against not only AChE but also SERT. We have not determined the reason for this striking improvement

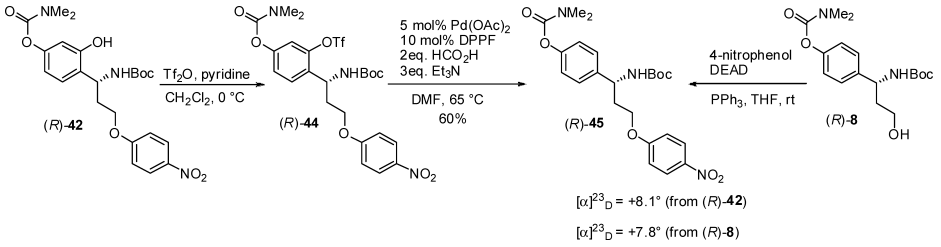
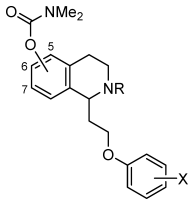


Chart 7. The Determination of the Absolute Stereochemistry of (*R*)-**40**

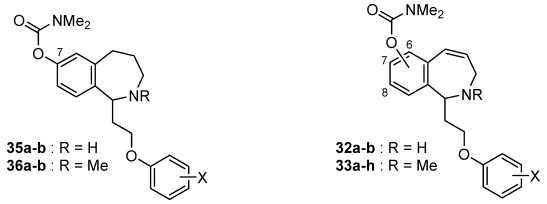
Table 7. *In Vitro* AChE and SERT Inhibition Activity of 1,2,3,4-Tetrahydroisoquinoline Derivatives



Compound[a]	Carbamate position	R	X	IC_{50} (nM)	
				AChE[b]	SERT[c]
21a	6-	H	4-NO_2	8	>1000
21b	6-	H	4-Cl	17	>1000
22a	7-	H	4-NO_2	101	>1000
22b	7-	H	4-Cl	219	>1000
24a	6-	Me	4-NO_2	11	940
24c	6-	Me	3-Me-4-NO_2	16	170
24d	6-	Me	3-NO_2	11	125
24b	6-	Me	4-Cl	33	660
24e	6-	Me	4-F	49	>1000
24f	6-	Me	4-Br	34	>1000
24g	6-	Me	4-OMe	20	>1000
23	5-	Me	4-NO_2	56	750
25a	7-	Me	4-NO_2	161	>1000
25b	7-	Me	4-Cl	265	520

a) Compounds were tested as their hydrochloride salts. *b*) From mouse brain. *c*) From rat synaptosome

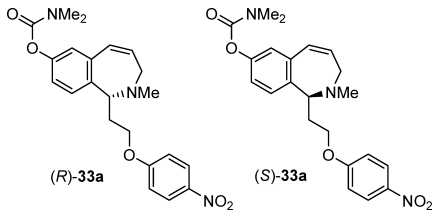
Table 8. *In Vitro* AChE and SERT Inhibition Activity of Derivatives with a Seven-Membered Ring



Compound[a]	Carbamate position	R	X	IC_{50} (nM)	
				AChE[b]	SERT[c]
35a	7-	H	4-NO_2	55	>1000
35b	7-	H	4-Cl	215	>1000
36a	7-	Me	4-NO_2	61	>1000
36b	7-	Me	4-Cl	116	>1000
32a	7-	H	4-NO_2	92	>1000
32b	7-	H	3-Me-4-Cl	153	>1000
33a	7-	Me	4-NO_2	66	63
33b	7-	Me	3-Me-4-Cl	103	61
33c	7-	Me	4-Cl	139	71
33d	7-	Me	4-F	135	850
33e	7-	Me	4-CF_3	285	62
33f	6-	Me	3-Me-4-Cl	146	900
33g	8-	Me	4-NO_2	>1000	>1000
33h	8-	Me	4-Cl	>1000	>1000

a) Compounds were tested as their hydrochloride salts. *b*) From mouse brain. *c*) From rat synaptosome.

Table 9. *In Vitro* AChE and SERT Inhibition Activity of **33a** Enantiomers



Compound[a]	IC_{50} (nM)	
	AChE[b]	SERT[c]
rac-**33a**	66	63
(*R*)-**33a**	14	6
(*S*)-**33a**	609	930

a) Compounds were tested as their hydrochloride salts. *b*) From mouse brain. *c*) From rat synaptosome.

in anti-SERT activities obtained by changing the secondary amine to a tertiary amine on the seven-membered ring. However, it is clear that the olefin moiety on the seven-membered ring is essential for the inhibitory potency of SERT as can be seen by comparing derivatives **33a**—**e** with **36a**, **b**. Compound **33a** was the most potent dual inhibitor of AChE (IC_{50}=66 nM) and SERT (IC_{50}=63 nM). Changing the substitution position of the dimethylcarbamate from 7- to 6-(**33f**) and 8-(**33g**, **h**) resulted in the loss of inhibitory potency for SERT.

The inhibitory activities of both enantiomers of **33a** were evaluated, as shown in Table 9. The enantiomer (*R*)-**33a** showed extremely potent inhibitory activity against both AChE (IC_{50}=14 nM) and SERT (IC_{50}=6 nM). Although both **5j** and its enantiomer showed similar levels of AChE inhibition, (*R*)-**33a** showed a 44 times stronger inhibitory activity against AChE than (*S*)-**33a**. This difference of AChE inhibition would result from the conformational rigidity of **33a**. Similarly (*R*)-**33a** exhibited a 155 times stronger inhibitory activity against SERT than (*S*)-**33a**. Compound (*R*)-**33a** showed extremely weak inhibitory activities against butylcholinesterase, choline acetyltransferase, norepinephrine and dopamine transporters. Compound (*R*)-**33a** was an extremely potent inhibitor of both AChE (IC_{50}=14 nM) and SERT (IC_{50}=6 nM).

12. Docking Study of Dual AChE–SERT Inhibitors

A conformational analysis and a docking study of derivatives **5j**, **11**, **12** and **33a** with AChE were performed in order to analyze their AChE inhibitory potencies.[51]

Each compound was model-built using the QUANTA system[52] in the protonated form with respect to the basic amine. A conformational analysis using molecular dynamics and molecular mechanics was performed using the CHARMm force field.[53] A docking study of the inhibitors was carried out with the QUANTA/CHARMm system based on several low energy conformations obtained by the conformational analysis. A series of initial positions and conformations of the inhibitors were manually set and stable complex structures were obtained by energy minimization with the enzyme structure being fixed.

From the results, comparable interaction energies were obtained for (*R*)-**5j** and (*S*)-**5j**. This is consistent with the fact that only a slight difference was observed for the IC_{50} values of the isomers of **5j**. On the other hand, the interaction energy of the most stable model of (*R*)-**33a** was about 6 kcal/mol more stable than that of (*S*)-**33a**, which is consistent with the observed potencies of the isomers of **33a**, *i.e.*, (*R*)-**33a** is around 40 times more potent than (*S*)-**33a**.

The most stable complex structure models of (*R*)-**33a** and (*S*)-**5j** are shown in Figs. 9A and B. As it can be seen for both cases, the carbamate moiety was located near catalytic Ser200 and the phenyl ring was located in the hydrophobic pocket near Phe330, which is located deep in the gorge. The proton of the tertiary ammonium group did not interact directly with the carboxyl group of Asp72. The positive charge is considered to be stabilized by the aromatic ring of Tyr334 through an aromatic–ammonium interaction.[54] This kind of interaction is observed in several complex crystal structures, such as those of donepezil.[55] The 4-nitrophenoxy moiety was located near the indole ring of Trp279 at the top of the gorge.

In the case of compounds **5j**, **11** and **12**, the length of the methylene chain between the benzylamine and nitrophenoxy moieties is an important factor affecting the potency. Compound **11** with a shorter tether group showed very weak inhibitory activity (IC_{50}>1000 nM). In contrast, compound **12** showed a slightly higher potency (IC_{50}=77 nM) than compound **5j** (IC_{50}=*ca.* 100 nM). In order to explain the difference among **5j**, **11** and **12**, we focused on the position of the nitrophenoxy group at the binding site. The nitrophenoxy group of **5j** was involved in the hydrophobic interaction with the indole group of Trp279 as mentioned above (Fig. 9A). In the case of **11**, the distance between the nitrophenoxy group and indole rings became 0.2 Å longer than that of (*S*)-**5j** (Fig. 9C). The shorter tether for **11** would prevent both phenyl and nitrophenoxy groups to simultaneously interact with the hydrophobic pocket at the bottom and top of the gorge, respectively. On the other hand, the distance between the nitrophenoxy group of **12** and the indole ring of Trp279 was 0.4 Å shorter than that of (*S*)-**5j**, leading to an enlarged ring overlap (Fig. 9D). Moreover, the interaction at the peripheral hydrophobic site seemed to be more favorable due to the additional hydrophobic methylene group of **12**, even though its larger number of rotatable bonds was more disadvantageous with respect to entropy compared to **5j**. This resulted in a slight improvement of the inhibitory activity of **12**.

13. Pharmacological Effects of Dual AChE–SERT Inhibitors

The pharmacological properties of (*S*)-**5j**, the most potent type A compound, and (*R*)-**33a**, the most potent type B compound, were evaluated in order to show efficacy as a drug for AD.[56] In the experiments, (*S*)-**5j** was used as a (fumaric acid)$_{1/2}$ salt (RS-1259) for solidification and (*R*)-**33a** was used as a hydrochloride salt.

***Ex Vivo* Inhibition of AChE and SERT** The result of the *ex vivo* inhibitory effects on AChE and SERT of RS-1259 in the mouse brain is shown in Fig. 10A. Table 10 summarizes the inhibition of AChE and SERT in mice and rats. The inhibition results of donepezil and fluoxetine are also included for comparison. After oral administration, compound RS-1259 inhibited both AChE and SERT in the mouse brains with a potency several fold weaker than donepezil and equiv-

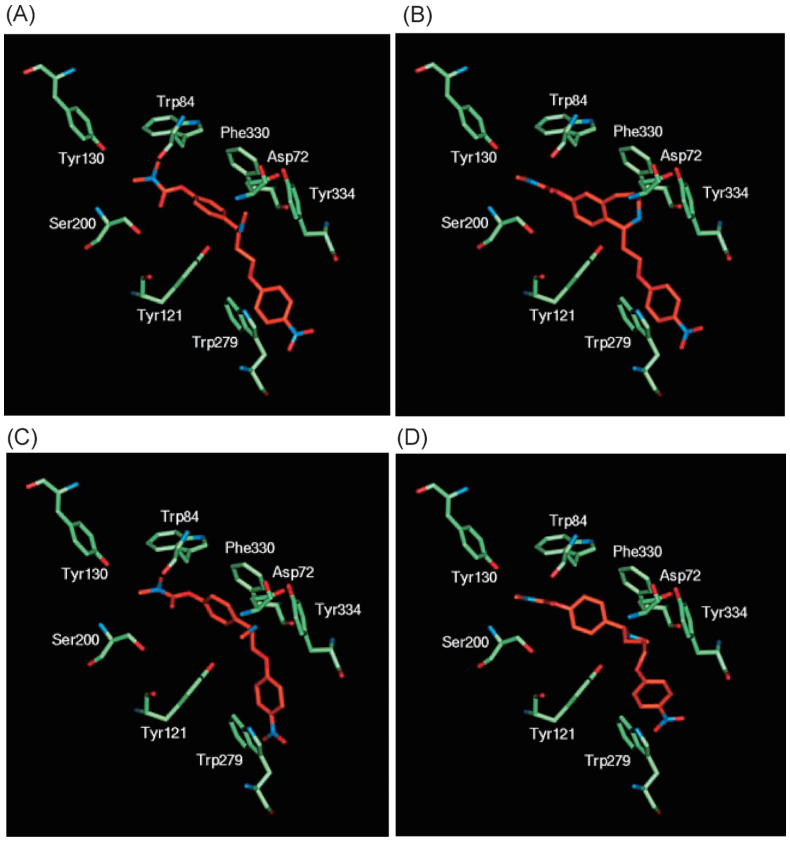


Fig. 9. Complex Structure Models of AChE and (*S*)-**5j** (A), (*R*)-**33a** (B), **11** (C), or **12** (D)

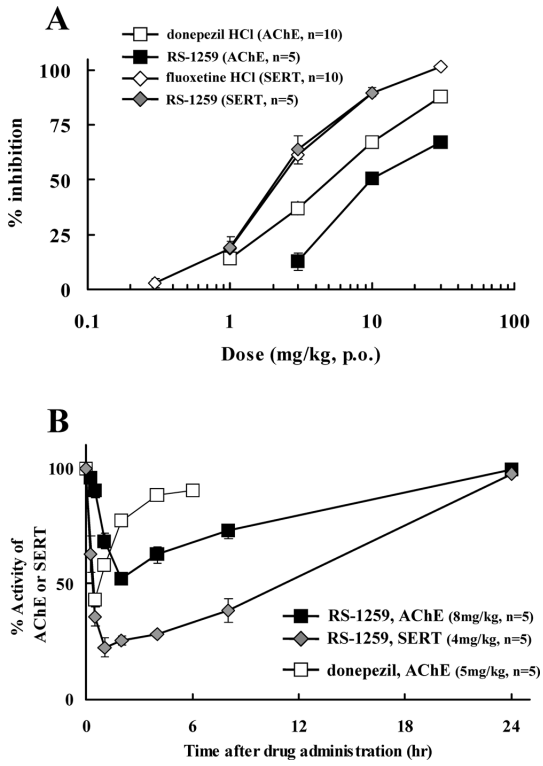


Fig. 10. Dose-Dependency (A) and Time Course (B) of Inhibition of AChE and SERT in the Mouse Brain Following Oral Administration of RS-1259, Donepezil and Fluoxetine

Ex vivo inhibition was measured at 60 min (A) and at other time points (B) after administration. Symbols represent the mean and S.E.M. of 5 animals.

Table 10. Inhibition of AChE and SERT in the Brain 60 min after Oral Administration of RS-1259, Donepezil and Rivastigmine in Mice and Rats

	ED_{50} values (mg/kg, *p.o.*)	
	Mice	Rats
AChE		
RS-1259	13 (11—15, $n=5$)	43 (31—75, $n=5$)
Donepezil	5.1 (4.6—5.6, $n=10$)	16 (12—21, $n=10$)
Rivastigmine	0.70 (0.64—0.77, $n=10$)	4.1 (2.7—7.5, $n=10$)
SERT		
RS-1259	2.4 (1.9—2.9, $n=5$)	<8 (66% inh. at 8 mg/kg, $n=5$)
Fluoxetine	2.2 (2.0—2.5, $n=10$)	Not determined
Fluvoxamine	4.6 (4.2—4.9, $n=10$)	Not determined

alent to fluoxetine, respectively. Compound (*R*)-**33a**, the most potent type B dual inhibitor, was not used in further experiments because a low penetration into the brain was predicted based on the *ex vivo* experiment of AChE inhibition in mice. The doses necessary for the 50% efficacy (ED_{50}) of RS-1259, donepezil and rivastigmine were larger in rats than those in mice, although the potency order of the compounds was the same in each species. RS-1259 was as potent as fluoxetine and more potent than fluvoxamine for SERT inhibition *ex vivo* in mice.

RS-1259 is the first compound that can facilitate both cholinergic and serotonergic transmission in the brain following oral administration. The balanced inhibition of AChE and SERT by RS-1259 demonstrated the unique property of this compound and its potentially high efficacy *in vivo*. A single oral administration of RS-1259 inhibited AChE and SERT in the central nervous system in a similar time frame,

as expected.

After oral administration, RS-1259 inhibited AChE and SERT in the mouse brains in a similar manner over time. The inhibition of AChE and SERT which reached a maximum at 1—2 h, was still observed at 8 h and fully disappeared at 24 h after administration. The time to reach maximum AChE inhibition and the duration of the inhibitory effect of donepezil were much shorter than that of RS-1259 in mice (Fig. 10B).

Microdialysis in the Rat Hippocampus An actual simultaneous elevation of extracellular levels of serotonin and ACh in the rat hippocampus due to the inhibition of both AChE and SERT was confirmed by microdialysis (Fig. 11). RS-1259 simultaneously elevated extracellular levels of ACh and 5-HT in the hippocampus of freely moving rats. Donepezil and fluoxetine only increased ACh and 5-HT levels, respectively. ACh was increased to a similar extent by RS-1259 and donepezil at doses of 64 and 16 mg/kg, respectively. At such high doses, all the rats showed cholinergic symptoms such as muscle fasciculation, salivation and lacrimation. The rate of 5-HT increase induced by fluoxetine at a dose of 32 mg/kg was slower and the overall 5-HT values were lower than those by RS-1259 at 64 mg/kg. The flat body posture elicited by fluoxetine could not be distinguished from the abnormal posture resulting from the cholinergic symptoms which was observed when rats were treated with RS-1259.

Both the *ex vivo* measurements of AChE and SERT inhibition and the microdialysis experiment indicated that RS-1259 was 3—4 times weaker than donepezil and almost equipotent to fluoxetine in exerting central cholinergic and serotonergic effects, respectively.

5-Hydroxytryptophan (5-HTP)-Enhancement in Mice RS-1259 enhanced 5-HTP-induced locomotor activity in a bell-shaped dose-response relationship with maximum enhancement at 8 mg/kg (Fig. 12A). The locomotor activity in mice treated with doses larger than 8 mg/kg was reduced as the cholinergic symptoms appeared. Fluoxetine (Fig. 12B) and fluvoxamine (Fig. 12C) were effective in the test with a longer duration of action for fluoxetine than for fluvoxamine.

RS-1259 was as effective as the serotonin-selective re-uptake inhibitors in the 5-HTP enhancement test in mice, suggesting an anti-depressant effect could be expected with the compound. It was somewhat surprising that RS-1259 was effective at doses lower than the effective dose of fluoxetine, since the potencies of both compounds were similar in the *ex vivo* measurement of SERT inhibition in the brain. Such low doses of RS-1259 alone did not increase the locomotor activity in mice and fluoxetine-induced 5-HTP enhancement was not affected by a co-administration of donepezil at doses that do not induce cholinergic symptoms. The above inconsistency may be due to the difference in the pharmacokinetic properties between RS-1259 and fluoxetine. The doses of RS-1259 and donepezil eliciting similar increases of ACh in the hippocampus correlated well with their ED_{50} for the *ex vivo* inhibition of brain AChE. An oral dose four times higher than donepezil was necessary for RS-1259 to achieve a similar inhibition level of brain AChE in mice and rats. Thus, it was concluded that the potency of RS-1259 following oral administration was approximately a quarter of that of donepezil with respect to AChE inhibition, whereas it was equipotent to fluoxetine with respect to SERT inhibition in the central nervous system *in vivo*.

Amelioration of Memory Deficits of Aged Rat It has been reported that not only cholinergic but also serotonergic impairments in the brain are involved in causing cognitive deficits in aged animals and in humans.[57,58] The effectiveness of RS-1259 in improving these deficits related to the central nervous system was compared to that of donepezil by evaluating two models, *i.e.* memory deficits in aged rats and the daytime consciousness level of rats with nocturnal habits.[59]

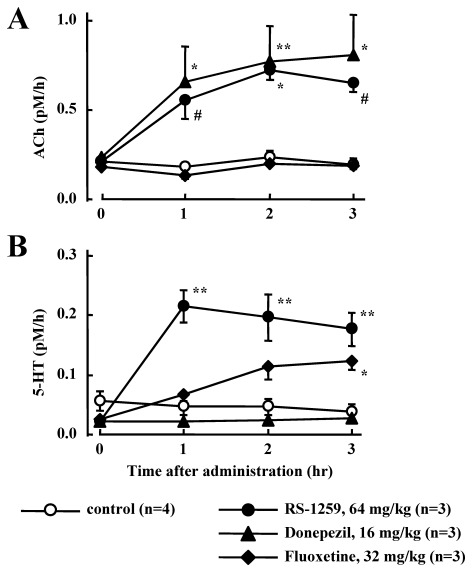


Fig. 11. Extracellular Concentrations of ACh and 5-HT in the Rat Hippocampus Increased by RS-1259, Donepezil and Fluoxetine

Compounds or the vehicle were orally administered just after the first sampling (−60—0 min). Each symbol represents the mean of 3—4 animals, with the S.E.M. indicated by a bar. The values indicated by #, *, and ** are statistically different ($p<0.1$, 0.05 and 0.01, respectively).

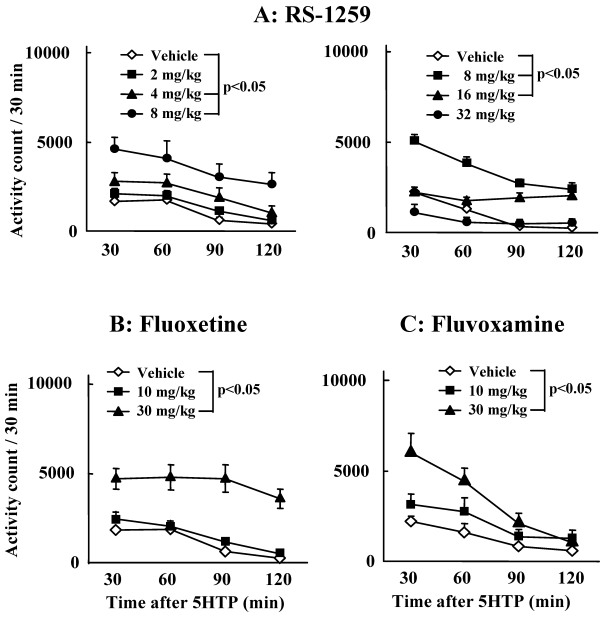


Fig. 12. 5HTP-Enhancing Effect of RS-1259 (A), Fluoxetine (B) and Fluvoxamine (C) in Mice

Each symbol represents the mean of 8 mice, with S.E.M. indicated by a bar. ANOVA of repeated measures and a *post-hoc* analysis, Fisher's PLSD, indicated that oral doses at 4, 8 and 16 mg/kg of RS-1259, and at 30 mg/kg of fluoxetine and fluvoxamine enhanced 5HTP-induced locomotor activity ($p<0.05$).

Short-term memory is severely affected early on in the course of Alzheimer's disease as well as in the normal aging process,[60] and it is reported that short-term memory is preferentially impaired in aged animals.[61] To examine whether RS-1259 could improve age-related cognitive decline, the spatial short-term memory was evaluated by using a two-platform task (TPT) using a water maze in aged rats. Briefly, two visible platforms of identical appearance (diameter: 12 cm), one fixed and the other floating, were simultaneously placed at the center of two non-neighboring quadrants (*e.g.* north and south) in the same pool as that in the pretraining. The surfaces of the platforms were set 0.5 cm above the water surface. The trial began by releasing a rat into the water facing the wall of the pool from one of the remaining two quadrants. The various patterns of platform locations and start positions are shown in Fig. 13A. One session consisting of 6 trials was given each day. The rats were released from the two start positions alternately and the trial ended when the rat reached either platform. At 24—25 months of age, 11 rats showing a significant decline in the TPT performance were orally administered AChE inhibitors or the vehicle 60 min before each test. In the first test, both donepezil (0.5 mg/kg) and RS-1259 (1 mg/kg, but not 0.5 mg/kg) significantly ameliorated age-related short-term memory impairment (Fig. 13B). In the second test, at half the dose, donepezil (0.25 mg/kg) also improved the memory impairment, although at twice the dose (1 mg/kg) it did not (Fig. 13C). A higher dose of RS-1259 (2 mg/kg) was also effective in recovering the memory deficit (Fig. 13C). The parameters to evaluate sensorimotor acitivities, *i.e.* the swimming distance, the swimming speed, the floating time, and the swimming time of rats, were not affected by the drug treatments (data not shown).

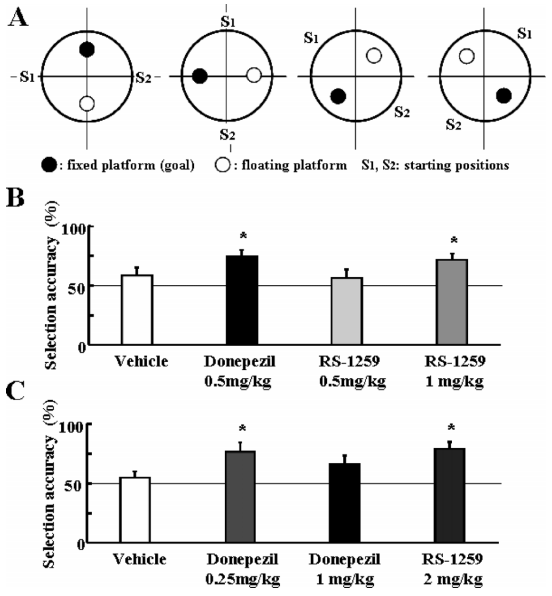


Fig. 13. Amelioration of Memory Deficits in Aged Rats by RS-1259 and Donepezil

(A) Four representative patterns of the platform locations and start positions are shown. The locations of the fixed platform and floating platform were randomly changed in each session. Closed circles, fixed platform; open circles, floating platform; S_1 and S_2, start positions. (B) and (C) represent the results of two separate drug tests. Animals were orally treated with AChE inhibitors or distilled water 60 min before a test session. Each column represents the mean accuracy of 11 rats, with the S.E.M. indicated by a bar. As determined by Wilcoxon's analysis, an amelioration of the reduced accuracy by RS-1259 and donepezil at doses of 1 and 2, and 0.25 and 0.5 mg/kg, respectively, was detected ($p<0.05$).

Both RS-1259 and donepezil ameliorated the low TPT performance of aged rats, but the effective dose of RS-1259 was approximately four times higher than that of donepezil, suggesting that no advantage was obtained by a dual activation of cholinergic and serotonergic transmissions over a solely cholinergic activation in the brain with respect to memory enhancement. TPT is reported to be highly sensitive to cholinergic deficits. We failed to observe any increase in the extracellular levels of ACh and 5-HT in the rat hippocampus by fluoxetine and donepezil, respectively, even at high doses. A positive interaction between cholinergic and serotonergic transmissions in the central nervous system may not exist, at least not in the hippocampus. A higher TPT score by RS-1259 was probably obtained solely by improvements in the cholinergic transmission and not the serotonergic one. The simultaneous serotonergic activation by RS-1259 seemed not to affect the cholinergic transmission involved in the memory system.

Elevation of Daytime Consciousness Level in Rats The daytime consciousness level of rats in a familiar cage was low. The total awake (10—20 min), slow wave sleep (35—45 min) and paradoxical sleep (about 5 min) episodes for every 60 min were stable during the recording period except for the initial 1 h when the awake episode increased and the other episodes decreased due to the handling necessary for oral administration. RS-1259 and donepezil, at all doses tested, increased the awake episode and decreased slow wave sleep as well as paradoxical sleep episodes in the initial 1 h following oral administration (Fig. 14). The consciousness level remained elevated up to 3 h after the highest dose of RS-1259 was administered, whereas it returned to control levels after 2—3 h in rats given the highest dose of donepezil. RS-1259 was also effective for the elevation of the daytime consciousness level in rats where the serotonergic neurons in the brain had been degenerated by an intracerebroventricular injection of 5,7-dihydroxytriptamine (5,7-DHT), except for the minimum effective dose of 4 mg/kg (Table 11).[62] No abnormal behavior was observed throughout the recording period at any dose tested.

The consciousness level is associated with several neurotransmitter systems in the brain, including the cholinergic and serotonergic systems. Dringenberg and Diavolitsis reported that co-activation of the cortical cholinergic and

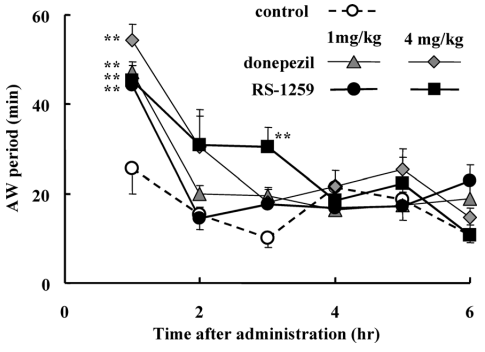


Fig. 14. Effects of RS-1259 and Donepezil on the Daytime Consciousness Level of Rats

The total time of awake episodes was calculated every 60 min after a single oral administration of AChE inhibitors or the vehicle. Each symbol represents the mean awake episode of 5 rats during 60 min, with the S.E.M. indicated by a bar. $**p<0.01$ *vs.* vehicle control (ANOVA).

Table 11. Effects of RS-1259 on the Daytime Consciousness Level in Intact Rats and Serotonergic Denervated Rats with 5,7-DHT

	Dose (mg/kg)	Awake episode (min)	
		Intact rats (n=5)	5,7-DHT treated rats (n=5)
Vehicle	—	51.0±5.4	101.0±7.4
RS-1259	1	76.2±6.0	Not tested
	2	82.4±9.3*	131.2±13.0
	4	106.6±9.8**	155.4±7.3***

Numbers represent the mean of total time of awake episodes in the period of 180 min after a single oral administration of RS-1259, with the S.E.M. $*p<0.05$, $**p<0.01$, *vs.* vehicle control in the intact rats. $***p<0.01$, *vs.* vehicle control in 5,7-DHT treated rats. The statistical differences were calculated in each group of intact and 5,7-DHT treated rats independently by a Dunnett's *t*-test.

serotonergic transmissions restored the electrocephalogram (EEG) activation abolished by a monoaminergic-cholinergic blockade.[63] The authors used a complicated pharmacological procedure to induce the blockade and a co-activation of cholinergic and serotonergic transmissions in the cortex, without considering any possible drug–drug interaction between the compounds used. Using the dual inhibitor of AChE and SERT, we were able to evaluate the pharmacological effects of a simultaneous activation of both cholinergic and serotonergic transmissions in the brain without having to take into account any drug–drug interaction. We compared the effect of RS-1259 with that of donepezil on the daytime consciousness level of rats, which is low when they are in a familiar surrounding. Both compounds increased the consciousness level at similar doses. Considering the fact that RS-1259 inhibited brain AChE with a potency four times weaker than that of donepezil, this result clearly indicates that a simultaneous activation of the central serotonergic system could reduce the dose of AChE inhibitor needed to maintain an active consciousness level. This notion was confirmed by the observation that a larger dose of RS-1259 was required for elevation of the consciousness level in rats subjected to serotonergic denervation than that in normal rats.

It was confirmed that the degree of cholinergic activation required in maintaining the brain active could be reduced by a simultaneous activation of the central serotonergic transmission, using the dual AChE–SERT inhibitor.

14. Conclusion

Dual AChE and SERT inhibition is considered to be a highly effective method for treating AD because a reduction in the progress of cognitive decline could be achieved and dose-related adverse effects caused by excessive AChE inhibition could be avoided. To create dual AChE–SERT inhibitors, a hypothetical model of the AChE active center was conceived from the complex crystal structure of AChE and donepezil. Subsequently, a hybrid compound of rivastigmine and fluoxetine was designed as a novel dual AChE–SERT inhibitor.

As a result of structure–activity relationship studies, compound (*S*)-**5j**, which possessed balanced inhibitory activities of AChE and SERT, was successfully obtained. Moreover, conformationally restricted compounds of (*S*)-**5j** were designed for further improvement of inhibition potency. Among them, (*R*)-**33a**, which was successfully synthesized using a ring-closing olefin metathesis reaction, showed the strongest inhibition of both AChE and SERT as anticipated.

Molecular modeling studies of several compounds, including **5j** and **33a**, were performed to analyze their inhibitory activities against AChE. The results corresponded well with their potencies and provided some useful information about enzyme-inhibitor interaction.

We confirmed that the degree of cholinergic activation required to maintain an active brain could be reduced by a concurrent activation of the central serotonergic transmission, using the dual inhibitor RS-1259, an (*S*)-**5j** $(\text{fumaric acid})_{1/2}$ salt. Thus, it was anticipated that cognitive disorders such as Alzheimer's disease could be effectively and safely treated with dual inhibitors of AChE and SERT.[64]

BTG International Ltd. has been developing RS-1259 as BGC20-1259, the most promising inhibitor of AChE and SERT. BGC20-1259 has completed Phase I clinical studies in healthy volunteers and BTG recently announced that it will now be is exploring partnering opportunities for the programme.

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