Donepezil Hydrochloride (E2020) and Other Acetylcholinesterase Inhibitors

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Abstract: A wide range of evidence shows that acetylcholinesterase (AChE) inhibitors can interfere with the progression of Alzheimer’s disease (AD). The successful development of these compounds was based on a well-accepted theory that the decline in cognitive and mental functions associated with AD is related to the loss of cortical cholinergic neurotransmission. The earliest known AChE inhibitors, namely, physostigmine and tacrine, showed modest improvement in the cognitive function of Alzheimer’s patients. However, clinical studies show that physostigmine has poor oral activity, brain penetration and pharmacokinetic parameters while tacrine has hepatotoxic liability. Studies were then focused on finding a new type of acetylcholinesterase inhibitor that would overcome the disadvantages of these two compounds. Donepezil hydrochloride inaugurates a new class of AChE inhibitors with longer and more selective action with manageable adverse effects. Currently, there are about 19 new Alzheimer’s drugs in various phases of clinical development.

Introduction

A number of studies has confirmed that the average life expectancy for individuals has increased from 50 years to about 75 years. Corollary with this increase, however, is the probability of mental health decline or dementia. Alzheimer’s disease (AD) is said to be the leading cause of dementia in elderly individuals. With the continuing increase of the elderly population, the prevalence of AD is likely to increase.

AD individuals exhibit retrogression in mental health functions rendering them incapacitated and unable to perform normal daily activities. Elderly persons are the ones commonly afflicted with this disease. However, evidence show that it can also afflict even individuals as young as 40 years of age. Ironically, the true nature or cause of the disease is still unknown making the development of treatment drugs a complex endeavor. Currently, the loss of cholinergic function is the only evidentiary finding responsible for cognitive decline. Hence, therapeutical development has focused on this theory.

Study Concept

Aging is often regarded as the main factor in memory impairment and decline in other mental functions or dementia. In this context, dementia is viewed as an inevitable phenomenon in old age. However, the presence of memory impairments in some elderly individuals does not necessarily make them demented. Some memory impairments are normal part of the aging process (“normal aging”). Therefore, memory loss and other neuropsychologic symptoms such as impairments of judgment, language, learning and abstract thinking, which are descriptive of AD, may be attributed to normal aging. In this context, the relationship between normal aging and AD is a debatable concept [1]. This relationship makes the diagnosis of AD most uncertain until the disease has progressed to a moderate to severe stage when treatment prospects become more formidable [2].

Alzheimer’s Disease

Definition

Alzheimer’s disease, discovered by Dr. Alois Alzheimer in 1907, is described as a degenerative disease of the central nervous system (CNS) characterized especially by premature senile mental
deterioration. AD patients exhibit marked decline in cognitive ability and severe behavioral abnormalities such as irritability, anxiety, depression, disorientation, and restlessness. AD is a progressive disease, i.e., the onset of the disease may show mild symptoms but these symptoms will sooner or later become more and more severe until the patient loses his or her capacity to handle normal daily activities. While AD is commonly regarded as a senile disease, the symptoms can also manifest in presenile individuals.

**Prevalence**

In Japan, the most prevalent type of dementia is caused by cerebro-vascular diseases (CVD) affecting about 42% of the population. Alzheimer’s Disease ranks only second, affecting about 32% of the Japanese population [3].

In the United States, however, statistics show that Alzheimer’s Disease is the leading cause of dementia affecting about four million of the U.S. population or 10% of Americans over the age of 65 [4].

**Impact on Health Care**

The annual healthcare costs of AD in the United States have been estimated to be as high at $100 billion [5]. In Japan, the annual healthcare cost of AD is probably much smaller compared to the U.S. However, with the increasing elderly population in Japan, the number of elderly individuals with AD may also be increasing in frequency. It is therefore inevitable that sooner or later, Japanese health authorities will have to confront increasing healthcare figures related to AD.

In addition to the impact on healthcare budget, there is also the emotional as well as physical stress brought to the family of the AD patient. While relatives and caregivers of elderly AD patients have the will to care for them, family care seems to break down sooner or later. Traditionally, the Japanese have had a unique attitude toward misfortune and burden. As a result, many caregivers in Japan endured the care burden because most have accepted it as their fate. But this traditional attitude is slowly fading as more and more younger caregivers in modern Japanese society are choosing not to undertake home care but opt for institutionalization [6].

**Pathogenetic Aspects**

Because the origin of AD is still unknown, a universal concept on the pathophysiology of the disease remains a debatable issue. However, two pathological hallmarks of the disease have been identified, i.e., neuritic plaques and neurofibrillary tangles (NFTs). Amyloid protein is a major component of the neuritic plaques. These plaques accumulate extracellularly in the brain. The neuritic senile plaque consists of a fibrillar amyloid core surrounded by dystrophic neurites and reactive microglia. NFTs develop within the soma of the neuron and after degeneration of the parent cell convert into extraneuronal structures, and are finally engulfed and degraded by astrocytes. The NFTs are composed of paired helical filaments microtubule. There is a major debate on whether amyloid plaques are the cause of neurodegeneration or simply an end-stage of amyloid fibril build-up [7].

At the cellular level, there is a marked reduction in the levels of neurotransmitters, e.g., acetylcholine, serotonin, noradrenaline, dopamine, glutamate and substance P. In this dramatic and global reduction of neurotransmitters, the depletion of acetylcholine is the most important event [8].

**Causes of Alzheimer’s Disease**

At present AD is considered a multifactorial disease with a combination of aging, genetic and/or environmental factors triggering the pathological decline. However, the precise mechanisms causing the disease are still unknown. Clinically, increasing age and a positive family history of dementia are the only definite risk factors for AD. And recent studies have shown that the presence of Apolipoprotein E (ApoE) type 4 allele is also a potential risk factor [9,10].

**Development of Treatment Drugs**

Numerous drugs have been studied as potential treatments for AD. But with the actual cause of the disease yet to be identified, drug development in this area has been confined to limiting the progression of the disease. At the moment, drug development has focused on (i) symptomatic treatments aimed at repleting deficient neurotransmitters, and (ii) etiologically based treatments aimed at slowing or halting the rate of progression [11].

**Cholinergic System and Alzheimer’s Disease**

The most consistent neurotransmitter-related change in the brain of an AD patient is the dramatic decrease in cholinergic innervation in the cortex and hippocampus due to the loss of neurons in the basal
forebrain. This fact has been confirmed in a large number of animal and human studies. This loss of cholinergic neurons and the associated decrease in levels of cholinergic neurotransmission have been associated with the cognitive impairment seen in AD patients [4].

Cholinergic Hypothesis

The above findings led to the development of the cholinergic hypothesis. Simply stated, the cholinergic hypothesis proposes that the cognitive loss associated with AD is related to decreased cortical cholinergic neurotransmission. Therefore, it is presumed that increasing cholinergic transmission may enhance cognitive function [4,10].

Cholinergic Enhancement Therapy

The cholinergic theory has provided the rational basis for therapeutic developments in AD. Based on this theory, six classes of drugs have been developed to enhance cholinergic deficit in AD patient. These are:

a) Cholinesterase inhibitors (ChEI), which block the AChE enzyme thereby invigorating cholinergic activity to enhance cognitive function.

b) Choline precursors, such as phosphatidylcholine, aimed at increasing the bioavailability of choline.

c) ACh releasers, which should facilitate the release of ACh from presynaptic end terminals.

d) M1 and M3 receptor agonists, which mimic ACh on postsynaptic end terminal receptors.

e) M2 and M3 receptor antagonists, generally presynaptic (autoreceptors), which regulate ACh release via negative feedback.

f) Nicotinic agonists or substances having nicotinic-like effects, which should enhance ACh release.

Among the above pharmacological agents, AChE inhibitors seem to be the most effective method to improve cholinergic deficit thus reducing the symptoms of the disease [12].

AChE Mechanism of Action

ACh is the most abundant neurotransmitter in the body and the primary neurotransmitter in the brain which is responsible for cholinergic transmission. The enzyme AChE plays a key role in the hydrolysis of the neurotransmitter ACh. AChE tends to become deposited within the neurofibrillary tangles and amyloid plaques associated with Alzheimer's Disease. In a study conducted by Inestrosa et al. [13] several cellular proteins, including AChE, have been found to co-localize with β-amyloid (Aβ) deposits and promote the assembly of Aβ peptide into amyloid fibrils. In this context, preventing the aggregation of Aβ into plaques is another way to combat AD (see Section - Anti-Amyloid Strategy).

In a related study by Alvarez et al. [13] it is reported that the incorporation of AChE into Alzheimer's amyloid aggregates results in the formation of stable complexes that change the biochemical and pharmacological properties of the enzyme and cause an increase in the neurotoxicity of the β-amyloid fibrils, suggesting that AChE could play a pathogenic role in AD by influencing the process leading to amyloid toxicity and the appearance of AD. Analysis of the catalytic activity of the AChE incorporated into these complexes shows anomalous behavior reminiscent of the AChE associated with senile plaques, which includes a resistance to low pH, high substrate concentrations, and lower sensitivity to anti-acetylcholinesterase agents.

AChE Inhibitors

Different types of AChE inhibitors have been studied for the treatment of AD. Some of the AChE inhibitors described below differ in their mechanism of action, metabolism and brain selectivity. Donepezil hydrochloride (E2020) is discussed in detail in Section 5.

Physostigmine

Physostigmine (PHY) (Fig. (1)), a cholinesterase inhibitor developed by Pfizer, is one of the first drugs to show cognitive improvements for some AD patients in 11 studies conducted. The results of an early review of these studies showed certain degree of improvements in 5 investigations using parenteral administration and in 4 of the 5 using oral administration [15]. However, further studies show that this is a short-acting AChE inhibitor. In November 1998, the US FDA issued a non-approval letter for PHY's Alzheimer's indication based mainly on a lack of efficacy as shown from results in Phase II and Phase III studies [16].

Tacrine

Tacrine (THA/Cognex) (Fig. (1)) is the first drug approved by the U.S. FDA for the palliative treatment of mild to moderate AD. Tacrine is an aminoacridine compound which is centrally active and a reversible
AChE inhibitor with a moderately long duration of action.

In an early study conducted by Summers et al. [17] in 1986, the patients receiving tacrine showed a small, but nonetheless significant reduction in the decline of cognitive performance (2.4 points in ADAS-cog) compared to placebo. These results led to a larger multicenter study carried out during the period 1990-1994 involving a total of 1905 patients enrolled in six major double-blind, placebo-controlled trials using similar methods of assessment of cognitive function [18-20]. On the basis of comparisons with placebo in cognitive scoring, mental deterioration was arrested by 2 to 12 months according to Mini Mental State Examination (MMSE) and 5 to 6 months according to the Alzheimer’s Disease Assessment Scale (ADAS) when tacrine treatments continued for 2 to 6 months. A dose-response effect (20 to 80 mg per day) was described with a 4-point improvement on the ADAS cognitive scale after 3 months’ treatment [21]. In a 30-week, randomized, controlled trial carried out with increasing doses of tacrine (up to 160 mg/day) [22], the resulting observation was a significant, dose-related improvement in objective performance-based tests, clinician- and caregiver-rated global evaluation and measures of quality of life. However, a high proportion of patients showed increased levels of serum alanine aminotransferase which is indicative of hepatocellular injury [23]. In summary, tacrine can modestly improve cognitive function in a portion of patients who are able to tolerate it. Given the propensity of this drug to cause marked increases on alanine aminotransferase levels, patients receiving this compound must be carefully monitored for ALT levels in order to prevent the possibility of hepatotoxicity.

Because of the positive effects observed in Tacrine, this compound has been used as the reference drug in the clinical development of other AChE inhibitors for both clinical efficacy and side effects [12]. Several tacrine analogs are under preclinical or clinical evaluation and some of these are presented below.

**Velnacrine**

Velnacrine (HP-029) (Fig. (1)) is a hydroxy metabolite of tacrine, with a shorter half-life. This compound acts biochemically as a potent AChE inhibitor. Pharmacodynamic trials with single doses of this compound in both healthy young individuals and patients with AD demonstrated statistically significant cognitive improvements compared to placebo [24]. However, as much as 20-30% of patients exposed to this drug developed hepatotoxicity [25].

**Amiridine**

Amiridine (NIK-247, Senita) (Fig. (1)), another tacrine-derivative AChE inhibitor, is now under Phase III study in Japan. Animal studies of this compound show increase acetylcholine levels as measured during in vitro and in vivo experiments [26-28]. In a passive avoidance test on rats with experimentally induced amnesia, Amiridine improved cognitive function at different levels of learning and memory processes. The compound was administered orally before or after training at doses ranging from 0.1 to 3 mg/kg. The results of this study show that amiridine may have more
than one memory-enhancing mechanism, one at low doses and the other at high doses.

A clinical study of this compound was conducted in a group of 88 patients with AD [28]. Amiridine was administered to 74 patients (at doses of 20, 40, or 60 mg/day) and the remaining 14 patients make up the control group. The treatment period lasted for 14 months, with a follow-up period that included clinical observation and testing. Results of this study show that amiridine had well-defined therapeutic effects in 30-40% of patients treated. Cognitive functions and general mental status of the patients continued to improve or were stabilized in 14 months while speech improved for a shorter period of time. Beneficial effects were produced at both the initial and marked stages of the disease, although the most significant results were obtained in patients with unmarked dementia.

**Eptastigmine**

Eptastigmine (heptylphysostigmine, MF 201, L-693487) (Fig. (1)) is an alkaloid derivative of physostigmine. Compared to physostigmine and tacrine, eptastigmine has a much longer duration of action. In a study conducted by Troetel et al. [29] it is found that the drug inhibits red blood cell AChE in a dose-dependent fashion. The mean AChE recovery half-life was about 10h, with a mean residual inhibition of 13% 24h after a 30 mg dose. Phase II studies in Alzheimer’s patients indicate that doses of 40-60 mg per day of eptastigmine are relatively safe and well tolerated and that moderate AChE inhibition (30-40%) is associated with maximal cognitive efficacy. In the first Phase III study carried out using doses up to 15 mg. t.i.d. for 25 weeks, the drug is still shown to be well tolerated in AD patients and appears to affect their cognitive and functional performance. The positive effects of eptastigmine compared to placebo appear to be greater in the more severely impaired patients.

**Galanthamine**

Galanthamine (Fig. (2)) is a tertiary alkaloid originating from botanical sources. As early as the 1950s, this compound was used by Bulgarian and Russian scientists in postsurgery reversal of tubocurarine-induced muscle relaxation, muscular dystrophy and traumatic brain injury. In 1972, Soviet researchers had demonstrated that galanthamine could reverse scopolamine-induced amnesia in mice. This finding was later on extended to humans. Despite the substantial and long-lasting history of clinical use of this compound in humans, it was not until 1986 when this compound was studied for the treatment of AD [30,31]. The limited use of this compound in clinical studies may be attributed to the fact that all supplies came from natural extracts and were only available in very limited amounts. It was not until 1960 when the chemical synthesis of this compound was accomplished leading to the industrial scale production and thus allowing drug development without the limitations imposed by the natural sources [32,33].

Animal studies identify galanthamine as a reversible AChE inhibitor [34] with differential activity on the central nervous system and hence an ability to cross the blood-brain barrier [35]. In human studies, the drug

![Fig. (2). AChE inhibitors-2.](image-url)
has shown promise in the treatment of patients with senile dementia of Alzheimer type (SDAT) [36]. The interim results obtained from Phase II study confirm earlier reports that galanthamine holds promise as an effective and well-tolerated treatment for cognitive impairment in patients with SDAT [37].

Galanthamine is approved in Austria for AD indication. It is currently in Phase III studies in Europe, USA, Australia, Canada and other countries [16].

**Rivastigmine**

Rivastigmine (SDZ-ENA-713/Exelon) (Fig. (2)) is a carbamate derivative of physostigmine. This compound is a carbamate type AChE inhibitor with a short kinetic half-life and a duration of action of approximately 10h [11]. This carbamate inhibit AChE by carbamoylating the serine residue of the catalytic triad in a pseudoirreversible manner.

In Phase III clinical studies conducted in the U.S., it was reported that patients receiving the highest doses of this compound (6-12 mg/day) showed average improvement of 0.79 points in the ADAS-cog scale, compared with an average decline of 4.15 points in the placebo group. The low-dose group showed a decline of 2.2 points, intermediate between placebo and high-dose groups. In the CIBIC-Plus scale, improvement was observed in 24% of the patients receiving the high dose, 25% on the low dose and 16% in the placebo group. About 25% of drug-treated patients showed side effects such as nausea, vomiting, diarrhea, loss of appetite, dizziness and fatigue. Based on these findings, a regimen of increasing dosage seems to be necessary in order to achieve maximum clinical benefits with minimal side effects [12].

Rivastigmine is approved in more than thirty countries worldwide under the trade name Exelon. It has been launched in Europe, Latin America, Asia, Afria and some Middle East countries. In July 1998, the US FDA requested additional analyses of data to confirm safety but no additional trials were requested. The launching of this product in the US is expected by the middle of this year (1999).

**Metrifonate**

Metrifonate (Fig. (2)) is an organophosphorous compound originally used as an anthelmintic drug. It is not an AChE inhibitor in itself but is time-dependently transformed into a physiologically active AChE inhibitor by dehydrochlorination. This compound is converted nonenzymatically to the active metabolite 2,2-dimethyl dichlorovinylphosphate (DDVP). DDVP is shown to increase ACh levels by stably binding to the active site of the AChE enzyme, leading to a sustained enzyme inhibition over time [38]. Metrifonate has a short plasma half-life but has a long duration of action in the brain. These characteristics make it a unique compound among the AChE inhibitors being used or studied for the treatment of AD.

Studies performed on laboratory animals show that metrifonate improve the cognitive performance of animals in various behavioural models. This compound is observed to readily enter the animal brains and dose-dependently inhibit AChE activity. As a consequence of AChE inhibition, metrifonate increases extracellular acetylcholine levels in the brain, local cerebral glucose utilization, and the cortical EEG activity in rat models [39-41].

The first application of metrifonate in AD therapy was studied by Becker et al. [42]. In this study, it was reported that metrifonate significantly improved cognitive function on the ADAS-cog scale and inhibited the erythrocyte AChE activity to 55.9% of control levels. In a 3-month dose-finding study [43], metrifonate improved the cognitive and global function of probably AD patients. In this study, a once-daily metrifonate dose of 30-60 mg, based on weight, improved ADAS-cog and CIBIC-Plus scores in the intent-to-treat patient population by 2.94 and 0.34 points, respectively, at 12 weeks of treatment in comparison with placebo. Adverse reactions observed during this study were generally transient and mild in intensity. A prospective 6-month study conducted by Morris et al. [44] confirms the finding of the earlier study and extends it by demonstrating that the efficacy of metrifonate was sustained over the longer treatment period.

The new drug application for this compound was filed in late 1997 in US and Europe. However, the Phase III trials being conducted in the US was halted for at least 3 months in September 1998 in agreement with the US FDA after some patients experienced muscle weakness with some requiring respiratory support. The European launch is also expected to be delayed because of this finding [16].

**(-)-Huperzine A**

(-)-Huperzine A (HupA) (Fig. (2)) is another kind of AChE inhibitor derived from natural extracts. It is an alkaloid isolated from the club moss, *Huperzia serrata*, which has gained popularity in Chinese herbal medicine. The compound's unique pharmacological features and relative lack of toxicity [45] makes it a potent compound for AD treatment. The structure of racemic Huperzine A shows some similarity to other known AChE inhibitors [46]. Animal studies reveal significant cognitive enhancement [47]. Initial clinical
trials have established the safeness of HupA and provided preliminary evidence for significant effects on patients exhibiting dementia and memory disorders [48]. In a recent study conducted by Koenig et al. [49], it was demonstrated that HupA decreases neuronal cell death caused by glutamate, particularly in primary cultures derived from hippocampus and cerebellum of embryonic rat. Because this compound is shown to increase acetylcholine levels in the brain and at the same time decrease neuronal death, HupA may be an important and promising drug for the treatment of AD.

Donepezil Hydrochloride (E2020)

Discovery of Donepezil Hydrochloride

Donepezil hydrochloride (E2020) (Fig. (3)) is the second drug approved by the U.S. FDA for the treatment of mild to moderate AD. It is a new class of AChE inhibitor having an N-benzylpiperidine and an indanone moiety which shows longer and more selective action. It is now marketed in the U.S. and in some European and Asian countries under the trade name of Aricept. In Japan, Aricept is now under application to the Ministry of Welfare.

The research on E2020 started in 1983. Following research developments of tacrine, our group in Eisai started to develop tacrine derivatives. However, we failed to develop a non-toxic tacrine derivative. Through random screening, we encountered N-benzylpiperazine derivative 1 which was then originally being synthesized in the study of anti-arterial sclerosis. Our tests showed that the acetylcholinesterase activity of N-benzylpiperazine derivative was 12.6µM at IC₅₀ in rat brain homogenate. This was not very strong but the compound’s novel structure was very promising. We decided to use N-benzylpiperazine derivative as the seed compound and synthesized about 700 derivatives.

Our succeeding experiments showed a dramatic increase in anti-acetylcholinesterase activity when N-benzylpiperazine was replaced with N-benzylpiperidine (Fig. (4)). It was a quantum leap in our investigation. Our
next challenge was to replace the ether moiety with an amide moiety. This process also increased anti-acetylcholinesterase activity. We thought that the introduction of a functional group at the para-position of the benzamide group might increase potency but removal of the nitro group at the para-position decreased the potency of the compound.

On the basis of these findings, we synthesized benzamide derivatives. We later on discovered that benzylsulfonyl derivative 4 was the most potent AChE inhibitor with an anti-AChE activity 21,000-fold greater compared to the seed compound 1 [50,51]. However, our excitement was shortlived because we found that this compound has a very poor bioavailability rate and has a short duration of action and therefore could not be a candidate for clinical testing. But this benzylsulfonyl derivative has a novel chemical structure and has a selective affinity to AChE, making it a very attractive lead compound. Immediately after these findings, we started the screening process again.

Our next strategy in drug design was the replacement of the amide moiety with ketone moiety 7. This approach maintained the AChE activity of the compound. Furthermore, this cyclic-amide derivative 6 showed enhanced inhibitory action. On the basis of these results, an indanone derivative 8 was designed. The resulting AChE activity was moderate, but we achieved longer duration of action. Subsequently, various indanone derivatives were synthesized and tested for anti-AChE activity. Among the indanone derivatives that were developed, donepezil was found to be the best balanced compound (Fig. (5))[52].

Structure-Activity Relationships (SAR)

The indanone derivatives were tested for in vitro inhibition of AChE. A rat brain homogenate was used as the AChE source and the activities were measured according to the method of Ellman et al[53].

The indanone derivative was divided into four parts as shown in Fig. (6): Part 1 (indanone moiety), part 2

![Chemical Structures](image-url)
Donepezil Hydrochloride (E2020) (linkage moiety), part 3 (piperidine moiety), and part 4 (benzyl moiety). All data were obtained from the racemic compounds.

Fig. (6). Four parts of indanone-piperidine derivative.

**Part 1.** Table 1 shows the anti-AChE activity of derivatives from the indanone moiety with various bicyclic rings. The effect of replacement of indanone ring with α-tetralone, 1-benzosuberone, 5,6-dimethoxy-1-indanone, 5,6-indanol, 5,6-dimetoxyindene was measured. The ring expansion of cyclic ketone (i.e. compounds 10, 11) greatly decreased the activity. But the introduction of the methoxy group to the 5,6-position of the indanone moiety increased the activity by 25-fold (E2020). The carbonyl group of the indanone moiety is essential to the activity since the indanol (12) and indene (13) derivatives both showed decreased potency.

**Table 1.** Anti-AChE Activity of Part 1 Substituted Compounds (1)

<table>
<thead>
<tr>
<th>Compound No.</th>
<th>X</th>
<th>Inhibition of AChE IC50 [nM] a</th>
</tr>
</thead>
<tbody>
<tr>
<td>9</td>
<td>O</td>
<td>150</td>
</tr>
<tr>
<td>10</td>
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<tr>
<td>12</td>
<td>OMe</td>
<td><img src="image" alt="12 structure" /></td>
</tr>
<tr>
<td>13</td>
<td>OMe</td>
<td><img src="image" alt="13 structure" /></td>
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</table>

a: Deviation of measurement of IC50 value is 10-20%.

The effect of introducing one or more methoxy groups in the indanone moiety is shown Table 2. We observed that the introduction of a methoxy group at the R3-position increased the activity by 20-fold (15). A methoxy substituent at the R4-position increased the activity by 10-fold (16) while substitution at the R2-

**Table 2.** Anti-AChE Activity of Part 1 Substituted Compounds (2)

<table>
<thead>
<tr>
<th>Compound No.</th>
<th>R1</th>
<th>R2</th>
<th>R3</th>
<th>R4</th>
<th>Inhibition of AChE IC50 [nM] a</th>
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<tbody>
<tr>
<td>9</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>150</td>
</tr>
<tr>
<td>14</td>
<td>H</td>
<td>OMe</td>
<td>H</td>
<td>H</td>
<td>81</td>
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<tr>
<td>15</td>
<td>H</td>
<td>OMe</td>
<td>H</td>
<td>OMe</td>
<td>6.4</td>
</tr>
<tr>
<td>16</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>OMe</td>
<td>12</td>
</tr>
<tr>
<td>E2020</td>
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<td>OMe</td>
<td>OMe</td>
<td>H</td>
<td>5.7</td>
</tr>
<tr>
<td>17</td>
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<td>H</td>
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<td>OMe</td>
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<td>OMe</td>
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</tr>
<tr>
<td>21</td>
<td>OMe</td>
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<td>OMe</td>
<td>H</td>
<td>13</td>
</tr>
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</table>

a: Deviation of measurement of IC50 value is 10-20%.
position (14) results in slightly increased activity. These results suggested that the methoxy group at the para-position in the carbonyl group of the benzoyl moiety greatly enhanced binding to the active site of the AChE enzyme. Among these derivatives, 5,6-dimethoxy-indanone derivative or the E2020, showed the highest activity.

Part 2. Various bridging groups between the indanone moiety and the piperidine moiety were tested. The results are shown in Table 3. Direct connection of the indanone and the piperidine rings dramatically decreased potency (22). The effect of the length of the bridging moiety on potency varied in the following order: propylene (26) > methylene (E2020) > pentylen (28) > ethylene (25) > butylene (27). The introduction of an exo-methylene double bond on both the indanone and piperidine moiety decreased the activity (23, 24).

Table 3. Anti-AChE Activity of Part 2 Substituted Compounds

<table>
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<th>Compound No.</th>
<th>Z</th>
<th>Inhibition of AChE IC50 [nM]a</th>
</tr>
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<td>5.7</td>
</tr>
<tr>
<td>22</td>
<td>=CH</td>
<td>3300</td>
</tr>
<tr>
<td>23</td>
<td>=CH</td>
<td>13</td>
</tr>
<tr>
<td>24</td>
<td>CH=</td>
<td>90</td>
</tr>
<tr>
<td>25</td>
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</tr>
<tr>
<td>26</td>
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<td>35</td>
</tr>
<tr>
<td>28</td>
<td>CH2CH2CH2CH2</td>
<td>14</td>
</tr>
</tbody>
</table>

a: Deviation of measurement of IC50 value is 10-20%.

Part 3. Table 4 shows the relationships between the location and the number of nitrogen atom and activity. The nitrogen atom at 1-position of the benzylpiperidine moiety was very important since the activity of 4-benzylpiperidine derivative (29) largely decreased activity. Replacement of the piperidine group with a piperazine group (30) also resulted in decreased potency. The distance between the carbonyl group in indanone ring and the nitrogen atom in piperidine ring might be critical for anti-AChE activity.

Table 4. Anti-AChE Activity of Part 3 Substituted Compounds

<table>
<thead>
<tr>
<th>Compound No.</th>
<th>Z</th>
<th>Inhibition of AChE IC50 [nM]a</th>
</tr>
</thead>
<tbody>
<tr>
<td>E2020</td>
<td>-</td>
<td>5.7</td>
</tr>
<tr>
<td>29</td>
<td>-</td>
<td>480</td>
</tr>
<tr>
<td>30</td>
<td>-</td>
<td>94</td>
</tr>
</tbody>
</table>

a: Deviation of measurement of IC50 value is 10-20%.

Part 4. Table 5 shows the relationships between the benzyl moieties. The 3-position-substituted benzyl derivatives showed the highest potency among the 2-, 3-, and 4-substituted regioisomers. Substitution of the benzene ring with an electron-donating methyl group and an electron-withdrawing nitro group showed a similar effect. The basicity of the nitrogen atom in the piperidine ring appear to have an important effect in increasing activity since the N-benzylpiperidine derivative (34) was almost inactive. Removal of the benzyl group (38) caused a great reduction in the potency of the compound but the activity was retained after replacement with cyclohexylmethyl group (39). The replacement of benzyl moiety with phenethyl (40) and 2-naphthyl group (41) decreased potency. Among the indanone derivatives, compound E2020 is one of the most potent compounds in terms of anti-AChE activity.

Rationale for Donepezil as a Selective AChE Inhibitor

The reversible inhibitor donepezil is the lead compound in a new class of AChE inhibitors having an N-benzylpiperidine and an indanone moiety which shows a greater selectivity for AChE than for butyrylcholinesterase, and is not expected to have any peripheral effects [50]. The enantiomers of donepezil exhibit near identical pharmacological profiles, including inhibitory effects, and the two enantiomers interconvert readily in aqueous solution, via a ketoenol
Donepezil Hydrochloride (E2020) Current Medicinal Chemistry, 2000, Vol. 7, No. 3 313

Table 5. Anti-AChE Activity of Part 4 Substituted Compounds

<table>
<thead>
<tr>
<th>Compound No.</th>
<th>R</th>
<th>Inhibition of AChE IC50 [nM]a</th>
</tr>
</thead>
<tbody>
<tr>
<td>E2020</td>
<td>-CH2-</td>
<td>5.7</td>
</tr>
<tr>
<td>31</td>
<td>-CH2-</td>
<td>10</td>
</tr>
<tr>
<td>32</td>
<td>-CH2-</td>
<td>2.0</td>
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<tr>
<td>33</td>
<td>-CH2-</td>
<td>40</td>
</tr>
<tr>
<td>34</td>
<td>-CO-</td>
<td>&gt;10000</td>
</tr>
<tr>
<td>35</td>
<td>-CH2-</td>
<td>160</td>
</tr>
<tr>
<td>36</td>
<td>-CH2-</td>
<td>4.0</td>
</tr>
<tr>
<td>37</td>
<td>-CH2-</td>
<td>100</td>
</tr>
<tr>
<td>38</td>
<td>H</td>
<td>5400</td>
</tr>
<tr>
<td>39</td>
<td>-CH2-</td>
<td>8.9</td>
</tr>
<tr>
<td>40</td>
<td>-CH3CH2-</td>
<td>180</td>
</tr>
<tr>
<td>41</td>
<td>-CH2-</td>
<td>2900</td>
</tr>
</tbody>
</table>

a: Deviation of measurement of IC50 value is 10-20%.

intermediate [54]. Consequently, donepezil is being developed as a racemic mixture. Tacrine became the first available agent for the treatment of Alzheimer's disease in the United States. However, the aminoacridines, in general, suffer from dose-limiting hepatotoxicity which is believed to be structure related [55]. Donepezil appears to be devoid of such an unfavorable side-effect profile probably because of its novel benzylpiperidine structure.

A number of compounds have been made and tested by Eisai in the quest for a potent and selective AChE inhibitor which has been achieved with donepezil. The corresponding structure-activity relationship (SAR) data has been used in computer-assisted molecular design (CAMD) studies [56-58] to develop guidelines for target synthesis and, retrospectively, to explain unusual SAR behavior. Some groups have undertaken the design and synthesis of donepezil-like compounds which incorporate biosisosteric replacements for the indanone ring [59,60]. A 3D-QSAR using comparative molecular field analysis, CoMFA, have been reported for a set of AChE benzylpiperidine inhibitors [61]. All of the CAMD studies have been receptor-independent in that the geometry of AChE had not been determined and was available for structure-based design studies. Over the course of this drug discovery study, we have developed an image of the active site of AChE based on a common active shape-pharmacophore hypothesis. We have postulated similar structural features between ACh and donepezil such as the carbonyl group and the tertiary nitrogen adjacent to bulky groups. We proposed an active conformation which is characterized by the indanone and piperidine rings being "perpendicular" to one another, with respect to a plane aligned along the larger of the two thickness dimensions of the piperidine ring [56, 62].

Five years after the discovery of donepezil, Sussman et al. have reported the crystal structure of AChE from Torpedo californica electric organ [63]. It was found that the active site of AChE lies close to the "bottom" of a deep and narrow aromatic gorge which is lined with the side chain rings of fourteen aromatic amino acid residues. It appears that the size of the active-site pocket is too small for donepezil, and its related analogs, to mimic the binding geometry of ACh. The availability of the geometry of an AChE presents the opportunity to extend the CAMD studies to inhibitor-enzyme docking analysis and to support, refute, and/or refine previous CAMD studies. The crystal structure of AChE has, in fact, already been used in nonenergetic docking studies of donepezil and some indanone isosteric analogs of donepezil and in the prediction of donepezil binding site on AChE [64,65].
Docking simulations may provide some guidelines for structural changes in the four features of donepezil. A primary target for structural changes may be the indanone ring. There are other bicycles that lead to active analogues [54,60] suggesting a structure-activity mapping may be possible by docking simulation.

Implementation of docking simulations in a design mode is predicated upon reliable calculations. The ability to predict reliable and accurate ligand-receptor binding energies remains elusive in intermolecular modeling. Perhaps the best chances for success in the donepezil analogue - AChE docking simulations is to combine X-ray studies to elucidate AChE-inhibitor complex geometries with free energy force field refinement [66] docking simulations. The X-ray structures can provide realistic initial geometries to the docking simulations for a training set of inhibitors for which the inhibition potencies have been measured. The terms in the force field used to compute the binding energies of the inhibitors are then scaled, in a QSAR fashion, to establish a correlation between inhibition potency and binding free energy.

### Lead Identification and Optimization

A benzylpiperazine compound 1 (Fig. (4)) which demonstrated weak, (its IC\textsubscript{50} value is 12.6 µM), but definite inhibitory activity against AChE was identified in blind screening. Continued screening was performed around compounds of similar structures. Another type of "seed" compound 3 was found which has both benzylpiperidine and benzamide moieties in its structure. This compound has strong AChE activity, its IC\textsubscript{50} being in excess of two orders of magnitude that of the original benzylpiperazine lead. Lead optimization synthesis, mainly involving modification of the benzamide moiety, led to N-[4-(1-benzyl)piperidinyl]ethyl-N-methyl-(4-benzylsulfonyl)benzamide 4. Compound 4 has an AChE IC\textsubscript{50} five orders of magnitude smaller (higher inhibition potency) as compared to the original benzylpiperazine compound, as well as selective AChE inhibitory activity. However, compound 4 has a very short half-life due to hydrolyzation by proteases in the liver, which is believed to lead to an N-disubstituted amide metabolite.

Metabolic resistance was sought by incorporating bioisosteric replacements for the N-disubstituted amide moiety. A series of benzamide analogs possessing high AChE activity and which are readily transported into brain became the focus in the lead optimization process.

Over the course of the lead optimization it appeared that methyl substitution on the amide nitrogen enhanced activity. Thus, trans and cis forms of benzamide were considered as models since methyl substitution at the amide nitrogen may change the ratio of cis and trans isomers of the amide. Overall, conformation was considered an important component in a QSAR analysis used to guide the initial lead optimization process. A good correlation equation, QSAR, was found between activity, as measured by IC\textsubscript{50}, and the difference in energy between the cis and trans isomers, \( \Delta E(\text{C-T}) \) and the bulkiness of substituents (see Fig. (7)). The QSAR suggests that the intrinsic activity of cis isomers of benzamide are higher than the trans isomers, since there is a negative correlation between IC\textsubscript{50} and \( \Delta E(\text{C-T}) \).

$$\log 1/C = 0.019 \text{MR} - 2.372 \Delta E(\text{C-T}) + 22.94$$

\( n = 19, s = 0.256, r = 0.833 \)

Table 6 contains the structure-activity behavior of \( N \)-alkylated amides which have higher activity compared to non-\( N \)-alkylated analogs. Conformational analysis of the \( N \)-alkyl substituted benzamides was performed using the MNDO quantum chemical method [67]. Non-substituted amides exist almost completely in the trans form, but \( N \)-substitution makes the cis conformation more stable. These results again indicate that AChE activity increases as the population of the cis isomer of the benzamide increases.

![Fig. (7). QSAR of para-substituted benzamides.](image)

**Table 6.** \( N \)-Alkyl Analogs of \( N \)-[4-(1-Benzyl)piperidinyl]ethylbenzamide

<table>
<thead>
<tr>
<th>R</th>
<th>IC\textsubscript{50} (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H</td>
<td>560</td>
</tr>
<tr>
<td>Benzyl</td>
<td>180</td>
</tr>
<tr>
<td>Methyl</td>
<td>170</td>
</tr>
<tr>
<td>Ethyl</td>
<td>130</td>
</tr>
<tr>
<td>Phenyl</td>
<td>35</td>
</tr>
</tbody>
</table>
The QSAR analysis provided the structural requirements for lead evolution. The specific structural requirements identified in the QSAR analysis are as follows:

1) The cis conformation of the benzamide is the active conformation.

2) Bulky groups at the para position of the benzamide increase activity.

3) The carbonyl oxygen of the amide is a proton acceptor for an intermolecular hydrogen bond.

A cis conformation rigid isostere by cyclization was attempted without success. However, flexible analogues having the cis conformation were made. One of these was donepezil which possess benzylpiperidine and indanone moieties (Fig. (8)). Donepezil was the first compound in which an indanone ring was part of the enzyme inhibitor.

**Donepezil as a Selective AChE Inhibitor**

Donepezil was initially thought to be a mimic of ACh by structural similarity, and, therefore, a competitive inhibitor of AChE. The N-benzylpiperidines show outstanding in vitro selectivity for AChE. The IC\textsubscript{50} of donepezil for AChE is 5.7 nM while the IC\textsubscript{50} for BuChE (butryrylcholinesterase), is about 7000 nM, a selectivity ratio in excess of three orders of magnitude. Tacrine and physostigmine show poor selectivity having the same order of magnitude IC\textsubscript{50}s for both AChE and BuChE. Inhibition of BuChE, which is abundant in plasma, may be associated with potentiating peripheral side effects [68]. Therefore, an AChE inhibitor which is essentially devoid of BuChE activity may display higher therapeutic indices than those which are also active BuChE inhibitors.

**X-ray Crystallography of Donepezil**

Fig. (9) shows the ORTEP [69] drawing of the racemic donepezil hydrochloride crystal structure. The ORTEP drawing shows a pattern of irregular thermal ellipsoids. There is an asymmetric carbon atom at the 2-position of indanone ring and the irregular thermal motions phenomenon occurs around this chiral center. This thermal behavior is observed both at 23 and -20 C and corresponds to two puckering structures of the indanone ring. Thus, the crystal structure of the indanone ring consists of two conformers (Fig. (10)). One conformer has a relative \( R \) configuration and the other has a relative \( S \) configuration. The most remarkable feature of \( R \) and \( S \) enantiomers is that they have the same molecular-shape except for small portions of the indanone and piperidine rings. The \( R \) and \( S \) enantiomers are so similar that they are not even recognized in the crystalline state. These two conformers also have almost equal heats of formation as computed by MNDO, and the crystal structure is one of the most stable intramolecular conformations of each
donepezil enantiomer. This high intramolecular stability suggests that these two conformations may not only exist in the crystal state, but also in solution.

Receptor Independent Molecular Modeling and QSAR Analysis

QSAR analyses have been performed on the substituted indanone and benzylpiperidine ring substructures of a set of donepezil. A set of QSARs were constructed and evaluated for substituents on the aromatic ring of the benzylpiperidine substructure. The most significant QSAR involves a representation of molecular shape, the largest principal moment of inertia, and the HOMO of the substituted aromatic ring. It appears that upon binding the receptor “wall” is closely fit around the benzyl ring, especially near the para position. Overall, the QSAR analysis suggests inhibition potency can be better enhanced by substitution on the indanone ring, as compared to the aromatic sites of the benzylpiperidine ring. Moreover, inhibition potency can be rapidly diminished, presumably through steric interactions with the
Fig. (10). Two conformations in crystal structure of racemic donepezil hydrochloride. The mirror image of a conformer set exists in an asymmetric unit. (left) $R$-form in the conformation A. (right) $S$-form in the conformation B.

receptor surface of AChE, by substitution of moderate to large groups on the benzyl ring, particularly at the para position.

Conformational analyses and molecular-shape comparisons were carried out on an analogue series of indanone-benzylpiperidine inhibitors of AChE. It was possible to define an active conformation with respect to the flexible geometry of the benzylpiperidine moiety, as well as an active conformation of the indanone ring-piperidine ring substructure for analogues having a single spacer group between these rings (Fig. (11)). No

Fig. (11). Molecular superposition of the active analogues under the criterium of maximizing overlap steric volume of each analogue with $R$-form of donepezil in its active conformation. $R$ is a postulated anionic receptor site, which is estimated to be 6.7 Å from each of the nitrogens of the compounds.
active conformation could be postulated for analogues having two or three spacer units between the indanone and piperidine rings. Still, a receptor binding model can be constructed for all indanone and piperidine ring substructures. The postulated active conformation for donepezil is close to the crystal structures with respect to the indanone-piperidine substructure, but differs from the crystal structures for the benzylpiperidine moiety. However, the crystal conformations and the postulated active conformation of the benzylpiperidine portion of the AChE inhibitor are estimated to be about equally stable.

Sussman and coworkers have reported the crystal structure of AChE from *Torpedo californica* electric organ [63]. The atomic coordinates of AChE have been deposited in the Brookhaven Protein Data Bank (PDB entry: 1ACE).

**Receptor Dependent (Docking) Molecular Modeling**

**Structure of AChE**

AChE which contains 537 amino acids is shown in Fig. (12) as ribbon model. AChE belongs to the class
of α/β proteins and consists of 12 β-sheets surrounded by 14 α helices. A close-up view of the enzyme active site from the enzyme surface is shown in Fig. (13). The active site serine residue can be seen from the surface of the enzyme. The active site consists of a catalytic triad composed of Ser-200, His-440 and Glu-327. The catalytic triad lies near the bottom of a long and narrow gorge, which is lined with the side chain rings of fourteen aromatic amino acid residues. These aromatic residues are color-coded orange in Fig. (13).

AChE catalysis involves an active serine residue, and the overall catalytic process proceeds in a three-step mechanism, as defined in Fig. (14), like transfer within a catalytic triad [61, 70]. The activated serine residue enables a nucleophilic attack on ACh, resulting in hydrolysis and acetate products being released. Some reversible AChE inhibitors show a mixed type of inhibition by blocking the deacetylation process [71-73]. The acetyl group is introduced into the esteratic site in this catalytic process. Therefore, acylated AChE can accept an external charged molecule at this vacant anionic site to form an EA-inhibitor complex (EAI). Donepezil appears to bind to both the free enzyme and the acylated enzyme to block both Michaelis complex formation and deacylation [74].

\[
E + S \rightarrow ES \rightarrow EA \rightarrow E + P1 + +
\]

\[
E + P2 E A I K_i
\]

\[
K_i^* E AI
\]

**Fig. (14).** Reaction scheme in which the inhibitor combines with E and EA. E, free enzyme; S, substrate; ES, enzyme substrate complex; EA, acylated enzyme; P, product; I, inhibitor; EI, enzyme inhibitor complex; EAI, acylated enzyme inhibitor complex; \(K_i\), dissociation constant for enzyme and inhibitor; \(K_i^*\), dissociation constant for EA and inhibitor.

Recent molecular dynamics simulations showed that reorientation of five aromatic rings leads to rapid opening and closing of the gate to the active site. The simulations watch the dynamic fluctuations in the channel. The channel walls can have openings large enough to admit solvent molecules, while the channel itself can open widely enough to admit substrate [75]. In other study the molecular dynamics trajectory is used to quantitatively analyze the effect of the gate on the substrate binding rate constant [76]. For a 2.4-Å probe modeling acetylcholine, the gate is open only 2.4% of the time, but the quantitative analysis reveals that the substrate binding rate is slowed by merely a factor of 2. They rationalize this result by noting that the substrate, by virtue of Brownian motion, will make repeated attempts to enter the gate each time it is near the gate. If the gate is rapidly switching between the open and closed states, one of these attempts will coincide with an open state, and then the substrate succeeds in entering the gate. However, there is a limit on the extent to which rapid gating dynamics can compensate for the small equilibrium probability of the open state. Thus the gate is effective in reducing the binding rate for a ligand 0.4 Å bulkier by three orders of magnitude. This relationship suggests a mechanism for achieving enzyme specificity without sacrificing efficiency.

It is known that anionic surface residues play a role in the long-range electrostatic attraction between AChE and cationic ligands [77]. Tara et al. [78] show that anionic residues also play an important role in the behavior of the ligand within the active site gorge of AChE. Negatively charged residues near the gorge opening not only attract positively charged ligands from solution to the enzyme, but can also restrict the motion of the ligand once it is inside of the gorge. They use Brownian dynamics techniques to calculate the rate constant \(k_{on}\) for wild type and mutant AChE with a positively charged ligand. These calculations are performed by allowing the ligand to diffuse within the active site gorge. Although a number of residues influence the movement of the ligand within the gorge, Asp-74 is shown to play a particularly important role in this function. Asp-74 traps the ligand within the gorge, and in this way helps to ensure a reaction.

Hydrated molecular dynamics simulations of isolated AChE (no inhibitor or substrate present) have identified a “backdoor” opening to the active site located along the active site gorge [79]. Our water-independent simulation studies of AChE, in the presence of ACh, or inhibitors, does not indicate that a backdoor can form a large-enough opening for a long-enough time period to permit the entry, or exit, of ligands and/or products to the active site [80]. However, an open "side door" in one of the AChE was recorded at 152 picoseconds of a large molecular dynamics simulation [75].

**Dipole Moments of ACh and Inhibitors**

Ripoll et al [81] have reported that AChE has a remarkably large dipole moment which is aligned directly along the axis defining the center of the aromatic gorge. Dipole-dipole interactions may play an important role in the long-range molecular recognition of AChE ligands. Molecules with complementing dipoles to AChE can be drawn toward, and down, the aromatic gorge, leading to the active site in an orientation in which a matching dipole-dipole interaction is formed. Thus, inhibitors that interact with
the enzyme in this orientation are expected to have potent activities, and some groups have proposed models for the binding of large dipole inhibitors to AChE [81]. It shows that electrostatic steering of ligands contributes to the high rate constants that are observed experimentally for AChE.

The calculated dipole of Ach, in its bound conformation, is about 23 Debye, while the extended conformation dipole moments of donepezil and its derivatives are on the order of 35-50 Debye. The direction of the dipole in each inhibitor runs through the molecule from the benzylpiperidine moiety to the methoxy groups on the indadone ring. Thus, the calculated large dipoles of Ach, donepezil and its derivatives support the dipole-dipole recognition model mentioned above [81, 82]. In essence, one has a tube (the active-site gorge) which possesses a positive charge on one end and a negative charge on the other. A successful inhibitor has a cylindrical shape which can fit into and slide along the inner surface of the tube. The cylindrical (inhibitor) possesses positive and negative charges on its ends so that the preferred orientation and position of the cylinder in the tube is dictated by electrostatic interactions.

**Binding Models of Donepezil to AChE and EA**

The atomic coordinates of AChE were obtained from the Brookhaven Protein Data Bank (PDB entry: 1ACE). Hydrogen atoms were added after deleting the crystalline water molecules present in the X-ray structure, and aliphatic carbon-hydrogen groups were treated as united atoms. Docking studies for an acylated form of the enzyme, EA, were also carried out [80]. The three-dimensional structure of EA has not been determined so that the EA model used in this study was generated from the X-ray structure of AChE.

The docking simulations show that stable conformations are adopted by donepezil and its derivatives upon binding to both AChE and the EA. Donepezil and its derivatives considered dock to both AChE and the EA in a similar manner. The docking suggests two common features of binding for these compounds;

1) The \( N \)-benzyl substituent forms a \( \pi \)-stacking interaction with the indole side chain of Trp-84.

2) The piperidine ring locates on the narrowest part of active-site cavity which is formed by four amino acid residues, Tyr-70, Asp-72, Tyr-121, and Tyr-334.

Donepezil exhibits five key binding interactions (KBI 1 to 5) which is identified in the binding models based on the interactions among AChE and five partial structures of donepezil. The definition of five KBI's are as follows;

KBI 1: the interaction between the indanone ring and the indole side chain of Trp-279.

KBI 2: the interaction between the methoxy group in the indanone and the main-chain carbonyl of Arg-289.

**Fig. (15).** (left) Superposition of donepezil-AChE binding models in the active-site cavity with the five key binding interactions (KBI's) residues defined. (right) Superposition of the docking models of donepezil in the acylated enzyme cavity shown in grid surface form.
KBI 3: the interaction between the carbonyl group in the indanone and the hydroxy group of Tyr-121 or Tyr-70.

KBI 4: the interaction between the NH group in the protonated piperidine and the carboxyl group of Asp-72, the phenyl ring of Phe-330 or the hydroxy group of Tyr-121.

KBI 5: the interaction between the phenyl ring in the benzyl piperidine and the indole ring of Trp-84.

The carbonyl oxygen interacts with Tyr-121 (KBI 3a) and Tyr-70 (KBI 3b). However, the $S$ enantiomer of donepezil only adopts the KBI 3b binding mode.

Superposition of the models reveals that the three binding classes, with respect to the piperidine ring, are similar in that they share five key interactions which are shown in Fig. (15). In a different structural model the indanone ring is parallel to the indole side chain of Trp-84 at a distance of 3.6 Å. The geometric binding difference between enantiomers is in the direction of the carbonyl in the indanone ring. The carbonyl group interacts in the KBI 3a mode ($R$ form only), and in the KBI 3b mode (both forms). The other four key interactions, namely the indanone ring with Trp-279, the methoxy group with Arg-289, the protonated piperidine NH with Asp-72 and the phenyl ring with Trp-84, are observed in all model.

The results of the docking study support the active conformation model discussed above which is characterized by the indanone and piperidine rings being "perpendicular" to one another, where the larger of the two thickness dimensions of the piperidine ring contains its plane relative to the indanone ring [56, 62]. The preferred mode of binding of donepezil to AChE is shown in Fig. (16). Overall, these results suggest that donepezil, and related analogs, are noncovalent AChE inhibitors which bind in the aromatic gorge, but do not interact with the catalytic residues. Consequently, the donepezil analogs can inhibit the Michaelis complex formation step and/or the deacylation step of the hydrolytic reaction of AChE.

Structure of Acetylcholinesterase Complex with Donepezil

Crystal Structure

Sussman and coworkers have reported the crystal structure of a complex of donepezil with *Torpedo californica* acetylcholinesterase (TcAChE) [83]. The atomic coordinates of AChE have been deposited in the Brookhaven Protein Data Bank (PDB entry: 1EVE). The X-ray structure, at 2.5 Å resolution, shows that the elongated donepezil molecule spans the entire length of the active-site gorge of the enzyme (Fig. (17)). It thus interacts with both the "anionic" subsite, at the bottom of the gorge, and with the peripheral anionic site, near its entrance, via aromatic stacking interactions with conserved aromatic residues. It does not interact directly with either the catalytic triad or with the "oxyanion hole".

They have also reported only the $R$ enantiomer is bound within the active-site gorge when the racemate is soaked into the crystal inspite of racemic compound

Fig. (16). Lowest-energy binding model of the $R$ enantiomer of donepezil with AChE in the active-site cavity.
whose enantiomers have similar affinity for the enzyme. The $R$ and $S$ enantiomers have the same molecular shape except for small portions of the indanone and piperidine rings as described at X-ray crystallography of donepezil section. Therefore, this behavior may be assumed to be caused by undistinguishableness between the $R$ and $S$ enantiomers in the crystal structure at 2.5 Å resolution.

**Comparison of Binding Modes in Simulation Model with That in Crystal**

In a docking study in donepezil, we proposed five key binding interactions (KBI 1 to 5) which is identified in the binding models based on the interactions among AChE and five partial structures of donepezil as described before. Three major KBIs are observed in the crystal structure of TcAChE complex with donepezil (Fig. (18)).

One face of the benzyl benzene ring stacks against the indole ring of Trp-84 at the anionic subsite near the bottom of the gorge in both model and crystal structure. On the opposite face, it makes a classic aromatic hydrogen bond with a water molecule (WAT1160). This water is held firmly by a hydrogen bond to another water molecule (WAT1161), in the "oxyanion hole", and to WAT1159.

In the constricted region, halfway up the gorge, the charged nitrogen of the piperidine ring makes a cation-π interaction [84] with the phenyl ring of Phe-330.

Although docking simulation predicted two more possible interactions between the charged nitrogen of the piperidine ring and the carboxyl group of Asp-72 and the hydroxy group of Tyr-121, the ring nitrogen makes an in-line hydrogen bond with WAT1159.

At the top of gorge, the indanone ring stacks against the indole ring of Trp-279 in a parallel π–π interaction. But, none of binding partner to the methoxy group at the indanone moiety is found in the crystal structure.

**Modeling of BuChE**

*Torpedo* acetylcholinesterase (TcAChE) and human butyrylcholinesterase (hBuChE) have 73% similarity and 53% identity of their amino acid sequences [85]. Both enzymes contain three internal disulfide loops with exactly the same chain lengths. Kyte-Doolittle amino acid hydrophyte plots are almost indistinguishable and the known structure of AChE suggests that cholinesterase are α/β-proteins with structural similarity to other crystallized proteins. This permitted modeling hBuChE (Fig. (19)) on the basis of the three-dimensional structure of TcAChE.

The modeled hBuChE [86] has a remarkably large dipole moment which is aligned directly along the axis defining the center of the aromatic gorge. The calculated dipole is about 354 Debye while that of
Fig. (18). Binding modes of donepezil to TcAChE. (left) Simulated binding modes of donepezil with KBIs defined. (right) Experimental binding modes of donepezil to TcAChE [80]. Donepezil is displayed as a ball-and-stick model (chiral center marked with black star); direct binding residues are represented as dark sticks; water-mediated binding residues as gray sticks; water molecules as light gray balls; standard H-bonds as heavy dashed lines; aromatic H-bonds, -cation and -stacking as solid lines.

TcAChE is about 361 Debye. The modeled hBuChE structure closely resembled that of TcAChE in overall features. However, six conserved aromatic residues that line the active-site gorge are absent in hBuChE. Modeling showed that two such residues, Phe-288 and Phe-290, replaced by leucine and valine, respectively, in BuChE. In the hBuChE model, the catalytic triad and Trp-84, the "anionic" site, do not move relative to its position in TcAChE. Hence the anionic-site-directed inhibitor tacrine can bind to BuChE as well as AChE.

The accessible surface areas at the active site cavity which includes the aromatic gorge and the catalytic triad in the hBuChE is 952.1 Å², and is larger than that of TcAChE, 694.9 Å² [84]. The waist of active site cavity in AChE have disappeared from the BuChE active site cavity (Fig. (20)). Consequently, the goodness of fit of donepezil into the active site cavity may decrease.

Trp-279, at the entrance of the active-site gorge in TcAChE, is absent in hBuChE. Modeling designated it as part of the "peripheral" anionic site, which is lacking in hBuChE (Fig. (21)). The indanone ring of donepezil stacks against the indole moiety of Trp-279, in the peripheral binding site, in a \(\pi-\pi\) interaction. The binding of donepezil is strongly dependent on interaction with Trp-279 and Phe-330, which are absent in hBuChE, may explain its high relative specificity for AChE versus BuChE. So tacrine or other small inhibitors which position in the anionic site near the catalytic triad could not acquire the selectivity against AChE.

Preclinical Pharmacology of Donepezil (E2020)

The following experiments were designed to evaluate the properties of donepezil, a new cholinesterase inhibitor, with respect to its effect on the central cholinergic system. The conventional ChE inhibitors such as tacrine and physostigmine were used as reference compound in some experiments.

Effects on Cholinesterase Activity

(a) The comparative specificity of donepezil, tacrine, and PHY for brain AChE activity

The initial experiments were designed to determine the relative in vitro inhibitory effects of donepezil on the activities of AChE and butyrylcholinesterase (BuChE, pseudocholinesterase) in comparison with two
recognized cholinesterase inhibitors, physostigmine (PHY) and tacrine. Rat brain homogenates were used as the source of AChE and rat plasma served as the source of BuChE. ACh was used as the substrate for AChE and butyrylthiocholine (BuCh) was the substrate for BuChE. Both enzyme preparations were incubated with several concentrations of each inhibitor. The results, expressed as IC\textsubscript{50} values, are shown in Table 7. More extensive kinetic studies indicated that donepezil, like tacrine, is a reversible noncompetitive inhibitor of AChE [74]. On the basis of this study, it is clear that, \textit{in vitro}, donepezil is a much more selective inhibitor of AChE than either tacrine or PHY [87]. Since donepezil was a more specific inhibitor of AChE \textit{in vitro}.

**Fig. (19).** Global view of the modeled hBuChE molecule looking down into the active site gorge.

**Fig. (20).** Comparison of solvent-accessible surfaces of TcAChE active-site cavity with that of modeled hBuChE. A calculation of the solvent accessible surface area at the active site cavity was made using the program GRASP. (Upper left) Side view of the active site cavity of AChE. (Upper right) Top view of the active site cavity of AChE. (Bottom left) Side view of the active site cavity of hBuChE. (Bottom right) Top view of the active site cavity of hBuChE.
In order to test the relative tissue specificity of the inhibitors, the compounds were administered orally to rats, the animals were sacrificed one hour after administration, and the blood and other tissues were removed and analyzed for ChE activity as in previous experiments. Since the peripheral tissues contain primarily BuChE and the brain contains AChE, this study provides evidence for the relative tissue specificity of the inhibitors given in vivo. The results indicate that PHY and tacrine inhibit the ChE from both brain and peripheral tissue at all doses tested. In contrast, donepezil significantly inhibits the AChE of brain, but does not inhibit the BuChE from heart and small intestine, and has only a marginal effect on the ChE from pectoral muscle (Fig. (23)). Although complete dose-response curves were not run for each agent, it is still clear that donepezil, when given orally, is more specific for AChE than either PHY or tacrine [88].

### Table 7. Inhibitory Effects of E2020 and Reference Compounds on Rat Brain AChE and Rat Plasma BuChE in vitro

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC50 (nM)</th>
<th>AChE Activity</th>
<th>BuChE Activity</th>
<th>Ratio of EC50s (BuChE/AChE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E2020</td>
<td>5.7 ± 0.2</td>
<td>5.7 ± 0.2</td>
<td></td>
<td>1252.0</td>
</tr>
<tr>
<td>Physostigmine</td>
<td>0.68 ± 0.02</td>
<td>8.1 ± 0.3</td>
<td></td>
<td>11.9</td>
</tr>
<tr>
<td>Tacrine</td>
<td>80.6 ± 2.5</td>
<td>73.0 ± 0.9</td>
<td></td>
<td>0.9</td>
</tr>
</tbody>
</table>

*Values represent the mean ± S.E. from 4 dose-response curves for each test drug.*
Fig. (22). Effects of oral administration of E2020, physostigmine (PHY) and tacrine on rat brain AChE activity ex vivo. Each column denotes ± S.E., *, **: p<0.05, p<0.01. The top numbers in each column represent the percent inhibition relative to control. The lower numbers represent the number of animals used.

Figures are mean ± S.E.

* *, p<0.05, 0.01 vs. respective control (Dunnett's t-test).

Table 8. Effects of E2020 on ACh Concentrations of the Cerebral Cortex, Hippocampus and Striatum of Rats

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose mg/kg</th>
<th>n</th>
<th>ACh concentration (nmole/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Cortex</td>
</tr>
<tr>
<td>E2020</td>
<td>-</td>
<td>6</td>
<td>15.8 ± 0.79</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>6</td>
<td>20.4 ± 0.69*</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>6</td>
<td>21.8 ± 1.44**</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>23.5 ± 0.74</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>25.6 ± 0.78</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>28.7 ± 1.14**</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>67.7 ± 3.52</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>87.8 ± 3.99</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>99.3 ± 8.95**</td>
</tr>
</tbody>
</table>

Concentration of ACh in the brain. This was tested in the following two different experiments (Fig. (23)).

**Effects of Donepezil on Brain Acetylcholine Concentrations**

In view of this clear-cut ability of donepezil to inhibit brain cholinesterase activity in vitro and in vivo, it is reasonable to assume that donepezil can increase the concentration of ACh in brain. This was tested in two different systems.

(a) The effect of donepezil on the concentration of ACh in rat brain

In the first set of experiments, donepezil was given orally to rats, and the animals were sacrificed by microwave irradiation of the head one hour later. The cerebral cortex, hippocampus and striatum were dissected, and acetylcholine was extracted and measured using an HPLC technique. The results (Table 8) indicate that oral administration of donepezil caused a dose-dependent increase in ACh in all three areas of the brain tested.

By using microdialysis technique, it was possible to carry out the effect of donepezil on the concentration of ACh in the extracellular space of the rat brain cortex [89]. A microdialysis probe was implanted into the cerebral cortex of conscious rats and perfused with buffer. The acetylcholine which was released into the extracellular space was collected with the perfusing buffer and analyzed by HPLC. Baseline release of ACh was measured for at least 60 minutes, and the amount of ACh in the perfusate was determined at 20-minute intervals. Donepezil was given intraperitoneally at doses of either 1 or 3 mg/kg, and 20-minute samples were analyzed over the next 2 or 3 hours. The results were expressed as a percentage of the average.
release measured during the control period (Fig. (24)). From this experiment, it is clear that E2020 increases ACh in the extracellular space of the rat cerebral cortex in a time- and dose-dependent fashion.

Fig. (23). Effects of E2020, Physostigmine and tacrine on ChE Activity in the Brain and Peripheral Tissues ex vivo.

Each column indicates mean, the vertical line indicating ± S.E.

*, **: p<0.05, P<0.01 vs saline control (Dunnett’s t-test).

The number of animals is 6, except for that indicated by @ (n=5).
It was concluded from the preceding experiments that donepezil is capable of decreasing AChE activity and increasing the ACh concentration in the cerebral cortex of normal animals.

(b) The effect of donepezil and tacrine on ACh concentration in the cerebral cortex of animals with cerebral cholinergic hypofunction

Since donepezil was designed for use under circumstances in which the concentration of ACh is below normal level, it was tested, along with tacrine, in a series of in vivo model systems in which the cortical cholinergic system is impaired.

In the first study, the neurotoxin ibotenic acid was injected into the nucleus basalis magnocellularis region of the rat brain. Destruction of this region, which innervates the cerebral cortex, causes a decrease in the concentration of acetylcholine in the cerebral cortex. Two to three weeks after injection, the animals
were given an oral dose of either donepezil or tacrine. One hour later, the animals were sacrificed by whole-head microwave irradiation, and the concentration of ACh in the cerebral cortex was determined by HPLC analysis. The results (Fig. (25)) indicate that exposure to ibotenic acid significantly decreased the concentration of ACh in the cerebral cortex, and that treatment with either donepezil (1.25 to 10 mg/kg) or tacrine (5 to 20 mg/kg), caused a dose-dependent increase in cortical ACh. In this model system, donepezil appears to be a more potent agent than tacrine.

In the second model, a peripheral injection of the cholinergic blocker scopolamine was used to cause a temporary decrease in the concentration of ACh in the brain. For this study, the test compounds, either donepezil or tacrine, were given orally, and 30 minutes later the animals were injected intraperitoneally with scopolamine. Thirty minutes after scopolamine, the animals were sacrificed by whole-head microwave irradiation, and the ACh concentration in the cortex was determined. The results (Fig. (26)) demonstrate that scopolamine produced the expected decrease in the ACh concentration of the cortex, and that this decrease was prevented by prior administration of either donepezil or tacrine. As in the first model, donepezil was more potent than tacrine under the conditions of this experiment.

In a third study, the neurotoxin AF64A was injected into both lateral ventricles of the brains of anesthetized rats. Two to four weeks after injection, the animals were given an oral dose of either donepezil or tacrine, and sacrificed one hour later by whole-head microwave irradiation. The concentration of ACh in the hippocampus was then determined by HPLC analysis. The hippocampus was chosen for analysis in this study because of its involvement in memory-related functions [88]. The results (Fig. (27)) show that the AF64A injection produced a decrease in the concentration of ACh in the hippocampus, and that an oral dose (5 mg/kg) of donepezil significantly reversed this effect.

On the basis of these three studies, it has been established that an oral dose of donepezil can increase the concentrations of ACh in the brain of animals with abnormally low level of ACh.

**Effect of Donepezil in Behavioral Models of Cholinergic Hypofunction**

On the basis of the previous studies, it is apparent that donepezil is a relatively specific inhibitor of AChE which can increase the concentration of ACh in both normal and ACh-deficient animals. The final set of studies was designed to determine the ability of donepezil to alter behavior which is impaired due to a deficiency in cortical ACh. Accordingly, donepezil was tested for its effect on several model systems of abnormal animal behavior.

(a) The effect of treatment with donepezil on the behavior of functionally impaired animals

The first study was designed to evaluate the relative effects of donepezil and tacrine on scopolamine-
induced impairment of 8-arm radial-maze performance, a spatial memory task in rats. A chocolate chip was placed at the end of each of the radial arms of the maze, and the rats were trained to find and eat the chips. On completion of training and subsequent initiation of the study, performance is rated by measuring the time required for the animal to find 8 out of 8 chips (total running time) and by noting the number of incorrect maze-arm selections (error number). When animals were pretreated with scopolamine, performance, as measured by running time and number of errors) was significantly impaired. However, when scopolamine-treated animals were pretreated orally with donepezil or tacrine, maze performance was improved (Table 9). Under these experimental conditions, donepezil appears to be more potent than tacrine in improving maze performance. These results indicate that E2020 can reverse the effects of central cholinergic impairment in the maze-running performance task in rats.

The second study was designed to evaluate the effects of donepezil and tacrine on a passive avoidance task in animals with lesions in the nucleus basalis.

Table 9. Effects of E2020 and Tacrine on Errors and Running Time in the Performance of the Radial Maze Task

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>Error Number (X ± SE)</th>
<th>Total Running Time (seconds (X ± SE))</th>
</tr>
</thead>
<tbody>
<tr>
<td>E2020</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline</td>
<td>10</td>
<td>0.7 ± 0.3</td>
<td>65.6 ± 6.78</td>
</tr>
<tr>
<td>Pre-scopolamine</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline/scopolamine</td>
<td>10</td>
<td>7.5 ± 1.57##</td>
<td>194.1 ± 18.62##</td>
</tr>
<tr>
<td>E2020 0.52 mg/kg / Scopolamine</td>
<td>10</td>
<td>5.2 ± 1.29</td>
<td>147.5 ± 21.12*</td>
</tr>
<tr>
<td>E2020 0.50 mg/kg / Scopolamine</td>
<td>10</td>
<td>3.3 ± 0.67*</td>
<td>127.5 ± 18.33**</td>
</tr>
<tr>
<td>Tacrine</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline</td>
<td>13</td>
<td>0.5 ± 0.14</td>
<td>47.2 ± 5.85</td>
</tr>
<tr>
<td>Pre-scopolamine</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline/Scopolamine</td>
<td>13</td>
<td>7.0 ± 1.17##</td>
<td>194.5 ± 21.40##</td>
</tr>
<tr>
<td>Tacrine 1.0 mg/kg / Scopolamine</td>
<td>13</td>
<td>5.3 ± 1.53</td>
<td>154.8 ± 22.68</td>
</tr>
<tr>
<td>Tacrine 2.0 mg/kg / Scopolamine</td>
<td>13</td>
<td>4.5 ± 1.28*</td>
<td>123.5 ± 18.88*</td>
</tr>
</tbody>
</table>

#: p < 0.01 when compared with value before scopolamine treatment.
*: p < 0.05 vs. saline control (Student's t-test).
**Donepezil Hydrochloride (E2020)**

The NBM was destroyed in test animals by bilateral injection of ibotenic acid. After one week, NBM-lesioned and sham-operated animals were placed in a passive avoidance box consisting of light and dark compartments, and trained, using electric shock, to avoid entry into the dark compartment. One hour prior to training, they were given either donepezil, tacrine or saline orally, and tested 24 hour later to determine whether they remembered their training. Retention (memory) was measured by the amount of time each animal waited before entering the dark compartment (response latency). Animals which retained the training, i.e., memory of the electric shock, had longer latency times. As shown in Fig. (28), sham-operated animals had a response latency of approximately 400 seconds, and lesioned animals treated with saline had a latency of approximately 100 seconds, indicating a decrease in their ability to retain the training. Lesioned animals treated with donepezil at doses from 0.125 to 1 mg/kg showed a statistically significant increase in latency. Animals treated with tacrine at doses from 0.25 to 1 mg/kg showed increases in response latency at 0.5 mg/kg, but this increase did not achieve statistical significance. These results indicate that donepezil is capable of enhancing the retention of training (memory) in animals with cholinergic hypofunction.

The third study was designed to determine the effect of donepezil and tacrine on the hyperlocomotion which occurs in animals with a lesion in the nucleus basalis magnocellularis. The NBM of the test animals was lesioned by electro-coagulation and the animals were tested for motor activity 2 weeks later. NBM-lesioned animals become exceptionally active at night or when placed in a new environment. When sham operated animals are placed in a new environment, they are most active in the first 20 minutes, and gradually become less active over the course of two hours. In contrast, when NBM-lesioned animals are placed in an identical environment, their activity is much greater than that of sham operated animals, and the increased activity is maintained for a longer period of time. When lesioned animals were given subcutaneous injections of either 2 mg/kg donepezil or 5 mg/kg tacrine immediately before placement in a new environment, their activity demonstrated a response pattern closer to that of the unlesioned animals. While initial activity remained high for the first 40 minutes in the observation chamber, donepezil animals acclimated quickly thereafter and showed statistically significant improvement over the untreated, lesioned animals from 40 to 120 minutes. Tacrine, while somewhat effective, did not produce a statistically significant reduction in locomotor activity until 80 minutes after placement of the animal in the chamber. Further, the average degree of inhibition of hyperlocomotion produced by tacrine was less than that for donepezil until the 120 minute observation point. NBM lesioning also resulted in nocturnal hyperlocomotion, even in a familiar environment. donepezil (2 mg/kg, s.c.) had a significant inhibitory effect [91].

These results indicate that both donepezil and tacrine can partially reverse the hypermotility shown by NBM-lesioned rats in a new environment and at night.

---

**Fig. (28).** Effects of E2020 and tacrine on the latency of passive avoidance response in NBM-lesioned rats. *,**, p<0.05, p<0.01 (Mann-Whitney's U-test). S: Saline, Sham: Sham-operated rats, NBM Lesion: NBM lesioned rats. Number of animals is given in each column.
Fig. (29). Effects of cholinergic drugs on hyperlocomotion induced by a new environment in rats with NBM lesions.

*: p<0.05, **: p<0.01 vs. saline control Dunnett's multiple range test. (n=5)

However, as in the other model systems studied, donepezil appears to be more potent than tacrine (Fig. 29).

Discussion

The neurochemical studies show that donepezil is a highly selective, reversible and noncompetitive inhibitor of acetylcholinesterase in vitro and the inhibitory effect of donepezil is relatively selective for brain and serum cholinesterase, i.e. AChE in vivo. In the systems used for these studies, donepezil was a more selective inhibitor of brain AChE than the reference compound, tacrine. donepezil is capable of increasing acetylcholine not only in the brain of normal rats and in the extracellular space of rat cerebral cortex, but also in the rat models of cholinergic hypofunction. In the behavioral studies, donepezil is capable of partially reversing the impairment in retention of a passive avoidance task produced by brain lesion and in maze performance caused by scopolamine. These results suggest that donepezil may be a candidate drug for AD.

Clinical Studies of Donepezil

U. S. Multicenter Study Phase II

A double blind, placebo-controlled, randomized trial 1, 3 and 5 mg donepezil in 141 patients was reported in 1996. A 12-week double-blind phase was followed by a-week single-blind placebo washout. Improvements in Alzheimer's Disease Assessment Scale (ADAS-cog) and Mini-mental State Exam (MMSE) scores were reported; no changes were found in this study of short duration on the clinical global impression of change. However, a statistically significant correlation between plasma concentrations of donepezil and AChE inhibition was demonstrated. Moreover, there appeared to be a possible correlation between plasma drug concentrations and cognitive scores. Treatment-related side effects were comparable with all three doses.

15-Week Phase III Study

In a Phase III study, approximately 150 patients each received either donepezil 5 mg/day, donepezil 10 mg/day or placebo once daily for 12 weeks followed by a single-blind placebo washout for 3 weeks [92]. The 10 mg/day dose was titrated using a blinded schedule in which subjects received 5 mg doses of donepezil for the first 7 days. Consistent with FDA guidelines, the principal outcome measures were ADAS-cog and CIBIC-Plus (Clinician's Interview-Based Impression of Change-Plus). Statistically significant improvements in ADAS-cog score were seen within 3 weeks continuing to study endpoint and were significantly different from the placebo group. Significant improvements in this study were also seen in the CIBIC-Plus at both 5 and 10 mg doses.

30-Week Phase III Study

In this study, which was similar in design to the 15-week study, approximately 150 patients each were entered into donepezil 5 mg, donepezil 10 mg or
placebo. The patients were followed for 24 weeks, followed by a 6-weeks washout [93]. Once again, there were statistical improvements in ADAS-cog in patients treated with both drug doses at 12 and 18 weeks. CIBIC-Plus scores also improved in both groups compared to placebo; Fig. (30), Fig. (31).

**Safety**

A high proportion of patients completed both of the Phase III studies [93]. Five percent of patients dropped out due to adverse events in placebo and low-dose donepezil groups, increasing to 13% in the higher, 10 mg dose groups. This greater drop-out at the 10 mg...
**Table 10. Number (%) of Patients with Treatment-emergent Signs or Symptoms**

<table>
<thead>
<tr>
<th>Adverse Event</th>
<th>Placebo (n = 162)</th>
<th>Donepezil 5 mg/d (n = 154)</th>
<th>Dopemizel 10 mg/d (n = 157)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fatigue</td>
<td>3 (2)</td>
<td>8 (5)</td>
<td>12 (8) *</td>
</tr>
<tr>
<td>Diarrhea</td>
<td>11 (7)</td>
<td>14 (9)</td>
<td>27 (17) *</td>
</tr>
<tr>
<td>Nausea</td>
<td>6 (4)</td>
<td>6 (4)</td>
<td>26 (17) *</td>
</tr>
<tr>
<td>Vomiting</td>
<td>3 (2)</td>
<td>5 (3)</td>
<td>16 (10) *</td>
</tr>
<tr>
<td>Anorexia</td>
<td>3 (2)</td>
<td>3 (2)</td>
<td>11 (7)</td>
</tr>
<tr>
<td>Muscle cramps</td>
<td>1 (1)</td>
<td>9 (6)</td>
<td>12 (8) *</td>
</tr>
<tr>
<td>Dizziness</td>
<td>9 (4)</td>
<td>15 (10)</td>
<td>13 (8)</td>
</tr>
<tr>
<td>Rhinitis</td>
<td>4 (2)</td>
<td>1 (1)</td>
<td>9 (6)</td>
</tr>
</tbody>
</table>

Dose was thought to be due to the rapid titration, since in open-label studies, the frequency of drop-out at 10 mg is much lower if the titration is taken over 4-6 weeks. Most of the treatment-emergent adverse events were mild in intensity and lasted less than 2 days. As with other cholinesterase inhibitors, the most common side effects were nausea, diarrhea, insomnia, muscle cramps, vomiting and fatigue (Table 10).

**N-Benzylpiperidine Derivatives**

The N-benzylpiperidine moiety originally found in the donepezil structure is shown to have superior efficacy, minimal side effect, and high brain selectivity compared to tacrine and PHY. This makes donepezil a very novel compound. Donepezil and other N-benzylpiperidine derivatives are shown to reversibly and specifically inhibit AChE by forming a complex in which the N-benzylpiperidine group presumably interacts with the anionic site which recognizes the quaternary ammonium group of ACh.

Clinical studies have shown that the inhibition of butyrylcholinesterase (BuChE) which is abundant in human plasma may be linked with potentiating side effects. N-benzylpiperidine compounds are shown to have high selectivity for AChE over BuChE demonstrating an exceptional safety profile of these compounds. Because of these findings, recent studies have focused on N-benzylpiperidines [50, 59, 94]. Two N-benzylpiperidine derivatives are presented below:

**TAK-147 (Takeda)**

TAK-147 is a 4-substituted benzylpiperidine compound currently in Phase II clinical trials in Japan. In vivo studies show that TAK-147 is more potent than tacrine and PHY in inhibiting AChE activity in rat cerebral cortex homogenates with an IC50 value of 51.2 nM. It is least potent in inhibiting BuChE activity in rat plasma with an IC50 value of 19.0 nM [60]. It is also shown to have superior potency over tacrine, PHY and donepezil in inhibiting noradrenaline (IC50 = 4.02 µM) and serotonin uptake (IC50 = 1.35 µM) into synaptosomal fractions of rat cerebral cortex and hippocampus.

An evaluation of the central cholinergic activity and selectivity of TAK-147 have been evaluated and the compound is shown to significantly inhibit apomorphine-induced circling behavior in rats with unilateral striatal lesions at a dose of 3 mg/kg p.o. (31.1% inhibition) with no significant peripheral cholinergic effects. It is also shown to significantly improve diazepam-induced memory impairment [60]. Using other animal models, TAK-147 was further evaluated for its enhancing effect on learning and memory. These studies show TAK-147 improved scopolamine-induced impairment of a delayed matching to sample task and in impaired differential reinforcement at low rate performance in AF64A-treated rats at doses of 0.3 – 3 mg/kg p.o. devoid of significant behavioral and peripheral effects [95]. Studies in aged rats indicated that the improvements observed in water maze and passive avoidance learning after repeated oral administration of TAK-147 are associated with improvement in impaired metabolism in brain regions involved in learning and memory processes.

**T-82 (SS Pharm.)**

In vitro studies show that the AChE activity of T-82 is 9.5 times higher than tacrine. In a two-compartment test with 8-week-old male Wistat rats which received oral doses of the compound 15 minutes after scopolamine (0.5 mg/kg i.p.), 24-h memory retention was improved in a dose-dependent manner, i.e. 0.03 mg/kg : 50% improvement; 0.1 mg/kg : 92%; 0.3 mg/kg : 96%).
There are strong indications that T-82 is also a 5-HT$_3$ receptor antagonist. As a consequence of the tonic inhibitory control which the serotonin system exerts over acetylcholine release, this type of activity could lead to enhanced presynaptic discharge of acetylcholine in cholinergic neurons. Because this type of serotonergic receptor does not seem to be significantly impaired in AD, a very high synergistic potential could result if AChE inhibition and 5-HT$_3$ antagonist could be combined in one molecule [96-98](Fig. (32)).

**Other Approaches in Alzheimer's Disease Treatment**

While AChE inhibitors can alleviate the symptoms of AD, one should give some thought on the limitations of these therapeutic agents. What is more important is to be able to develop a therapeutic agent that can actually cure the disease. However, the development of curative compounds is markedly limited by the fact that the etiology and pathogenesis of AD is still unclear. Yet the research should continue.

Briefly discussed below are other approaches currently being pursued in the treatment of AD, such as anti-inflammatory agents, anti-oxidants, estrogen replacement therapy, galanin receptor antagonist, and the anti-amyloid strategy.

**Anti-inflammatory Agents**

The use of anti-inflammatory agents in the treatment of AD was pursued based on the large body of pathological evidence suggesting the presence of inflammations in AD brain. These inflammations can generate a self-propagating process in which different molecules (complement, cytokines, acute phase proteins) act, potentiating β-amyloid toxicity. A significant observation derived from a study conducted on the use of non-steroidal anti-inflammatory drugs (NSAIDs) in treating elderly arthritic patients, is the low-incidence of AD among these patients suggesting NSAIDs may delay neuronal degradation and limit the progression of the associated inflammation in AD [99-102].

**Anti-oxidants**

Several central nervous system (CNS) disorders including AD are thought to be related to changes in oxidative metabolism. Normally, the interaction of these compounds with molecules crucial for cellular viability is inhibited by endogenous antioxidant enzymes and free radical scavengers. However, when anti-oxidant defenses are inadequate, free radicals will produce cytotoxic damage to the cell membrane, enzymes and DNA by altering the electron number, the structure and the function of lipids, proteins, nucleic acids and other cellular constituents [8]. Studies show that oxygen radicals initiates amyloid build-up leading to neurodegeneration [103,104]. On the basis of this observation, anti-oxidants as a therapeutic approach to Alzheimer's disease has been pursued.

Vitamin compounds with anti-oxidant properties such as retinol (Vitamin A), ascorbic acid (Vitamin C) and tocopherol (Vitamin E) are now being studied as a potential therapeutic approach to AD.

**Estrogen Replacement Therapy**

Animal studies have provided evidence that estrogen stimulates nerve growth production. In overiectomized rats, ERT has been shown to: (1) restore and even enhance learning abilities; (2) prevent the decrease of neuronal choline uptake and choline acetyltransferase; (3) reduce the decline in nerve growth factor and brain-derived neurotrophic factor mRNA in those brain areas, and (4) exert neuroprotective effects in a tissue culture model [105].

These observations led to the application of estrogen replacement therapy (ERT) to prevent neuronal degeneration and cognitive decline. Studies conducted by Honjo et al. and Hagion et al. [106, 107] all confirmed the evidence that estrogen-responsive subjects exhibited osteoporosis and lower serum estrogen levels prior to beginning estrogen replacement, which suggests that AD in some women...
may be associated with systemic estrogen deficiency. Long-term prospective trials of estrogen in normal perimenopausal women and in women with AD are now needed to substantiate these hypotheses.

Galanin Receptor Antagonist

Galanin is a 29-amino acid peptide abundant in the brain and peripheral tissues. This peptide and its receptors have been implicated in a variety of physiological processes such as food intake, pain, anxiety and depression, memory and neuroendocrine functions. Tatamoto et al. [108] were the first to isolate this polypeptide from porcine intestine. Galanin appears to co-exist with several other neurotransmitters including ACh in the ventral forebrain [109]. The interaction between galanin and acetylcholine has been a major focus in galanin research. Axonal plexuses innervating the ventral hippocampus and nucleus basalis have been described in rat, monkey and human [110] and galanin inhibits the release of acetylcholine in these regions [111]. In AD brain tissue, galanin hyperinnervation of cholinergic neurons in the nucleus basalis has been observed suggesting that cholinergic dysfunction in AD patients may be depressed further by plasticity in the galanin system [112]. It is therefore attractive to speculate that galanin has a crucial role in the regulation of cholinergic function in forebrain pathways relevant to memory and may be involved in the cognitive dysfunctions associated with Alzheimer's disease [113].

Anti-Amyloid Strategy

As discussed in Section - AChE Mechanism of Action, β-amyloid deposits are found to co-localize with cellular proteins leading to the assembly of Aβ peptides and its aggregation into amyloid plaques. Because of its close relationship with the plaques and neurofibrillary tangles, β-amyloid has long been considered to be responsible for the development and the progression of the neurodegeneration in AD. But the processes that transform β-amyloid into potentially neurotoxic amyloid and its accumulation as senile plaques are still unclear. However, the discovery of its presence in AD brains, provides insight as to possible therapeutic targets for AD. Research has focused on interventions to prevent the destruction of neurons and the disruption of brain function by β-amyloid. These include the administration of antioxidants and free radical scavengers to reduce further neural damage from deposits of β-amyloid, the activation of various growth factors to repair damaged cells and restore their functions, and the stimulation of the normal processing of the precursor protein not only to aid in neural repair but more importantly to prevent the formation of additional β-amyloid [114].

Conclusions

Given the fact that the etiology and pathogenesis of AD is still unclear, the development of a curative compound is markedly limited. This limitation is even more compounded by the unavailability of a true animal model of AD. The screening of new AChE inhibitors is performed with a battery of pharmacological testings. But the question remains on whether the animal models are valid [1]. Appropriately validated animal models are important in the efficient and rational development of AChE inhibitors and other treatment compounds for AD.

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