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CHOLINESTERASE INHIBITION BY DERIVATIVES OF 2-AMINO-4,6-DIMETHYLPYRIDINE

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Derivatives of 2-amino-4,6-dimethylpyridine, aryl(alkyl)carboxamides, thiocarbamides and amidrazones, already known for their anti-inflammatory properties, were found to be moderately active inhibitors of acetyl and butyrylcholinesterase. Quantitative structure-activity relationships showed that the binding affinity was enhanced by the following structural modifications: (1) increase in molecular volume, (2) decrease in the energy of the lowest unoccupied molecular orbital, (3) insertion of a methylene group between the amide carbonyl and the aromatic ring, (4) replacement of the amide oxygen by sulfur. The affinity remained, however, weaker than that of the specific inhibitor 9-amino-1,2,3,4-tetrahydroacridine (tacrine). The association of anti-inflammatory and cholinesterase inhibiting activities within the same compound may prove useful for the treatment of Alzheimer's disease.

Keywords: 2-amino-4,6-dimethylpyridine; cholinesterase; inhibition; Alzheimer's disease; anti-inflammatory; QSAR.

INTRODUCTION

Derivatives of 2-amino-4,6-dimethylpyridine exhibit interesting anti-inflammatory properties.[1–5] It seemed useful to investigate the cholinesterase inhibiting potency of these compounds, for at least two reasons: (1) inhibition of serum cholinesterase may potentiate the action of associated drugs, such as aspirin, which are hydrolysed by this enzyme[6], (2) anti-inflammatory drugs, as well as cholinesterase inhibitors, are considered useful in the management of Alzheimer's disease[7] and it is therefore of interest to seek compounds having both activities. In this work we have studied the

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binding of some amides, thioamides and amidrazones derived from 2-amino-4,6-dimethylpyridine to purified forms of horse serum butyrylcholinesterase (BuChE, EC 3.1.1.8) and electric eel acetylcholinesterase (AChE, EC 3.1.1.7).

MATERIALS AND METHODS

Chemistry

Synthesis

Amides **1–6**, **16**, **17** and **18** were prepared by reacting the corresponding acid chloride with 2-amino-4,6-dimethylpyridine in the presence of triethylamine (method A) in excellent yields (70–85%.)[1–3]

In the heterocyclic series, activation of the acid by formation of a salt of acyloxyphosphonium using the couple Ph_3P/CCl_4, or $Ph_3P/CBrCl_3$ constituted a mild method (B) affording satisfactory yields (~60%) of amides **7–15**.[4] 3-Thienylacetamide **19** was obtained in a 59% yield by this method and the yield increased to 69% by recourse to imidazolide formation (method C), and up to 90% when using 2-chloro-1-methylpyridinium iodide to activate the acid (method D).

Sulfur/oxygen exchange of amides **1** and **9** into thioamides **20**[4] and **21** was carried out with phosphorous pentasulfide in pyridine (method E); yields were moderate being 60 and 26%, respectively. Recourse to Lawesson's reagent in HMPA, heated to 90–95°C (method F) afforded only 19% of **20**[4] and failed with the carboxamide **9**.

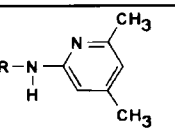
Amidrazones **22** and **23** were obtained in satisfactory yields, 81 and 71% respectively[5], by reaction of the corresponding thioamides with hydrazine hydrate, at room temperature (method G).

The chemical structures[1–5] of the previously synthesised compounds **1–18**, **20**, **22** and **23** are shown in Table I. Physicochemical data for acetamide **19** and thiocarboxamide **21** are described in the Experimental.

Experimental

Melting points were determined on a Tottoli-Büchi apparatus without correction. IR spectra were run using KBr pellets on a Beckman IR4230 infrared spectrophotometer. ^1H NMR spectra were recorded on a Bruker AC 250 spectrometer (250 MHz) with $CDCl_3$ as solvent. Chemical shifts (δ) are reported in parts per million (ppm) downfield from tetramethylsilane as the internal standard. Microanalyses were performed on a Perkin-Elmer CHN240 apparatus. Analyses, indicated by the symbols of the elements, were within ±0.40% of theoretical values.

TABLE 1 Chemical structures of *N*-(4,6-dimethylpyridine-2-yl)aryl(alkyl) carboxamides (**1–19**), thiocarbamides (**20**, **21**) and amidrazones (**22**, **23**).



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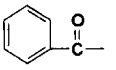
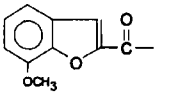
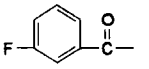
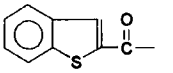
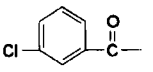
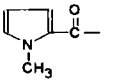
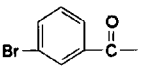
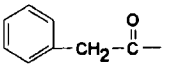
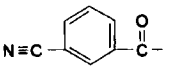
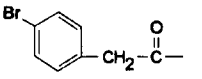
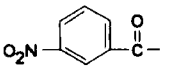
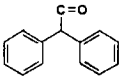
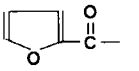
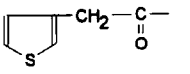
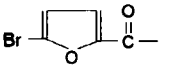
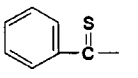
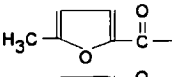
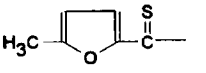
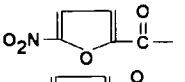
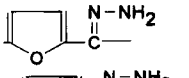
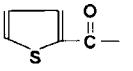
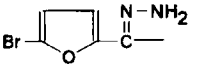
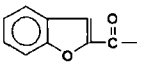
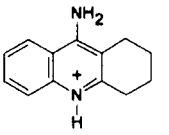
	1		13
	2		14
	3		15
	4		16
	5		17
	6		18
	7		19
	8		20
	9		21
	10		22
	11		23
	12		
	Tacrine		

Method A:

See Reference 1 for amides **1**, **3**, **4** and **6**, Reference 2 for amides **16** and Reference 3 for amides **2**, **5**, **17** and **18**.

Method B: N-(4,6-dimethylpyridin-2-yl)3-thienylacetamide 19

Triphenylphosphine (7.4 g, 28.1 mmol), bromotrichloromethane (5.55 ml, 56.2 mmol), 3-thienylacetic acid (4 g, 28.1 mmol) and 2-amino-4,6-dimethylpyridine (6.9 g, 56.2 mmol) were dissolved in 80 ml of THF and the solution heated under reflux for 3 h. After cooling, the 2-aminolutidine halohydrate formed was filtered off, the solvent evaporated and the residue purified by column chromatography with CH_2Cl_2 as eluent. Recrystallization from diisopropyl ether afforded **19** as white crystals (4 g yield 59%), mp 133–134°C. (Found: C, 63.27; H, 5.76; N, 11.34. $C_{13}H_{14}N_2OS$ requires C, 63.40; H, 5.73; N, 11.38%). IR(KBr) cm^{-1}: 3235 (νNH); 1670 (νC=O); 1620 (νCN); 1580 (δNH); ^1H NMR ($CDCl_3$) δ ppm: 2.30 (s, 3H, 4-CH_3); 2.36 (s, 3H, 6-CH_3); 3.74 (s, 3H, CH_2); 6.72 (s, 1H, pyr. H^5), 7.06 (dd, 1H, $J_{H^5H^4}$ = 4.9 Hz, $J_{H^2H^4}$ = 1.2 Hz, H^4); 7.20 (dd, 1H, $J_{H^5H^2}$ = 3Hz, H^2); 7.35 (dd, 1H, H^5).

Method C: 19

3-Thienylacetic acid (2.15 g, 15 mmol) was placed into THF (20 ml) and CDI (2.45 g, 15 mmol) was progressively added. The reaction mixture was stirred for 1 h at room temperature and 2-amino-4,6-dimethylpyridine (1.85 g, 15 mmol) was added. Stirring was maintained overnight. The imidazole formed was filtered off and the solvent was evaporated under reduced pressure. The crude amide **19** was purified using column chromatography and eluting with CH_2Cl_2, progressively enriched in ethanol (up to 2%). Yield: 69%.

Method D: 19

3-Thienylacetic acid (4 g, 28.1 mmol) was dissolved in anhydrous CH_2Cl_2 (70 ml) and 2-amino-4,6-dimethylpyridine (3.4 g, 28 mmol), 2-chloro-1-methylpyridinium iodide (7.2 g, 28 mmol) and triethylamine (8.8 ml, 70.3 mmol) successively added. The reaction mixture was maintained under reflux for 2 h. The cooled solution was washed with water (20 ml) and the organic phase was dried (Na_2SO_4). The solvent was evaporated under reduced pressure and the crude amide left was purified by column chromatography and recrystallized from diisopropyl ether. Yield: 90%.

Method E: N-(4,6-Dimethylpyridin-2-yl)-5-methylfuran-2-thiocarboxamide 21

N-(4,6-Dimethylpyridin-2-yl)-5-methylfuran-2-carboxamide **9** (6.9 g, 30 mmol) was dissolved in anhydrous pyridine (60 ml) and the solution was then heated to 100°C. P_4S_{10} (10.2 g, 23 mmol) was added and the mixture was refluxed for 80 min. and then poured into ice-water (300 ml) and stirred vigorously for 20 min. The precipitate formed was separated by filtration and dried. This crude product, purified by column chromatography gave pure yellow thiocarboxamide **21** (1.9 g, yield 26%) mp: 89°C (CH_2Cl_2). (Found: C, 63.58; H, 5.79; N, 11.41. $C_{13}H_{14}N_2OS$ requires C, 63.40; H, 5.73; N, 11.38%). IR (KBr) cm^{-1}: 3350 (νNH); 1620 (νC=N); 1530 (ν_{as}NC=S); 1345 (combined NC/CS); 1040 (ν_sNCS). ^{1}H-NMR ($CDCl_3$) δ ppm: 2.36 (s, 6H, 5-CH_3 and 4-CH_3); 2.46 (s, 3H, 6-CH_3); 6.14 (d, 1H, J=3.6 Hz, H^4); 6.83 (s, 1H, pyr H^5); 7.37 (d, 1H, H^3); 8.83 (s, 1H, pyr H^3); 9.89 (s, 1H, NH).

pK_a Determination

The pK_a of these compounds was determined by spectrophotometry in buffer solutions (citrate 0.01 M/K_2HPO_4 0.02 M for pH 3.0–7.4; Citrate 0.02 M/NaOH 0.02 M for pH 2.6–5.1). Stock solutions of each compound, at about 6.5×10^{-3} M, were prepared in methanol. They were diluted, first in methanol then in buffer, so that their final concentration was about $5 \times 10^{-6} - 1 \times 10^{-5}$ M, in 4% methanol. The spectrum of each solution was recorded on a Shimadzu UV-1205 spectrophotometer with the solvent mixture (buffer + methanol) in the reference cell. All measurements were carried out at 25°C in thermostated cells. Twelve spectra were recorded for each compound, using the pH range 2.6–5.1 for the least basic compound (nitrofurancarboxamide, **10**) and the pH range 3.0–7.4 for the other compounds (Table II). Absorbance values were recorded at nanometer intervals from 240–600 nm and transferred to a microcomputer by means of a RS-232 interface. The pK_a was determined as described previously, using our own program.[8] Tacrine (9-amino-1,2,3,4-tetrahydroacridine hydrochloride) was a kind gift drom Parke-Davis laboratories (Ann Arbor, Michigan, USA). Its pK_a, 9.8, was taken from the literature.[9]

Enzyme Inhibition

All reagents were from Sigma. The enzymes were acetylcholinesterase from electric eel (specific activity: 1270 U/mg) and butyrylcholinesterase from horse serum (specific activity: 580 U/mg). The enzymatic hydrolysis of acetylthiocholine and butyrylthiocholine was measured at 37°C by a modified Ellman's method[10], using dithiobis-2-nitrobenzoate (DTNB) as chromogenic agent. The reaction

TABLE II Physico-chemical properties and cholinesterase-inhibiting activities of lutidine derivatives and tacrine. E_{HOMO}, E_{LUMO}: Orbital energies (eV); μ: Dipole moment (D); V: Molecular volume (Å^3); K_i, K_i': Competitive and uncompetitive inhibition constants (mol/L).

Compound	E_{HOMO}	E_{LUMO}	μ	V/100	log P	pK_a	pK_i (AChE)	pK_i' (AChE)	pK_i (BuChE)	pK_i' (BuChE)
1	–8.90	–0.27	1.62	7.33	3.78	4.43	3.21	3.28	3.38	3.40
2	–9.04	–0.55	0.80	7.40	3.42	4.26	3.94		3.82	
3	–9.02	–0.53	0.74	7.74	4.30	4.20	3.66	3.13	3.41	3.55
4	–9.01	–0.57	0.71	7.95	4.58	4.25	3.99		3.82	3.04
5	–9.04	–0.87	1.85	7.90	3.82	4.17	4.06		3.54	
6	–9.21	–1.36	3.94	7.93	3.74	4.05	4.09	4.41		
7	–9.00	–0.39	1.39	6.85	2.73	4.29	3.36	2.94	3.14	2.80
8	–9.08	–0.66	0.47	7.51	3.17	4.19	3.92	3.60	4.46	
9	–8.94	–0.33	1.99	7.42	2.75	3.78	3.58	3.92	3.72	3.27
10	–9.25	–1.70	3.84	7.50	2.63	4.43	4.26			4.34
11	–8.90	–0.68	1.83	7.03	3.08	4.33	3.06	3.17	3.66	3.48
12	–8.93	–0.79	1.35	8.15	3.50	4.10	4.13		4.37	
13	–8.93	–0.82	0.57	8.82	3.25	4.00	4.27		4.40	
14	–8.70	–0.66	1.76	8.33	3.84	4.08	4.36	3.89	4.36	
15	–8.93	0.02	0.85	7.42	2.80	4.93	3.72		3.49	3.64
16	–8.96	0.06	1.86	7.86	3.72	4.55	3.89		3.85	
17	–9.02	–0.30	1.35	8.48	4.51	4.35	4.39		5.06	
18	–8.86	0.07	1.60	9.82	5.50	4.22	3.92		4.77	
19	–8.92	0.00	1.36	7.64	3.60	4.41	3.89			2.88
20	–8.42	–0.80	2.49	7.60	4.43	3.54	5.10	5.49	5.01	4.19
21	–8.53	–1.05	2.57	7.57	3.40	3.61	5.14		4.15	4.17
22	–8.47	–0.11	2.81	7.13	2.89	5.55	3.70		4.87	5.22
23	–8.59	–0.35	3.43	7.99	3.32	5.54	3.68	3.48	4.05	3.89
Tacrine	–12.66	–4.81	0.96	6.36	1.78	9.80(a)	7.21 7.40(b) 7.15(c)	7.25 6.89(b)	8.04 7.77(b) 7.52(c)	8.32 7.33(b)

(a) Ref. 9. (b) Ref. 16. (c) Ref. 17.

mixture contained 2 ml of a solution of DTNB (0.4 mM) in phosphate buffer (0.05 M, pH 7.5), 0.1 ml of a solution of inhibitor in methanol, and 0.2 ml of an enzyme solution (about 1 U/ml). This mixture was incubated at 37°C for 2 min and the reaction was started by adding 0.2 ml of a substrate solution. The final concentrations were: methanol 4%, acetylthiocholine 0.02–0.2 mM, butyrylthiocholine 0.05–0.25 mM. The progress curve of the reaction was followed by monitoring the absorbance increase ar 412 nm, due to the liberation of the thionitrobenzoate anion (ε = 13600 L.mol^{-1}cm^{-1}), in a Shimadzu UV-1205 spectrophotometer. Absorbance values were recorded every 2 s during 1 min and transferred to a microcomputer. Initial rates were computed by fitting a third-order

polynomial to each progress curve. The competitive (K_i) and uncompetitive (K_i') inhibition constants were determined by unweighted nonlinear regression, using our own program. Only the inhibition constants significant at the 5% level were retained.

Molecular Modeling

The compounds were modeled with the HyperChem software[11], using the AM1 semi-empirical molecular orbital method with geometry optimization. The starting geometry of the pyridine derivatives was fixed close to that of the crystal structure of the compounds[12], and, in agreement with their low pK_a values (see Results), only the neutral form was considered. Tacrine, however, exhibited a much higher pK_a and was therefore modeled as the protonated form. The protonation site was considered to be the acridine nitrogen since only this form yielded a stable structure on modeling. For all compounds, the following properties were computed on the refined structure:

- Dipole moment (μ)
- Van der Waals volume (V).
- Energies of highest occupied (HOMO) and lowest unoccupied (LUMO) molecular orbitals.
- Octanol/water partition coefficient (log P), a common lipophilicity parameter, was computed within HyperChem by summation of atomic contributions; hence, the given value is only a rough approximation.

Quantitative Structure-activity Relationships (QSAR)

QSAR studies were performed by linear regression with automatic selection of the most significant variables, using the S-plus software.[13] In addition to the above-mentioned electronic properties and pK_a, indicator variables were used to account for the presence of a CH_2 group between the amide function and the aromatic ring, and for the replacement of the amide group by thioamide (C=S) or amidrazone (C=N-NH_2). Only those variables with regression coefficients significant at the 5% level were retained in the final equations.

RESULTS

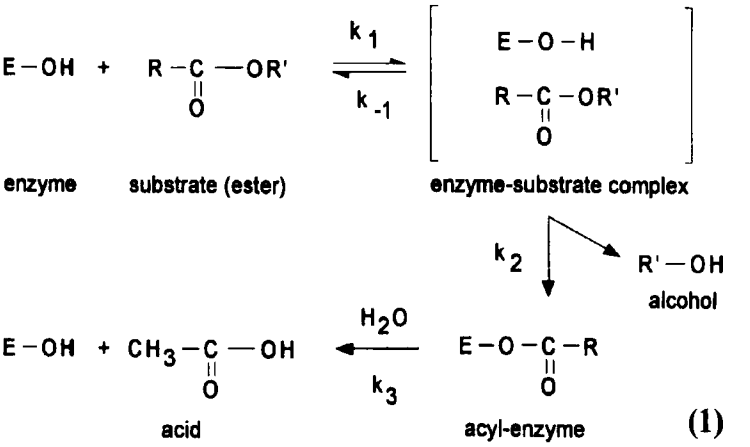
pK_a Determination

The physicochemical properties of the compounds are summarized in Table II (a detailed analysis of the influence of structure upon these properties will be

published elsewhere). All pyridine derivatives were weakly basic. While the enzyme inhibition experiments were carried out at 37°C, the basicities were evaluated at 25°C in order to minimize the risk of decomposition at the most extreme pH values. Since the pK_a's are much lower than the pH of the inhibition mixture (pH 7.5), the concentration of the protonated species may be neglected so that only inhibition by the neutral forms of the compounds needs to be considered. The pK_a of tacrine, however, was much higher than the pH of the inhibition mixture, so that inhibition by the protonated form of the compound must be considered.

Enzyme Inhibition

It is well known that the hydrolysis of an ester catalysed by a cholinesterase proceeds through an acyl-enzyme intermediate which reacts with water to regenerate the enzyme[14]:



The competitive inhibition constant (K_i) is the dissociation constant of the enzyme-inhibitor complex. It has been shown that for the hydrolysis of choline esters that $k_2 \gg k_3$, i.e. deacylation is rate-limiting, so that the enzyme-substrate complex does not accumulate. Hence, the uncompetitive inhibition constant (K_i') is usually regarded as reflecting the binding of the inhibitor to the acyl-enzyme only.[15]

Statistically significant K_i values were found for all pyridine derivatives with AChE and for 20 (out of 23) of the derivatives with BuChE; statistically significant K_i', values were found for about half of the compounds. These inhibition constants

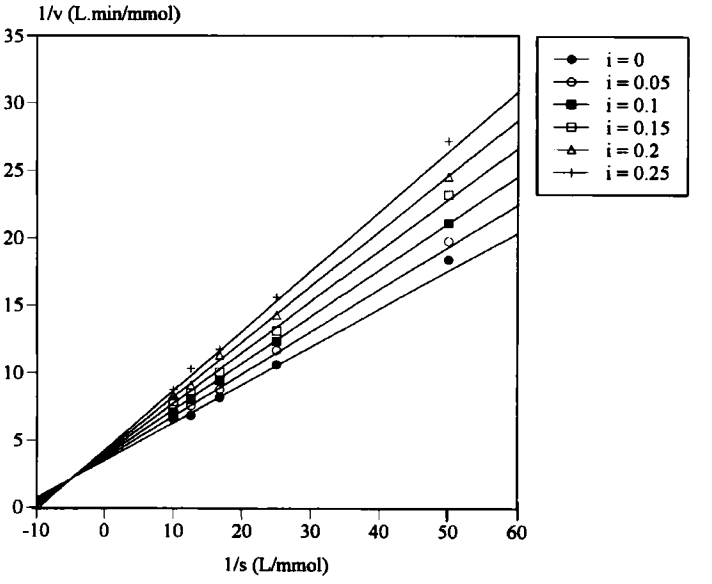


FIGURE 1 Lineweaver-Burk plot for inhibition of acetylcholinesterase by compound **7**. s = initial substrate concentration; v = initial reaction rate; the inhibitor concentrations in mmol/L are shown in the inset.

are presented in Table II, where they are expressed as $pK_i = -\log K_i$ and $pK_i' = -\log K_i'$, where K_i and K_i' are the dissociation constants of the complexes, expressed in mol/L (this convention facilitates the comparison of compounds since pK_i and pK_i' increase when the inhibition increases). The absence of a particular inhibition constant in the table does not mean that the corresponding binding step does not exist, but merely that it could not be characterized with sufficient precision in our eperimental conditions due to, for example, poor solubility or stability of the inhibitor. A representative Lineweaver Burk plot for inhibition of AChE is shown in Figure 1.

The data in Table II allow us to discuss the influence of structure on the inhibition potency of the compounds (in what follows, the pK_i of the example compounds are shown in brackets, the first set being relative to AChE and the second set to BuChE):

(1) Benzamide derivatives of 2-amino-4,6-dimethylpyridine were moderately active inhibitors of both cholinesterases, mostly of the mixed type, although in some cases the difficulty, in estimating one of the inhibition constants

could result in an apparent competitive (e.g. **2**) or uncompetitive (**10** and **19** with BuChE) inhibitor.

(2) Replacement of the phenyl ring by a 5-membered heterocycle had little influence on the binding affinity (**1/7/11** [3.21 / 3.36 / 3.06] [3.38 / 3.14 / 3.66]).

(3) Substitution of the phenyl or heterocyclic ring by a lipophilic or electron-withdrawing substituent enhanced the binding affinity (e.g. **1/5** [3.21 / 4.06] [3.38 / 3.54], **7/8** [3.36 / 3.92] [3.14 / 4.46]).

(4) Annelation of the heterocycle by a phenyl ring resulted in a marked increase in affinity (**7/12** [3.36 / 4.13] [3.14 / 4.37], **11/14** [3.06 / 4.36] [3.66 / 4.36]).

(5) Intercalation of a methylene group between the amide group and the aromatic ring also enhanced the affinity (**1/16** [3.21 / 3.89] [3.38 / 3.85], **11/19** [3.06 / 3.89]).

(6) Replacement of the amide group by thioamide increased the binding affinity, mainly for AChE (**1/20** [3.21 / 5.10] [3.38 / 5.01]). Indeed, these thioamide derivatives were the most potent AChE inhibitors in the series.

(7) Replacement of the amide group by amidrazone had little effect on AChE inhibition but increased the affinity for BuChE (**7/22** [3.36 / 3.70] [3.14 / 4.87], **8/23** [3.92 / 3.68] [4.46 / 4.05]).

Tacrine was found to be a mixed inhibitor of both AChE and BuChE. The inhibition constants reported here were in agreement with the previous results of Radic *et al.*[16] (using mouse cholinesterases) and Chelliah *et al.*[17] (using the same cholinesterases as here).

Quantitative Structure-activity Relationships

Statistically significant relationships were observed for AChE:

$$\begin{aligned} pK_i &= (0.31 \pm 0.08)V/100 - (0.44 \pm 0.12)E_{\mathrm{LUMO}} + (0.39 \pm 0.16)I_{\mathrm{CH_2}} \\ &\quad + (1.21 \pm 0.18)I_{CS} + 1.13 \\ &\quad n = 23,\ r = 0.91,\ s = 0.23,\ F_{4,18} = 21.4 \\ pK_1' &= -(0.92 \pm 0.41)E_{\mathrm{LUMO}} + (1.75 \pm 0.41)I_{CS} + 3.00 \\ &\quad n = 10,\ r = 0.90,\ s = 0.38,\ F_{2,7} = 14.3 \end{aligned} \tag{2}$$

where V is the molecular volume (Å^3), E_{LUMO} is the energy of the lowest unoccupied molecular orbital (eV), $I_{\mathrm{CH_2}}$ and I_{CS} are indicator variables denoting the presence or absence of the corresponding group, n is the number of compounds,

r the correlation coefficient, s the residual standard deviation and F the ratio of explained variance to residual variance.

Binding to the free enzyme (pK_i) was influenced by the molecular volume and the electron-accepting ability of the compound (characterized by $-E_{LUMO}$). This result explained some of our previous qualitative observations:

(1) Replacement of the phenyl ring by a 5-membered heterocycle increased the electron-accepting ability but reduced the molecular volume, thus showing little influence on the binding affinity.
(2) Substitution of the phenyl or heterocyclic ring, as well as annelation of the heterocycle, enhanced the binding affinity by increasing both molecular volume and electron-accepting ability.

Two additional factors were found to strengthen the inhibition independently of the previously noted volumic or electronic effects:

(1) The presence of a methylene group between the amide group and the aromatic ring enhanced the affinity, maybe by increasing the flexibility of the molecule, thus facilitating the transition to a more tightly bound conformation. This phenomenon, however, occured at the expense of a decrease of the electron-accepting ability, probably due to an impairment of the electronic delocalization between the phenyl ring and the amide group.
(2) The replacement of the amide oxygen by sulfur increased the affinity. This may be due to a local increase in lipophilicity, suggesting that this part of the molecule interacts with a lipophilic amino acid of the enzyme.

The absence of the lipophilicity parameter (log P) in the structure-activity relationship may seem surprising but it must be noted that, (1) the precision of the estimation of log P from atomic contributions may be poor, and (2) a great part of the lipophilicity of the inhibitor is already accounted for by its molecular volume.[18]

Binding to the acyl-enzyme of AChE (pK_i') seemed to depend on the same factors although the influence of V and I_{CH_2} was not found statistically significant. This may be due, however, to the small number of compounds for which pK_i' could be measured.

Tacrine had a much lower value of E_{LUMO} than the pyridine derivatives, due to the presence of a positive charge. This may explain its higher affinity for AChE. However, trying to extrapolate the observed structure-activity relationship to tacrine resulted in an underestimation of its binding affinity by two orders of magnitude (estimated $pK_i = 5.22$ instead of 7.21). This result suggests that tacrine and the pyridine derivatives have different binding sites.

The statistical significance of the relationships for BuChE was less good:

$$pK_i = (0.50 \pm 0.15)V/100 + (0.77 \pm 0.33)I_{CS} + (0.66 \pm 0.33)I_{CNNH_2} + 0.01$$
$$n = 20, r = 0.71, s = 0.44, F_{3,16} = 5.5$$
$$pK_i' = (0.45 \pm 0.15)\mu + 2.79$$
$$n = 13, r = 0.68, s = 0.52, F_{1,11} = 9.3 \quad (3)$$

However, it is interesting to notice the absence of E_{LUMO} in the equation for BuChE, as well as the presence of different factors in the equations for pK_i and pK_i', which suggests diffierent binding modes in the enzyme and acyl-enzyme. In addition, the positive influence of the amidrazone group was confirmed.

DISCUSSION

Crystallographic studies have shown that the active site of cholinesterases is located at the bottom of a deep and narrow 'gorge' which is lined with hydrophobic residues.[19] The active site itself consists of three subsites: the 'acyl pocket', which binds the acyl moiety of the substrate by hydrophobic interactions, the 'esteratic site' which contains the 'catalytic triad' (Ser, His, Glu) and the 'anionic site', which contains a tryptophan residue and binds the quaternary ammonium part of the substrate mainly by charge-transfer (rather than electrostatic) interactions. The enzyme-substrate complex is also stabilized by a hydrogen bond between the carbonyl group of the substrate and the peptide chain. In addition, there exists a 'peripheral anionic site', located near the rim of the gorge, which contains both anionic (Asp) and aromatic (Trp) residues; a second substrate molecule can bind to this site and influence the reactivity of the active site by allosteric interaction, resulting in an inhibition by excess substrate in the case of AChE and an activation by excess substrate in the case of BuChE.

The crystallization of the tacrine-AChE complex[20] has shown that this compound binds to the 'anionic' site by forming a charge-transfer complex with the aromatic amino acid tryptophan. Our results suggest that the 2,4-dimethylpyridine derivatives bind to a different site. The most likely candidates are the hydrophobic amino acids which line the walls of the gorge. This would explain, (1) the influence of molecular volume in inhibitors of the two enzymes, which reflects the hydrophobic interactions, (2) the influence of the electron-accepting ability of the inhibitor with AChE but not BuChE, since most of these hydrophobic amino acids in AChE are aromatic (thus able to act as π-electron donors) while about half of them are replaced

by aliphatic ones in BuChE, (3) the interaction of the inhibitor with both enzyme and acyl-enzyme, since the walls of the gorge are accessible in these two enzyme forms, while the presence of the acyl group probably restricts their accessibility in the acyl-enzyme, particularly for the amino acids located near the active site. As noted by Soreq *et al.*[19], the multiplicity of potential binding sites may result in complex inhibition patterns. This would explain why for some of our inhibitors the affinity for the acyl-enzyme is greater than that for the enzyme, while it is the inverse for most of them. So, in spite of the common structure-activity relationship, different binding modes may exist within the same series of compounds.

The binding affinity (as K_i) of the most active pyridine derivatives (the thioamides) is about 100 times lower than that of tacrine for AChE, and about 1000 times lower for BuChE. Hence, it seems unlikely that the cholinesterase-inhibiting activity of these compounds may by itself be useful in Alzheimer's disease. However they could exert a beneficial effect, either by their anti-inflammatory properties or by an interaction with a typical cholinesterase inhibitor such as tacrine. For instance, these compounds could potentiate the action of tacrine by displacing it from its binding sites on plasma cholinesterase (BuChE), thus increasing the concentration of the free (active) form. In addition, their activity *in vivo* may be higher than expected since these neutral compounds may diffuse in the central nervous system more easily than the charged tacrine molecule, as evidenced by the fact that they inhibit experimental rat brain edema after IP administration.[4] For all these reasons, it seems justified in future work to search for increased anticholinesterase activities within this series and structural modifications according to the structure-activity relationships that we have established may prove useful in this respect.

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