REVIEW

From Molecular Structure to Alzheimer Therapy

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ABSTRACT—Clinical trials in the USA, Japan and Europe have confirmed the hypothesis that a steady state increase of acetylcholine resulting from cholinesterase inhibition in the brain results in an improvement of cognitive function in mild to moderate Alzheimer disease (AD) patients. During the last decade, a systematic effort to develop a pharmacological treatment for AD has resulted in two drugs being registered for the first time in the USA and Europe for this specific indication. Both are cholinesterase inhibitors (ChEI). Based on these first positive results, several second generation ChEI are being developed. An additional effect of certain ChEI is to maintain cognitive function at a constant level during a 6 months to one year period of treatment as compared to placebo. It is possible that the drug effect is one of slowing down cognitive deterioration. Comparison of clinical effects of 5 ChEI demonstrates a rather similar magnitude of improvement. For some drugs, this may represent a limit, while for others it may be possible to increase the benefit further. To maximize and prolong positive drug effects, it is important to start early and adjust the dosage during the treatment. Other strategies may involve combinations with other cholinergic drugs such as muscarinic or nicotinic agonists. A second important class of drugs which is being developed is that of muscarinic m1 agonists. However, their clinical use is still limited by side effects. The increased knowledge and recognition of the beta-amyloid molecule as a central focus of AD pathology has strongly stimulated research with the hope of finding ways of influencing its processing and deposition. At this point, no product in this line of development has reached clinical trial level. Other pharmacological approaches are related to preventive and neuroprotective interventions (estrogens, anti-oxidants and anti-inflammatories). In conclusion, given the relatively short time of research in this field, results are encouraging.

Keywords: Alzheimer disease, Cholinesterase inhibitor, Beta-amyloid

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Abbreviations used are (in alphabetical order): ACh, acetylcholine; AChE, acetylcholinesterase; AChEI, acetylcholinesterase inhibitor; AD, Alzheimer disease; ADAS-cog, Alzheimer disease assessment scale-cognitive subscale; APOE, apolipoprotein E; APP, amyloid precursor protein; BuChE, butyrylcholinesterase; CGIC, clinician global impression of change; ChE, cholinesterase; ChEI, cholinesterase inhibitor; CIBIC, clinician interview-based impression of change; CNS, central nervous system; CSF, cerebrospinal fluid; DA, dopamine; ITT, intention to treat; KPI, Kunitz-type protease inhibitor; NE, norepinephrine; PKC, protein kinase C; physo, physostigmine; THA, tetrahydroaminoacridine; tacrine.

1. Introduction: Drug therapy for Alzheimer disease

An attempt to develop systematically drugs to treat Alzheimer disease (AD) was initiated on a large scale ten years ago following the publication in the New England Journal of Medicine of the first successful results obtained with the cholinesterase inhibitor (ChEI) tacrine (THA, tetrahydroaminoacridine) by Summers et al. (1). Tacrine is not the first ChEI being tested clinically for AD. Numerous studies (2) had been performed previously, particularly in the USA, in small groups of patients, with physostigmine (physo) alone or in combination with lecithin. Physo, like tacrine, produced definite but only shortlasting improvements of cognitive symptoms (attention, concentration, memory) that were accompanied with severe peripheral and central cholinergic side effects. These consisted mainly of gastrointestinal symptoms and drowsiness, but in the case of tacrine, also of liver toxicity. It was soon realized that inspite of this first encouraging result, both physo and tacrine were far from an ideal drug for AD treatment. However, physo and tacrine represent important milestones in AD therapy as they supported for the first time in patients, the pharmacological hypothesis formulated in experimental animals (3–7) that a treatment improving the function of the central cholinergic system obtained through an increase of brain acetylcholine (ACh) would also improve cognition in AD patients. Targeting the cholinergic system for AD therapy does not necessarily limit itself to the use of a ChEI. Two other classes of cholinergic drugs might represent valid alternatives such as nicotinic and muscarinic agonists alone or in combination with ChEI (Fig. 1).

A second, non-cholinergic approach, is based on the classic pathological landmarks of the disease, aiming to decrease beta-amyloid (beta-A4) deposition and amyloidogenic APP (amyloid precursor protein) release in the brain (Fig. 1). A third way to AD therapy aims to correct events that are probably secondary to the disease process by means of estrogens, anti-oxidants, free-radicals scavengers and anti-inflammatory agents (Fig. 1).

Fig. 2. Processing of the beta-amyloid (beta-A4) precursor protein (APP), its regulation in Alzheimer disease and its relation to dementia and apoptosis. ACT=alpha-antichymotrypsin, VLDL=very low density lipoprotein receptor, APOE=apolipoprotein E, ALG-3=apoptosis-linked gene, TAU-P=phosphorylated tau protein, presen. 1 and 2=presenilin 1 and 2.

TREATMENT OF ALZHEIMER DISEASE WHICH WAY TO GO?

Fig. 1. Treatment of Alzheimer disease. Which way to go?

Fig. 3. Five pharmacological interventions to selectively decrease extracellular concentration and deposition of beta-A4 in brain and to slow down dementia.

Based on the present knowledge of beta-A4 synthesis, processing, accumulation and deposition in the cortex and related genetic factors (8–11) (Fig. 2), we can think of at least five pharmacological interventions to selectively decrease extracellular concentration and deposition of beta-A4 in the brain and to slow down the disease process (Fig. 3). In theory, the same result could be achieved by blocking beta-A4 processing or slowing down secretion or deposition of amyloidogenic forms of APP. Processing and secretion of APP could be modified by inhibiting specifically one or several involved proteases (alpha, beta or gamma secretases) (8). One might also think of reducing aggregation by enhancing removal of deposited beta-A4 (Fig. 4). There is evidence for considering apolipoproteins such as apolipoprotein E (APOE) 3 and APOE2 as factors reducing beta-A4 aggregation and APOE4 as promoting it (Fig. 4) (12, 13). Presenilin 1 on the other hand, promotes both clearance and degradation of beta-A4 (Fig. 4) (9, 14). The final goal is to block the progressive and irreversible degeneration of synapses and neurons that is the main feature of the disease. A better understanding of the mechanism of action of presenilins and APOE could lead to new therapeutic strategies developing drugs interacting with these processes (12) or pre-

ventive and protective measures based on early identification of genetic risk factors (see low-fat diet and control of hypertension for cardiovascular diseases). Recent data suggests an involvement of the cholinergic system in beta-A4 processing both experimentally and clinically (15) (Fig. 5). Chemical cholinergic lesions in the nucleus basalis of the rat forebrain decrease ACh cortical levels and release and increase accumulation of APP in the cerebrospinal fluid (CSF) and cortex (Table 1) (16, 17). The effect of the lesion is mimicked by muscarinic antagonists and reversed by treatment with ChEI. The data of Soininen et al. (18) and Poirier et al. (19) show that the APOE4 genotype exerts a deteriorating effect on the cholinergic system and might facilitate selective neuronal damage. This cholinergic hypofunction might condition the response to cholinergic drugs in AD patients (19) (Fig. 5). Other data suggesting an interaction between cholinesterase inhibition and brain metabolism as clinical evidence of neuroprotection are summarized in Table 2. These include the effect of a ChEI (tacrine) on cortical glucose metabolism and cerebral blood flow monitored in AD patients with imaging techniques such as PET (positron emission tomography) and SPECT (single photon emission computed tomography) utilizing FDG

Fig. 4. Effect of apolipoproteins (APOE) and presenilin 1 (PRS1) on beta-A4 aggregation.

Fig. 5. Relation between apolipoprotein E4, cholinergic lesions and cholinomimetic drug response to ChEI.

(fluoro-deoxyglucose) and ^{11}C -nicotine as biological markers of metabolic and cholinergic activity, respectively (20, 21). A delay in time to nursing home placement is chosen as a clinical marker of delayed deterioration of the patient (22). Although multiple non-cholinomimetic interventions are feasible (Fig. 1), this review will focus mainly on ChEI compounds for which we have most detailed basic and clinical information.

Table 1. Cholinergic lesions and APP processing

a chemical lesion in the nucleus basalis of the rat produces (76):

- A) a decrease in ACh cortical level and release
- B) a rapid and persistent accumulation in cortical and CSF APP

this response is:

- A) age-dependent
- B) mimicked by muscarinic antagonists
- C) reversed by treatment with ChEI (16, 17)

Table 2. Cholinesterase inhibitors: Clinical evidence of neuroprotection

1. Increased cortical glucose metabolism and rCBF
18 month THA, PET FDG (20, 77)
2. Increased cerebral nicotinic binding
12 month THA, PET- ^{11}C nicotine (77)
3. Stable regional cerebral rCBF
14 month THA, SPECT 133 Xe (78)
4. Delay in time to nursing home placement
500 days THA (22)

rCBF = regional cerebral blood flow.

2. Molecular design of new cholinesterase inhibitors

The ChEI tacrine was developed for purposes other than therapy of AD, such an anti-delirium and morphine antagonist as well as an adjunct in anesthesia (23). However, tacrine became the first drug to be registered in the USA in 1994 for this indication. Several tacrine and physio-analogues were developed subsequently which are presently being evaluated for AD therapy in Europe and Japan. A list of drugs presently in clinical trials in Japan is reported in Table 3. Several ChEI are included. In analogy with tacrine and physio, they act at a site close to

Table 3. Drugs for AD treatment currently in the clinical phase in Japan (1996)

Cholinesterase inhibitors

NIK-247	Nikken
ENA-713	Sandoz
TAK-147	Takeda
E-2020	Eisai

Acetylcholine releasers

T-588	Toyama Kagaku
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M1 agonists

YM-976	Yamanouchi
AF-102B	Snowbrand

Neuropeptides, peptidase inhibitors

TRH	Japan Tobacco
JTP-4819	Japan Tobacco
Epibizatide	Hoechst

Other: dibenzodiazepine inverse agonists, nootropics,

Ca²⁺-uptake inhibitors

TRH	Daiichi
S-5810	Shionogi
Nimodipine	Bayer

Table 4. Cholinesterase inhibitors: AD clinical trials in the USA and Europe (1997)

Compound	Country	Company	Clinical phase****	Side effects Comments
Physostigmine slow release	USA	Forest	III	N.A.
ENA-713	USA/Europe	Sandoz	III	Low side effects
Eptastigmine	USA/Italy	Mediolanum	III	Low side effects
E-2020	USA/Japan	Eisai	III Regist.	Low side effects
MDL-73,745	USA/Europe	Marion Merrell Dow	II	Low side effects
Metrifonate	USA/Germany	Bayer/Miles	III	Low side effects
Tacrine (THA)*	USA/Europe	Warner-Lambert	IV Regist.	Hepatotoxicity
Velnacrine (HP029)**, ***	USA/Europe	Hoechst-Roussel	II	Hematology
Suronacrine (HP-128)**				Hepatotoxicity
Galanthamine	Germany	Shire Pharm.	II	Low side effects
	USA	Ciba-Geigy		
Huperzine A	China	Chinese Acad. Sci.	III	N.A.
NX-066	England/USA	Astra Arcus	II	N.A.
CP-118,954	USA	Pfizer	II	N.A.
KA-672	Germany	Schwabe	I	N.A.
NIK-247	Japan	Nikken	III	Low side effects
TAK-147	Japan	Takeda	III	Low side effects

*other indications: HIV, tardive dyskinesia; **withdrawn; ***neutropenia or agranulocytosis. ****clinical phase in the USA. Regist.=registered in the USA and in some European countries. N.A.=data not available.

the catalytic triad of the enzyme. The acetylcholinesterase (AChE) catalytic triad is made up by one glutamic, one histidine and one serine residue (24). Reacting with the histidine residue, the glutamic carboxylic group activates the serine hydroxy group, which then hydrolyzes the ACh ester function by nucleophilic attack. When the substrate is physo or a physo-analogue such as eptastigmine (Table 4), phenserine, ENA-713, RO-46 or HP-029, the intermediate carbamic acid is hydrolyzed very slowly. These compounds all inhibit ChE by pseudo-irreversibly carbamoylating the serine residue of the catalytic triad. The x-ray crystal structure of the complex between tacrine and AChE has been studied in detail (24). As a consequence, the interaction between the enzyme and ACh is known, likewise its mechanism of action. This molecular model of interaction represents an important step in designing new inhibitors by means of rational molecular modelling. Models include either AChE or butyrylcholinesterase (BuChE)-selective compounds (25). An example of application of molecular modelling to drug design is the synthesis of a large number of physo-analogues in which the methylcarbamic group has been substituted with alkyl- and dialkylcarbamic groups of different length and lipophilicity (25). This has resulted into compounds with increased oral absorption, higher brain penetration, increased plasma half-life, and reduced toxicity and severity of side effects (25). A good example of such an analogue is eptastigmine. In such a case, the 50% reactivation time of the enzyme is increased from 15 min to 3 hr as com-

pared to physo. Reactivation is also faster for the erythrocyte-derived enzyme than for the cerebral enzyme which might represent an advantage as it probably increases duration of effect and drug efficacy (26).

Knowledge about the molecular structure of the catalytic site and those of the two anionic sites of AChE would allow the synthesis of inhibitors highly selective for either AChE or BuChE. This property is particularly interesting since it is known that many cholinergic side effects are not associated with inhibition of BuChE. Moreover, AChE is strongly reduced in the brains of AD patients, while BuChE is intact or even increased (Table 5). Therefore, an ideal inhibitor for AD treatment should be selective not only for membrane-anchored AChE but should also inhibit BuChE that hydrolyzes the ACh extrasynaptic pool. In addition, the hydrophobic tetrameric-tailed G4 form of AChE anchored to the plasma membrane is dominant in brain synapses (Table

Table 5. Percent variation in enzyme activity in human cortex of AD patients compared to normal controls

Enzyme	Activity (% of normal)	Molecular form
AChE	10–15	mainly G4
BuChE	120	mainly G1
ChAT	10–15	

Ref.: 28, 48, 50, 51, 53. ChAT=choline acetyltransferase.

Table 6. Differences in clinical potential and side effects between ChEI and muscarinic agonists suggest a differential action on subtypes of receptors and specific pre- and post-synaptic interactions

Class	Clinical effects	Side effects
Cholinesterase inhibitors	Predominantly cognitive	low incidence with second generation drugs
Muscarinic agonists	Predominantly behavioral	significant cardiovascular and gastrointestinal side effects

5) (27). Based on our pharmacological knowledge, a ChEI selective for this molecular form could also be designed (28). Compounds having such characteristics may constitute future alternatives of new ChEI molecules.

3. Cholinergic therapy: Which way to go? Cholinesterase inhibitors or muscarinic agonists? Future development

A parallel line of research has aimed to develop drugs such as muscarinic agonists to stimulate selectively postsynaptic M_1 -receptors or antagonists to inhibit the effect of M_2 presynaptic receptors to improve ACh synaptic release (Fig. 1, Table 3). Neither approach has produced so far highly selective drugs; therefore, compounds have seldom reached clinical trials or are still at early clinical phases. One major obstacle is represented by the severity of cholinergic toxicity, particularly gastrointestinal and cardiac side effects. Due to these limitations, future muscarinic agonists need to demonstrate higher brain receptor selectivity. The number of muscarinic agonists presently in clinical trial is lower than that of ChEI, but some differences are emerging with regard to the clinical potential of these two classes of cholinergic drugs (Table 6). Cholinesterase inhibitors seem to exert predominantly cognitive effects (improve attention, memory, and concentration), while muscarinic agonists seem to act also on behavioral symptoms. A combination of these properties may prove to be of benefit. In spite of differences in severity of toxicity in the two groups of drugs, for muscarinic agonists, the main obstacle is to overcome autonomic side effects. For acetylcholinesterase inhibitors (AChEI), both severity and frequency of side effects seems to be less significant, particularly for the second generation of ChEI (Table 6).

4. Cholinesterase inhibitors: Do they work in Alzheimer disease? How do they work? Is there a difference?

A 1997 list of ChEI presently in clinical trials in Japan, USA and Europe includes more than a dozen drugs, most of which have already advanced to clinical phase III (Tables 3 and 4). The next two-year period (1997–1999)

will be crucial for selecting efficacious drugs. The second generation of ChEI to replace tacrine will have to fulfill specific requirements (13). Analyzing results from numerous trials throughout the world, the first question to be answered obviously is: do ChEIs work in the patient and if so, how do they work? The second question is: are there major differences between various compounds? These two fundamental questions can be examined and partially answered for the first time by comparing recent clinical data (Table 7). Table 7 shows the effect of five ChEI on ADAS-cog (AD Assessment Scale-cognitive subscale) test using ITT (intention to treat) criteria. The duration of the trials varied between 12 and 30 weeks. Based on these data, the answer to question one is affirmative. All five ChEI produce statistically significant improvements evaluated with specific scales of standardized and internationally validated measures of both cognitive and non-cognitive function. The cognitive items have been most widely used in these investigations. One first observation is the similarity in effect for all five drugs when expressed as a difference between drug- and placebo-treated patients. Differences consist generally of 2–5 points (ADAS-cog) depending on the type of analysis performed and on less than 0.5 on CIBIC (Clinician Interview-Based Impression of Change). The difference varies from a maximal gain of 5.3 points (tacrine high dose) to a minimum of 3.9 points (ADAS-cog, E-2020 high dose). Does this trend suggest a ceiling effect of 4–5 ADAS-cog points average for all ChEI? Or does it indicate the fact that drugs have not been used yet at their maximal potential capacity? The high percentage of drop-outs and side-effects for tacrine seem to indicate a limit in drug effect. For other drugs (e.g. metrifonate), using a dosage that produces high ChE inhibition (up to 80%), severity of side effects do not seem to be a limiting factor. In general, the percentage of improved patients varies from 18% (ENA-713, low dose) to 50% (tacrine, high dose) with an average of 30% for most drugs. This indicates that approximately one third of treated patients demonstrate a positive response to these ChEI. This not very impressive figure becomes more relevant if we consider that the number of improved patients could still be increased to 50% by using ChEI with less severe side-

Table 7. Effect of five ChEI on the ADAS-cog test (ITT)

Drug	Ref.	Dose (mg/day)	Duration (weeks)	Treatment difference (from placebo)	Improved patients (%)	Drop-out (%)	Side-effects (%)
Tacrine	(30, 31)	80–160	30	4.0–5.3	30–50	50–73	40–58
Eptastig	(79, 80)	45	25	4.7	30	12	38
E-2020	(32)	5	12	3.9	25	12	36
ENA-713	(29)	12	26	4.9	18*	22	28
Metrifonate	(33)	30	12	4.6	35	2	2

*considering 4 point improvement.

effects. Looking at the 6-months data one could say that patients treated with the active compound do not change significantly from the baseline determined at the beginning of treatment (6 months earlier). As an example in a US study with ENA-713, patients given placebo for 26 weeks would deteriorate approximately 4 points on the ADAS-cog compared to only 0.3 in patients given 6–12 mg/day of the drug (29). The difference seen after 6 months between placebo-treated and drug treated groups seems to depend more on the deterioration of the placebo group (3–4 points) than on true additional improvement. This interpretation implies an anti-deteriorating and slowing rather than a purely symptomatic effect of the drug. This presumed “anti-deteriorating effect” could be related to an improvement of cholinergic function as reflected by the cognitive improvement measured by ADAS-cog. Tacrine, velnacrine and E-2020, on the other hand, seem to produce a small but real initial improvement (2–3 points) as compared to the placebo group lasting 4–24 weeks, depending on the dose (30–32). Whether or not this represents a real difference among drugs remains to be demonstrated. As indicated by studies of longer duration (up to 18 months) (33), it is possible that the difference between placebo- and drug-treated subjects could be maintained beyond the 6 months limit to at least one year. This would represent a significant gain for both patient and caregiver. Drugs could differ also in this respect. In comparing the results of clinical trials and the effect of different drugs, one should take into consideration the fact that studies may differ one from another, due to differences in selection criteria, age of subjects, severity of disease, concomitant illnesses, variable instruments of assessment and side-effect evaluation. Although using “completers” instead of ITT analysis could produce somewhat higher effects. Thus, studies are not totally comparable and conclusions at this stage can only be indicative. Given these limitations, the immediate next goal to achieve for a ChEI would be an improvement of 5–6 ADAS-cog points and a 0.6–0.7 point on CIBIC during a 6-months treatment period. Most of this effect should still be present at 12 months and the

drop-out should be no more than 20%. Is this goal achievable?

5. What causes the difference between various cholinesterase inhibitors? Is there a tolerance?

The relation between percent of peripheral ChE inhibition and cognitive (ADAS-cog) or global impression of change rated by the clinician (CGIC) effect is a relevant factor which is reported in Table 8. The data presented in Table 8 support the concept of an optimal brain ChE inhibition and functional ACh levels that might vary for each drug and relate to an optimal gain in cognitive and therapeutic effect (13, 34, 35). This hypothesis is in accordance with pharmacological data in animals (3, 36) and in humans (34). The level of peripheral enzyme inhibition, which has been measured in patients (AChE activity in erythrocytes or plasma BuChE activity), producing maximal effect on cognitive testing varies between 30% and 60% depending on kinetic and pharmacological characteristics of the compound (Table 8). However, for some drugs, (see E-2020 and metrifonate), this can be as high as 80%. As predicted by pharmacological and behavioral data, there is a clear correlation between ChE inhibition (or drug plasma concentration) and cognitive effect (36, 37). Drugs producing only mild side effects at high dosage and causing high brain ChE inhibition have the advantage that they can be tested clinically within their full range of therapeutic potential. For some drugs (physo, eptastigmine and metrifonate), the relation between ChE inhibition and cognitive effect is inverse-U shaped, while for others (such as tacrine and E-2020), this relation seems to be linear. The U-shaped form can be explained by the fact that by increasing the dose of the inhibitor, one sees increasing efficacy for as long as adverse effects do not become a limiting factor. Other reasons for the U-shaped curve depend on specific inhibition kinetics of the inhibitor- and substrate-induced saturation effects of ChEs. The level of ACh brain elevation varies according to brain ChE inhibition (13, 35, 38). With increased brain concentrations of ACh, substrate

Table 8. Relation between percent ChE inhibition and effect on ADAS-cog or CGIC

Drug	Ref.	Dose (mg/day)	Steady state (% inhibition)	Optimal (% inhibition)	Correlation ChEI/ADAS-cog or CGIC
Physostigmine	(81)	3–16	40–60 (BuChE)	30–40	U-shaped
Eptastigmine	(82, 79)	30–60	13–54 (AChE)	30–35	U-shaped
Metrifonate	(83)	30	35–75 (AChE)	65–80	U-shaped
E-2020	(32)	5	64 (AChE)	60	linear
Tacrine	(30, 31, 84)	160	40 (BuChE) 60 (AChE)	30	linear

inhibition of enzyme activity is a phenomenon of particular importance that is observed *in vitro* and is probably present also *in vivo* (38). Plotting velocity of enzymatic reaction against substrate (ACh) concentration, a bell-shaped curve with a peak in the case of AChE and a sigmoid curve in the case of BuChE are observed. Thus, AChE is inhibited by a large excess of ACh such as it can be produced by a sudden high inhibition of brain AChE. This substrate elevation has the effect of decreasing the catalytic potency of the enzyme and subsequently its pharmacological (and perhaps therapeutic) effect. From this relationship, it can be predicted that a high ChE inhibition reached rapidly in time during treatment (rapid passage of the drug into the brain and its accumulation) will not increase but reduce efficacy and increase central nervous system (CNS)-dependent side effects (drowsiness, nausea, vomiting, etc). It should be an advantage to use a slow-release type of ChEI, inhibiting the brain enzymes at a slow pace and slowly reaching steady-state levels of brain ACh. Kinetic mechanisms may explain the inverse-U-shaped relationship seen for some drugs (physo, eptastigmine and metrifonate). There is an excellent agreement between clinical and animal data for both physo and tacrine with regard to dose/behavioral effects relationships. Rupniak et al. (39) using two primate models (rhesus monkeys) found that both tacrine and physo improved visual recognition memory significantly. Both drugs showed a clear inverse U-shaped relationship with a maximal effect at around 0.0010–0.02 mg/kg, *i.m.* for physo and 0.8–1 mg/kg for tacrine. Lower or higher doses did not improve performance. Central cholinergic side effects, which may develop early in the treatment, are not related directly to brain AChE inhibition but mainly to elevation of ACh levels (35, 38). In addition, peripheral side-effects may occur depending on a rapid distribution of the drug between extra-CNS (peripheral organs) compartments and CNS. A combination of pharmacokinetic and pharmacodynamic effects (possible down regulation of muscarinic and nicotinic receptors) as well enzymatic changes may be responsible for the tolerance to therapeutic effects developed by the patient after

prolonged treatment. Progressive, disease-related deterioration of synaptic function (such as ACh release and receptor function) may also contribute to a time-dependent reduction of clinical effects resembling the “wearing off” phenomenon observed in Parkinson patients treated with dopaminergic drugs (Table 9). This tolerance phenomenon has been observed for most ChEI, but has not been studied in depth either in animal models or in the patient. Due to a combination of factors, following a prolonged (months or years?) period of treatment, the therapeutic effect slowly decreases and eventually disappears. It is important to know whether this tolerance effect depends on upregulation of AChE activity due a change in the rate of synthesis of the brain enzyme, changes in drug metabolism or in uptake of the drug in the brain with time. Preliminary results in the rat indicate that after a few weeks of daily treatment at moderate doses, AChE inhibition in rat brain progressively decreases. This phenomenon may constitute the base of tolerance. In order to prolong therapy, the dosage needs to be either adjusted (increased) to new levels of inhibition or an intermittent schedule with wash-out periods adopted. This dose-adjustment procedure should begin already in the initial phase of treatment in order to avoid starting “toxic concentrations” of CNS ACh. This can be achieved by starting with a lower dose and then increasing

Table 9. Parkinson and Alzheimer: Pharmacological strategies: Differences and similarities

	Parkinson	Alzheimer
Transmitter	dopamine	acetylcholine
Precursor	<i>l</i> -dopa*	choline
Release	amantadine*	M2 antagonists*
Degradation	MAO A-B, COMT inhibitors*	AChE inhibitors*
Postsynaptic receptor	D1, D2 agonists*	M1 agonists*
Transm. uptake	uptake inhibitors*	vesamicol

*=utilized in therapy. MAO=monoamine oxidase. COMT=catechol-*o*-methyl-transferase.

Table 10. Parkinson and Alzheimer: Therapeutical strategies: A comparison

	Parkinson	Alzheimer
	Levodopa	ChEI
Decreased effect	after prolonged use	after shorter use
Most effective	2–5 years	6 months–1 year
Side effects	dyskinesia	cholinergic
Effectiveness (% of patients)	75	25–30

dosage progressively to a maintenance dose. However, this procedure has the disadvantage, for the more toxic ChEI, of delaying the clinical effect. Later during treatment, in order to maintain effective ACh levels in the brain, the dose of the ChEI may need to be increased again or its effect potentiated with a combination of muscarinic or nicotinic agonists. This strategy implies that the dose of ChEI to be administered at each stage of the disease should be adjusted in order to reach and maintain maximal clinical effects. This therapeutic strategy reminds us of the management of Parkinson patients with *L*-dopa and other dopaminergic drugs throughout the disease (Table 9). The decrease in effect of both therapies is also striking (Table 10).

In developing new ChEI, a broad therapeutic index is important for the reason of safety. Likewise, a long duration of AChE inhibition in brain is important in order to reach steady state levels of brain AChE inhibition producing sufficiently high ACh levels to stimulate hypoactive cholinergic synapses. For some ChEI, (see epastigmine) (26) the inhibited brain enzyme recovers more slowly than the red blood cell (RBC) enzyme. This effect should also reduce the dosage to one or maximally two doses daily, which is most convenient from the practical point of view. In general, we can say that the potential

efficacy of a ChEI cannot be fully tested until side-effect free inhibition can be maintained at a steady state. This condition may be realized only with some of the second generation ChEIs.

Last but not the least problem of ChEI therapy is the early identification of those patients most likely to benefit from therapy. The correlation of therapy to risk factors (APOE-alleles) represents an attempt in this direction (Fig. 5). Choosing the proper and possibly early stage of the disease at which to start medication may be crucial for the success of the therapy.

6. Effects of cholinesterase inhibitors on cortical neurotransmitters

Clinical as well as experimental evidence indicate involvement and interactions between the cholinergic system and the biogenic amine system in the cognitive impairment observed in AD (40, 41). We have devoted particular attention to this problem (37).

Table 11 compares the effects on ACh, norepinephrine (NE) and dopamine (DA) levels as well as AChE inhibition after systemic administration of six clinically tested ChEIs studied in our laboratory. Our results show a significant increase in cortex for all three neurotransmitters and for all six ChEIs investigated.

The results reported in Table 11 suggest that extracellular ACh levels in the cortex are related not only to ChE inhibition, supporting results of previous microdialysis studies showing comparable elevations of ACh levels in spite of different magnitudes of ChE inhibition (42). This consideration may be of importance in predicting clinical effects and side effects (see dopaminergic effects) and setting dosages of various ChEIs. The development of combinations of ChEI and monoamine-receptor agonists and antagonists is suggested by these studies (13, 37).

Table 11. ChEI effects on ACh, NE, DA levels and ChE activity in rat brain cortex after s.c. administration

Compound	Ref.	Dose (mg/kg)	ChE (max. % inhib.)	ACh	NE	DA
Physostigmine	(55)	0.3	60	4000	75	120
Heptyl-physostigmine	(55)	2	75	2500	25	75
E-2020	(55)	2	35	2100	100	80
MF-268	(85)	2	40	2500	100	60
MDL 73,745	(86)	2	65	1020	120	370
Metrifonate	(87)	80	70	1700	60	75

E-2020 = (*R,S*)-1-benzyl-4-(5,6 dimethoxy-1-idanone)-2-yl-methylpiperidine (55); MF-268 = 2,6-dimethylmorpholin-octyl-carbamoyl eseroline (85); MDL 73,745 = 2,2,2-trifluoro-1-(3-trimethylsilylphenyl)ethanone (86); Metrifonate = 0,0-dimethyl-(1-hydroxy-2,2,2 trichloroethyl-phosphate) (87).

Fig. 6. Inhibition curve for rat brain homogenate using Mipaflox as an inhibitor and two partially purified enzymes from electric organ (AChE) and Cohn serum fraction (BuChE) as reference. Ordinate: percent ChE activity of control. Abscissa: negative log molar concentration of inhibitor (pI). The inhibition curve for rat brain fits between the two curves. Substrate: acetylthiocholine iodide, 4 μ M. (modified from Giacobini and Holmstedt (44)).

7. Should a cholinesterase inhibitor be selective for brain acetylcholinesterase?

The brain of mammals including man, investigated so far, has been shown to contain two major forms of ChEs, AChE and BuChE (43). Using specific substrates and inhibitors, it has been shown that in contrast to AChE, BuChE hydrolyzes preferentially butyrylcholine (44). Butyrylcholine is not a substrate in human brain.

Combining specific substrates with selective inhibitors, it has been demonstrated that in rat brain, approximately 80% of the activity is AChE and 20% is BuChE (44) (Fig. 6 and Table 12). In rat brain, BuChE activity is mainly

localized to glial cells, while AChE activity is mainly concentrated in nerve cells (45, 46). In the human brain, BuChE is found both in neurons and glia as well as in neuritic plaques and tangles in AD patients (47).

Depending on the region, human brain AChE activity is 1.5 (temporal and parietal cortex)- to 60 (caudate nucleus)-fold higher than BuChE activity (36, 48–53).

An important feature distinguishing BuChE from AChE is its kinetics toward concentrations of ACh. An excess of this substrate (μ M) will inhibit only AChE but not BuChE. Therefore, because of the difference in K_m of the two enzymes, glial BuChE is less efficient in hydrolyzing ACh at low substrate concentrations (sub- μ M) than neuronal AChE. The mechanism of inhibition caused by the excess of substrate has been clarified by Shafferman et al. (54). It depends on a change in conformation of the Tyr 337 amino acid that lines the upper part of the catalytic gorge of the enzyme which represents in part the peripheral site overlapping the substrate-inhibitory site. A change in conformation of Tyr 337 or a mutation in a single amino acid will induce allosteric changes in the peripheral site that are responsible for this inhibition (54). In the case of BuChE in which alanine is replacing tyrosine no substrate inhibition is observed. Not only is cholineacetyltransferase activity significantly decreased in AD (48) but also both AChE and BuChE activities are altered.

The proportion of the two enzymes present in human brain is strongly altered in the course of AD. In the cortex of patients affected by AD, AChE activity decreases progressively to 10–15% of normal values, while BuChE activity is unchanged or even increased by 20% (Table 5) (36, 48, 50, 51, 53). As an example, BuChE/AChE ratio will increase from 0.6 to 0.9 in the frontal cortex and from 0.6 to 11 in entorhinal cortex. This may be a consequence of a combination of reactive gliosis and of an accumulation of BuChE in neuritic plaques (47). It is likely that in conditions of a strongly decreased concentration of synaptic AChE, particularly

Table 12. In vitro activity of ChEI in the rat (IC_{50} , nM)

Compound	AChE*	BuChE**	BuChE/AChE
E-2020	5.7	7138	1252
Huperzine A	58.4	58.900	1008
Physostigmine	0.68	8.1	11.9
Galanthamine	2000	12.600	6.3
ENA-713	57.000	16.000***	3.6
DDVP (Metrifonate metab.)	1600	1500	0.9
THA	80.6	73.0	0.9

*striatum, **plasma, ***heart.

the membrane-anchored G4 form (27, 28), in the presence of a ChEI, ACh concentrations could reach μM levels which are inhibitory for AChE activity. This increase may trigger glial BuChE to hydrolyze ACh, resulting in compensation of the loss of neuronal AChE activity. Due to the close spatial relationship between the glial cell protoplasm and synaptic gap, it is likely that extracellularly diffusing ACh could come in contact with glial BuChE and be effectively hydrolyzed as demonstrated by our experiments (55).

To study the function of glial BuChE and in particular its effect on the regulation of ACh extracellular concentrations, we perfused intracortically in the rat the highly BuChE selective inhibitor MF-8622 (Table 13) at concentrations varying between 15 and 170 μM (55). We measured simultaneously extracellular ACh levels (55) without using a second ChEI by means of a sensitive microdialysis method (56). With the highest MF 8622 concentration (170 μM), ACh level increased 15-fold (from 5 nM baseline value to 75 nM). This implies that ACh concentration in and around cholinergic synapses in the cortex might have increased from nM to low μM levels (56). These values approach inhibitory concentrations for AChE (57) which may affect glial BuChE activity. For comparison, we tested eptastigmine, a non-selective ChEI, under similar conditions and found that at 15- μM concentration, it elevated ACh 20-fold (from 5 nM baseline value to 100 nM). Thus, the dose-dependent steady-state elevation of cortical ACh seen with the BuChE selective inhibitor MF 8622 is comparable in magnitude with the effect of a mixed AChE-BuChE inhibitor such as eptastigmine. However, to reach the same ACh elevation a tenfold higher concentration of MF 8622 is necessary (170 μM). This suggests that, as expected, under normal conditions AChE inhibition is more efficient than BuChE inhibition in elevating cortical ACh. In spite of the high ACh eleva-

tion in brain seen after the BuChE inhibitor, the animals perfused with MF 8622 did not show cholinergic side effects. These results represent the first study in which a selective BuChE inhibitor has been shown to significantly elevate extracellular levels of cortical ACh without producing side effects in the animal. The results of this experiment support the concept of two pools of functional ChE in rat brain, one neuronal (AChE) acting mainly under normal conditions and one glial (BuChE) acting under conditions of decreased AChE activity. The two pools show different kinetic properties with regard to regulation of brain ACh.

8. Is inhibition of butyrylcholinesterase clinically important?

From a clinical point of view, this mechanism implies that a selective BuChE inhibitor may produce significant increases of brain ACh without producing peripheral or central cholinergic side effects. From the experimental point of view, a wide range of inhibition (measured in rat brain and plasma) of the two types of ChEI is seen for various ChEIs (Table 12). As an example, the BuChE/AChE ratio of inhibition in rat brain is above 1000 for E-2020 and Huperzine A, but less than 1 for tacrine and metrifonate. Several other inhibitors such as physo, galanthamine and ENA-713 show intermediate values of this ratio.

Particularly interesting from the clinical point of view is the relative rate of inhibition shown by several ChEIs toward human erythrocytes (AChE) and plasma (BuChE), which are reported in Table 13. We can observe that most inhibitors presently utilized for AD therapy are not selective for AChE; however, they all show various degrees of clinical efficacy. Considering the drastic decrease in AChE activity taking place in the brain of AD

Table 13. In vitro activity of ChEI in humans (IC_{50} , nM)

Compound	AChE*	BuChE**	BuChE/AChE
BW 284 C51	18.8	48.000	2.553
Phenserine	22.2	1552	70
Galanthamine	0.35	18.6	53
DDVP (Metrifonate metab.)	800	18.000	22.5
Physostigmine	5.4	35	6.5
THA	190	47	0.25
Eptastigmine	20	5	0.25
Hetopropazine	260.000	300	0.001
Bambuterol	30.000	3	0.001
MF 8622	100.000	9	0.00009

*human erythrocytes, **human plasma.

patients (reaching 5% AChE levels at autopsy in some regions) and the large pool of BuChE still available both in glia and neurons, it may not constitute an advantage for a ChEI to be selective for AChE. On the contrary, a good balance between AChE and BuChE inhibition should result in higher therapeutic efficacy.

The present knowledge of the molecular configuration of the two enzymes would allow researchers to design compounds possessing well-balanced AChE-BuChE specificity, high CNS penetration and low peripheral and central cholinergic toxicity. Some of the second generation ChEI have demonstrated some of the advantages of these characteristics.

9. Cholinesterase inhibition as a possible mechanism to slow down deterioration

The beta-A4, one of the major constituent proteins of neuritic plaques in the brain of AD patients, originates from a larger polypeptide denominated APP (58). APP is widely distributed throughout the mammalian brain including rat brain with a prevalent neuronal localization (59). APP can be processed by several alternative pathways. A secretory pathway is believed to generate non-amyloidogenic soluble derivatives (APPs) following cleavage within the beta-A4 segment (60). Cholinergic agonists regulating processing and secretion of APPs by increasing, as demonstrated *in vitro*, protein kinase C (PKC) activity of target cells (61–64) could decrease potentially amyloidogenic derivatives. We suggested that long-term inhibition of ChE via increasing levels of synaptic ACh may result in activation of normal APP processing in AD brain (38). This effect could slow down the formation of amyloidogenic APP fragments. Lahiri et al. (65) using nerve cell cultures found that the level of secretion of APP derivatives into conditioned media were inhibited by treating them with 100 µg/ml tacrine (Table 14). Chong and Suh (69) found a dose-dependent effect of

tacrine on APP processing (Table 14). Tacrine at low concentrations (0.02–0.5 mM) enhanced whereas concentrations above 0.5 mM blocked APP 770 processing *in vitro* (Table 14). Haroutunian et al. (17) reported that one-week treatment with the ChEI phenserine normalized the levels of secreted beta-APP in the CSF of forebrain cholinergic lesioned rats, suggesting that secretion of beta-APP into the CSF and neurons can be modulated by ChEI (Table 1). To determine whether ChEI could alter the release of APP in the brain, we used superfused cortical slices of the rat (67) following the method described by Nitsch et al. (68). Both short- and long-acting ChEI were tested for their ability to enhance the release of non-amyloidogenic soluble derivatives of APP (67). These included physo, heptastigmine and DDVP (dichlorvos, a metabolite of metrifonate) at concentrations producing ChE brain inhibitions ranging from 5% to 95%. All three ChEIs elevated APPs release significantly above control levels (Table 15).

Using two different doses of tacrine (0.5 and 0.1 µM), we found that only the lower dose elevated the release of APPs in the cortical slices, which supports the data of Chong and Suh (66) of a dose-dependent modulation of APP secretion by AChE inhibition (Table 14). In our study, electrical field stimulation significantly increased the release of APPs within 50 min. A similar increase was observed after muscarinic receptor stimulation with bethanechol, supporting the results from *in vitro* experiments (63). Tetrodotoxin completely blocked the effect of electrical stimulation (67).

The level of total APP mRNAs in rat cortical slices did not change after incubation with bethanechol, DDVP and physo, but activation of PKC with phorbol 12-myristate-13-acetate (100 nM) increased the level of total APP mRNA by 50% (Table 15) (70). Physo and DDVP administration (0.3 mg/kg and 80 mg/kg s.c., respectively) for 3–48 hr did not significantly change the levels of APP 695 and APP-KPI (Kunitz-type) protease inhibitor

Table 14. Effect of ChEI on beta-amyloid processing *in vitro* and *in vivo*

Drug	Ref.	Conc. (mM)	APP accumulation	APP secretion	Preparation
Tacrine	(66)	500	—	+	APP 770
Tacrine	(66)	< 500	+	—	APP 770
Tacrine	(65)	100 mg/ml	+	—	Cell lines
Phenserine	(17)	2.5 mg/kg	=	—	Rat CSF
Tacrine	(67, 87)	0.5	+	—	Rat cortical slices
Tacrine	(67, 87)	0.1	—	+	Rat cortical slices
Physostigmine	(67, 87)	0.1	—	+	Rat cortical slices
Eptastigmine	(67, 87)	0.1	—	+	Rat cortical slices
DDVP (Metrifonate metab.)	(67, 87)	0.02	—	+	Rat cortical slices

Table 15. Drug-stimulated changes of basal APPs release and APP-KPI mRNA from rat brain (67)

Drug	Conc. (μ M)	APPs increase (% of basal)	ChE activity (% Inhib.)	APP-KPI mRNA (% of basal)
Bethanechol	1	48	0	—
	100	53	0	—
Physostigmine	0.1	48	25	—
Heptyl-physostigmine	0.1	41	61	−35*
Dichlorvos	0.02	33	95	—
Phorbol myristate	0.1	—	—	+50

*5 mg/kg, s.c., 48 hr (70).

mRNAs (Table 15). Heptastigmine administration (5 mg/kg, s.c., 3–48 hr) decreased by 35% the level of APP-KPI mRNA in rat cerebral cortex (70). AD pathology has been associated with an increase of the KPI-containing forms of APP and the propensity across species to develop neuritic plaques in cortical regions (71). Our findings suggest that administration of ChEI to AD patients by increasing secretion of APP and inhibiting formation of specific APP mRNAs may exert a neuro-protective effect, activating normal APP processing through a muscarinic mechanism and decreasing amyloid deposition in brain cells.

This effect of ChEI on APP processing could be reflected clinically by slowing down cognitive deterioration as suggested by clinical data of short- and long-term treatments discussed in previous sections of this paper. In order to understand the mechanism of action of ChEI on APP processing, it is important to consider in addition to the muscarinic mechanism mentioned above, some new data related to specific properties of human AChE as summarized in Table 16.

Inestrosa et al. (72) demonstrated that recombinant human AChE accelerates beta-A4 formation from wild-type beta-A4. This effect is not shared by BuChE. Thus, in vitro data suggest that AChE could play a role in beta-amyloid deposition. It is also interesting to note that this effect is blocked by ChEIs binding to the peripheral anionic binding site of AChE. This could represent an interesting property for future development of peripheral-site blocker ChEIs.

Table 16. Effect of human AChE on APP processing

1. AChE (but not BuChE) promotes beta-A4 formation (72).
2. This effect is blocked by ChEIs binding to the peripheral anionic binding site of AChE (72).
3. AChE accumulates within senile plaques and has non-intrinsic proteolytic activity on APP-770 in vitro (74).
4. AChE and its associated protease promotes amyloidogenic processing of APP-770 (69).

It has been suggested that the proteolytic activity associated with AChE could play a role in APP processing acting as an alpha-secretase and participating in the non-amyloidogenic cleavage of APP (73). According to the recent work of Funes (74), only the AChE derived from the brain of Alzheimer patients has such a proteolytic activity. Inhibition of the esteratic activity, however, does affect the proteolytic activity of AChE as well as amyloidogenic cleavage of APP (75). These results do not suggest a protective effect of ChEI per se in AD related to inhibition of AChE-associated proteases.

10. Is there a future for cholinesterase inhibitors in Alzheimer disease therapy?

Cholinesterase inhibitors are the only drugs presently demonstrating efficacy in AD treatment. We are beginning to discern advantages as well as therapeutical limitations of these drugs. We should also consider new possibilities for a wider clinical application and possible improvement of the cholinergic approach. As previously discussed, selectivity for AChE does not seem to be a prerequisite for clinical efficacy. On the contrary, it would be interesting to test a selective BuChE inhibitor in view of possible interactions with beta-A4 accumulation, plaque formation (47) and lack of side effects. The implication of a proteolytic activity directly associated with AChE is still controversial. Recent evidence implies that a classic ChEI would not be effective on APP metabolism unless associated in its molecule with a selective protease inhibitor function. This would be possible with a bifunctional compound. Another interesting concept is the combination of two cholinergic functions in the same molecule, such as a ChEI with muscarinic (M1 or M3) agonist or antagonist (M2) properties or nicotinic agonist (13).

Analysis of the outcome of several clinical trials poses new questions and outline new research goals: 1. How to improve the effect on cognitive tests (ADAS-cog) beyond the magic “four-five point and 0.5 CIBIC level”. 2. How

to improve drug delivery and dosage to avoid or delay "wearing off" in long-term treatment. 3. To understand why some ChEI show symptomatic improvement and others seem to maintain patient conditions at baseline level? Understanding this difference in pharmacological effect between drugs may be the key to develop new and more efficacious drugs. 4. To understand the relationship between cognitive improvement and ChE inhibition and whether there are major differences between drugs in this respect. This is not an "academic" question as argued by some clinicians.

11. Conclusions: The future of Alzheimer disease therapy

Cholinesterase inhibitors are presently the drugs of choice for AD. In less than ten years, starting from non-specific first generation drugs, they have been developed into a second generation of more selective molecules. The latest data from clinical trials suggest that optimization and maintenance of clinical effects for one year or more is possible, and this will depend on our knowledge of the pharmacodynamic effects of long-term treatment. Cholinesterase inhibitors, particularly second generation, (post-physo and post-tacrine compounds), affect cortical as well as sub-cortical neurotransmitters other than ACh. Their effects on NE and DA are of particular clinical interest. A newly demonstrated feature of ChEI, is their ability to enhance the release of non-amyloidogenic soluble derivatives of APPs *in vitro* and *in vivo* and possibly to slow down formation of amyloidogenic compounds in the brain. This process might also slow down cognitive deterioration of the patient as indicated by the analysis of recent clinical data. A critical question is how long the effect of ChEI will be clinically significant. Clinical trials extending beyond 36 month duration should be able to demonstrate whether or not the pharmacological effect on APP metabolism is of significance. Cholinomimetic alternatives other than ChE inhibition are being explored pharmacologically and clinically. Drugs most investigated are those showing direct stimulation of postsynaptic M1 and M3 muscarinic receptors. This line of therapy has not produced convincing results so far, but may be more promising as a combination therapy.

Depending on the success of ChEI, one can see potential indications for applications to different stages of AD such as: a) preclinical presymptomatic stages in at risk individuals with MCI (minimal cognitive impairment); b) early AD patients with manifested symptoms (clinical dementia rating, CDR: 0.5–1), presently the most treated group; c) late AD (CDR2) patients with behavioral symptoms. Combination of ChEI with muscarinic or nicotinic agonists or with beta-A4 processers or APP-releasers (Figs. 1, 2 and 3) represent interesting alternatives in case

of tolerance to ChEI monotherapy.

Acquisition of future clinical knowledge may provide other indications to expand the use of ChEIs in other areas of dementia such as: Vascular Dementia (multiple infarct dementia, MID), Lewy body disease Dementia, Parkinson Dementia, Frontal lobe Dementia and Down syndrome Dementia. For some of these applications, it will be necessary to demonstrate that the ChEI does not promote extrapyramidal side-effects (see tacrine) and that it has cognitive effects as powerful as those demonstrated for AD.

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