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Velnacrine Thiaanalogues as Potential Agents for Treating Alzheimer's Disease

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Abstract—The only therapeutic drugs for combating dementia disease are acetylcholine esterase inhibitors (AChEI). However, the use of tacrine, the first AChEI to be launched as an Alzheimer's disease (AD) drug, has been limited by serious side effects. Therefore, efforts to search for more potent and selective inhibitors of AChE still remain highly significant in the therapeutic treatment of AD. In this work we modified the cyclohexyl ring of velnacrine, a less toxic analogue of tacrine, by synthesizing a series of thiopyranoquinolines in which the C-3 methylene unit was replaced by a sulphur atom. The anti-AChE data show that the activity was maintained with the bioisosteric substitution carried out. The introduction of a chlorine atom at different positions of the aromatic ring resulted in an array of different activities. In an attempt to understand the different behaviours displayed by the chlorine-substituted derivatives, a molecular docking study was performed.

Introduction

Alzheimer's disease (AD) is a progressive and neurodegenerative disorder of the CNS that is the major cause of dementia among elderly people. The social and economic consequences of AD are alarming due to the notable increase in life expectancy.

In spite of the existence of several theories regarding the pathogenesis of AD, the molecular causes of this condition remain unknown.

The most widely accepted biochemical theory, known as the cholinergic hypothesis,[1] is that the decline in cognitive and mental functions associated with AD is related to the loss of cortical cholinergic neurotransmission. Consequently, potentation of the activity of the central cholinergic pathway is one strategy for the symptomatic treatment of cognitive disfunction in AD. One rational way to enhance cholinergic neurotransmission is to inhibit acetylcholine esterase (AChE),[2] an enzyme responsible for the metabolic breakdown of acetylcholine (ACh). This concept has led to the development and commercialization of several drugs in the USA and Europe for the symptomatic treatment of AD: tacrine (THA, Cognex®),[3] donepezil (Aricept®),[4] rivastigmine (Exelon®),[5] and the latest, galanthamine (Reminyl®)[6] which was recently approved by the FDA and launched in the UK (Fig. 1). These products are known as acetylcholinesterase inhibitors (AChEIs). The latter product, galanthamine, differs from the other AChEIs in that it has a dual mode of action, acting also on the nicotinic receptors in the brain. Modulation of these receptors amplifies neurotransmission of the acetylcholine signal.

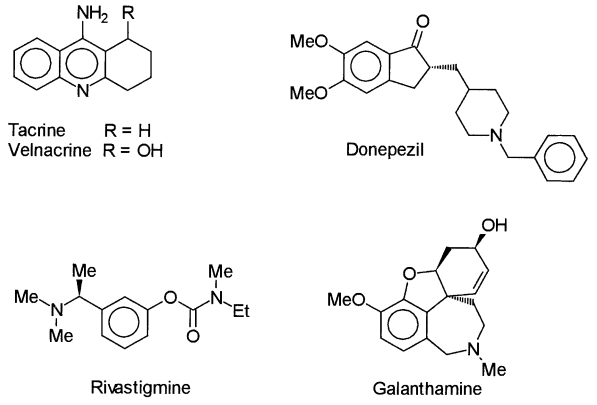

Figure 1. Structural formulas for AChEI.

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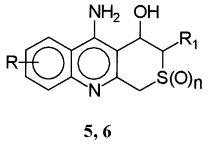
It has also been speculated that the long-term administration of these products may interfere with the progression of AD.[7]

THA was the first AChEI to be approved for therapeutic use having demonstrated a moderate but significant efficacy in alleviating the symptoms of mild to moderate AD.[8] However, the use of THA in AD has been limited by serious side effects such as hepatotoxicity, which often forces patients to discontinue treatment. In single dose studies,[10] analogues of THA such as velnacrine,[9] the 1-hydroxy metabolite of THA, has been well-tolerated, justifying research efforts aimed at improving the pharmacological profile of the prototype.

THA and its analogues act on a site close to the esteratic site of the enzyme (the catalytic triad formed by Ser-200/His-440/Glu-327). The X-ray crystallography structure of the THA–AChE complex has been determined.[11] Examination of this structure revealed that the 4-aminoquinoline portion of the THA seems to be responsible for the drug binding to the enzyme, while the cyclohexyl ring prevents the substrate from entering the active site.

In an attempt to find new potent AChEIs, we modified the cyclohexyl ring by introducing a sulphur atom at the C-3 position of velnacrine. Velnacrine, the 1-hydroxy metabolite of THA, was chosen as a template because it is less toxic than its analogue, THA. Thus, a series of thiopyranoquinolines **5** and **6** (Fig. 2), having various substitutents on the aromatic ring, were synthesized as velnacrine thiaanalogues and assayed for their ability to inhibit AChE. To evaluate the importance of 4-hydroxyl function, the keto derivative **4ag** and the oxime derivative **7ag** were also synthesized and assayed (see Table 4).



5, 6

Figure 2. Thiopyranoquinolines prepared in this study (for R, R_1 and n, see Scheme 1).

Chemistry

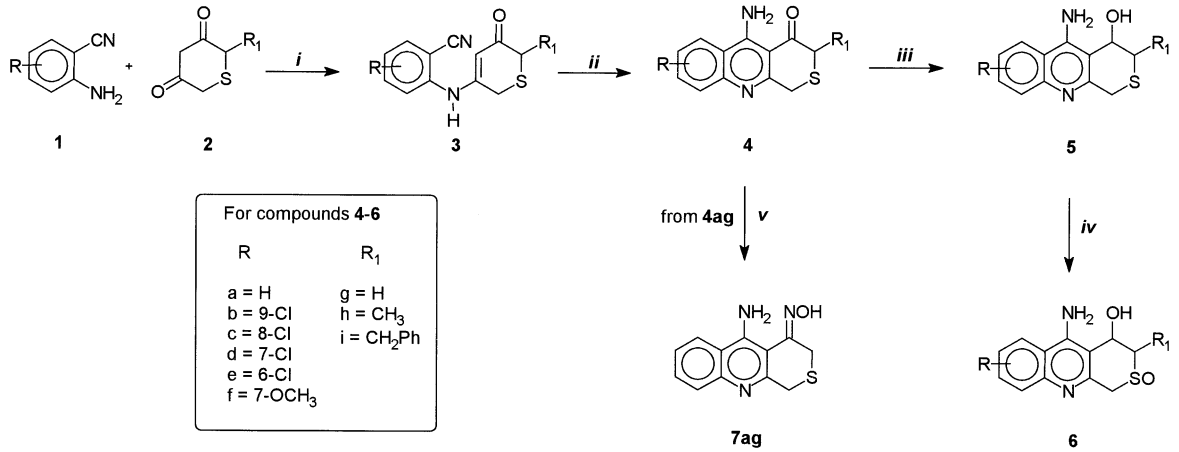
The synthesis of thiopyranoquinolines **5**–**6** is shown in Scheme 1. The condensation of substituted anthranilonitriles **1**[12] with thiopyrandiones **2**,[13] under typical conditions for enamine formation (refluxing benzene in the presence of catalyst such as *p*-toluenesulphonic acid and subsequent azeotropic removal of water), gave the enamino ketones **3** (Table 1). When thiopyrandiones **2i** and **2h** were used, the condensation reaction occurs in regiospecific manner giving **3i** and **3h** as the only isomers. The enamino ketones **3** were then cyclized in DMF in the presence of K_2CO_3 and CuCl, to give thiopyranoquinolinones **4** (Table 2). In contrast to what is reported in the literature,[9,14] it was found that CuCl has to be used in a stoichiometric rather than in a catalytic amount in order to obtain a higher yield.

Reduction of compounds **4** with $LiAlH_4$ resulted in the thiopyranoquinolin-4-ol target derivatives **5** (Table 3). In addition, selected compounds, **5ag**, **5cg**, and **5eg**, were then converted into the corresponding sulphoxide derivatives **6ag**, **6cg**, and **6eg** (Table 3) by oxidation with *m*-chloroperbenzoic acid (MCPBA) in methanol. Finally, ketone **4ag** was converted into the corresponding oxime derivative **7ag** by reaction with hydroxylamine hydrochloride (Scheme 1).

Results and Discussion

The AChE inhibition of the velnacrine thiaanalogues synthesized in this study, were evaluated in vitro using human red blood cells (AChE hRBC) and a slightly modified Ellman's method.[15] The results are presented in Table 4.

The first observation is that the bioisosteric substitution of one methylene unit in the cyclohexyl ring with a sulphur atom maintains the activity. In fact, compound



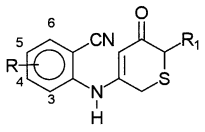
Scheme 1. Reagents: (i) dry benzene, TsOH, reflux; (ii) K_2CO_3, CuCl, DMF, 90 °C; (iii) $LiAlH_4$, dry THF; (iv) 50% MCPBA, MeOH; (v) NH_2OH, pyridine, EtOH.

5ag displays the same inhibitory activity ($IC_{50} = 3.27$ μM) as the reference compound velnacrine. The 4-keto intermediate **4ag** also possessed good activity ($IC_{50} = 2.56$ μM), which was slightly higher than that of the 4-hydroxyl analogue **5ag**. In contrast, activity declined noticeably when the 4-hydroxyl group was substituted with the oxime group as in **7ag**.

Compounds in which a methyl or a benzyl group was introduced at the C-3 position of the thiopyranic ring were devoid of any inhibitory activity (see **5ah** and **5ai**).

The introduction of a chlorine atom in different positions on the aromatic ring of the thiopyranoquinolines resulted in an array of different activities. The presence of a chlorine atom at C-8 or C-6 position improved the activity; compounds **5cg** ($IC_{50} = 0.45$ μM) and **5eg** ($IC_{50} = 0.32$ μM) displayed similar activities that were 10 times greater than that seen with the dechloro analogue **5ag**. In contrast, the C-9 and C-7 chlorinated derivatives **5bg** and **5dg** were inactive. These results are in agreement with what was already reported for 6- and 7-chloro-velnacrine derivatives[9] and the 6-chloroTHA derivative.[16]

Table 1. Data for enamino ketones **3**



Compd[a]	R	R_1	% yield[b]	Mp (°C)	Formula[c]
3ag	H	H	50	198–200	$C_{12}H_{10}N_2OS$
3ah	H	CH_3	90	130–131	$C_{13}H_{12}N_2OS$
3ai	H	CH_2–Ph	77	165–166	$C_{19}H_{16}N_2OS$
3bg	3-Cl	H	55	78–80	$C_{12}H_9ClN_2OS$
3cg	4-Cl	H	86	204–206	$C_{12}H_9ClN_2OS$
3dg	5-Cl	H	75	149–152	$C_{12}H_9ClN_2OS$
3eg	6-Cl	H	80	194–197 (dec)	$C_{12}H_9ClN_2OS$
3fg	5-OCH_3	H	100	155–157	$C_{13}H_{12}N_2O_2S$

[a]All compounds were purified by crystallization with Et_2O.
[b]Yields were not optimized.
[c]All compounds had elemental analyses within ±0.4% of theoretical value.

In contrast to the activity displayed by the 7-methoxytacrine (7-MEOTA),[17] our 7-methoxy derivative **5fg** was completely inactive.

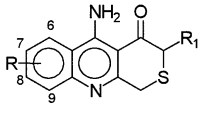
The oxidation of the thiopyranic sulphur atom gave contrasting results; a slight increase in activity was observed when no substitution was present in the aromatic ring (**6ag** vs **5ag**) and continued to be inactive when a chlorine atom was present at C-9 position (**6bg** vs **5bg**). A marked decline in activity was observed with the C-8 chlorine derivative (**6cg** vs **5cg**).

In an attempt to understand the reasons for the different activities displayed by the chlorine substituted derivatives, **5bg**, **5cg**, **5dg** and **5eg**, we performed a molecular docking study to evaluate the possible binding modes inside the THA binding site. For the molecular modeling study, we chose the X-ray structure of the AChE-THA complex (**acj** Protein Data Bank entry, resolution 2.8 Å)[18] since the analogous complex with velnacrine was not available. Semi-empirical optimized structures of the inhibitors were used. The relatively high basicity of THA ($pK_a = 9.8$ for the endocyclic nitrogen) indicates that at a pH = 7 this compound is protonated. We therefore performed geometry optimization and docking calculations for the *N*-endocyclic protonated derivatives.[19]

The SYBYL molecular modeling software was used to build and optimize the molecular structures of the inhibitors. The program was also used as the graphical interface for the GRID docking program, which calculated the form of binding between the inhibitors studied and the AChE structure. Finally, we explicitly considered the presence of water in the active site, because of its well-known role in reinforcing the binding of THA molecule.

The results of the docking study indicated that only the binding of the C-6 and C-8 chloro derivatives (**5eg** and **5cg**), is practically superimposed to the orientation assumed by the THA molecule in the active site (Fig. 3a). In this way, the analogues **5cg** and **5eg** can maintain all the interactions which are considered to be important: staking interactions of the aromatic rings of the inhibitors

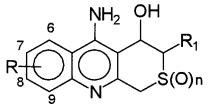
Table 2. Data for thiopyranoquinoline-4-ones **4**



Compd	R	R_1	Purification method[a]	% yield[b]	Mp (°C)	Formula[c]
4ag	H	H	A	60	198–200	$C_{12}H_{10}N_2OS$
4ah	H	CH_3	B	45	170–171	$C_{13}H_{12}N_2OS$
4ai	H	CH_2–Ph	B	50	143–145	$C_{19}H_{16}N_2OS$
4bg	9-Cl	H	A	40	208–210	$C_{12}H_9ClN_2OS$
4cg	8-Cl	H	C	40	200–204	$C_{12}H_9ClN_2OS$
4dg	7-Cl	H	D	45	>270	$C_{12}H_9ClN_2OS$
4eg	6-Cl	H	D	15	122–125	$C_{12}H_9ClN_2OS$
4fg	7-OCH_3	H	C	31	220–222	$C_{13}H_{12}N_2O_2S$

[a]Purification by column chromatography eluting with: (A) $CHCl_3$/MeOH, 99:1; (B) CH_2Cl_2/EtOAc, 90:10; (C) gradient of CH_2Cl_2/EtOAc, 80:20 to CH_2Cl_2/EtOAc, 50:50; (D) EtOAc.
[b]Yields were not optimized.
[c]All compounds had elemental analyses within ±0.4% of theoretical value.

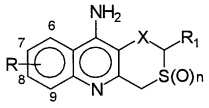
Table 3. Data for thiopyranquinoline-4-ols **5** and **6**



Compd	R	R_1	*n*	Purification method[a]	% yield[b]	Mp (°C)	Formula[c]
5ag	H	H	0	A	90	204–206	$C_{12}H_{12}N_2OS$
5ah[d]	H	CH_3	0	A	50	171–175	$C_{13}H_{14}N_2OS$
5ahI[e]	H	CH_3	0	B	15	186–190	$C_{13}H_{14}N_2OS$
5aiI[e]	H	CH_2–Ph	0	A	55	198–201	$C_{19}H_{18}N_2OS$
5bg	9-Cl	H	0	C	60	205–208	$C_{12}H_{11}ClN_2OS$
5cg	8-Cl	H	0	C	80	212–215	$C_{12}H_{11}ClN_2OS$
5dg	7-Cl	H	0	A	60	195–198	$C_{12}H_{11}ClN_2OS$
5eg	6-Cl	H	0	C	45	187–190	$C_{12}H_{11}ClN_2OS$
5fg	7-OCH_3	H	0	A	40	195–196	$C_{13}H_{14}N_2O_2S$
6ag	H	H	1	A	70	244–247	$C_{12}H_{12}N_2O_2S$
6bgI[f]	9-Cl	H	1	D	30	>300	$C_{12}H_{11}ClN_2OS$
6bgII[g]	9-Cl	H	1	D	20	>300	$C_{12}H_{11}ClN_2OS$
6cgI[f]	8-Cl	H	1	D	30	>300	$C_{12}H_{11}ClN_2O_2S$
6cgII[g]	8-Cl	H	1	D	25	>300	$C_{12}H_{11}ClN_2OS$

[a]Purification by column chromatography eluting with: (A) CH_2Cl_2/MeOH, 90:10; (B) CH_2Cl_2/EtOAc, 90:10; (C) CH_2Cl_2/MeOH, 95:5; (D) CH_2Cl_2/MeOH, 98:2 to CH_2Cl_2/MeOH, 90:10.
[b]Yields were not optimized.
[c]All compounds had elemental analyses within ±0.4% of theoretical value.
[d]A 1:1 mixture of two diastereoisomers; see Experimental.
[e]Pure diastereoisomer; see Experimental.
[f]First eluted diastereoisomer; see Experimental.
[g]Second eluted diastereoisomer; see Experimental.

Table 4. In vitro AChE inhibition of thiopyranoquinolines prepared in this study



Compd	R	R_1	X	*n*	IC_{50} (μM)[a]
4ag	H	H	CO	0	2.56
5ag	H	H	CHOH	0	3.27
5ah[b]	H	CH_3	CHOH	0	100
5ahI[c]	H	CH_3	CHOH	0	>100
5aiI[c]	H	CH_2-Ph	CHOH	0	>100
5bg	9-Cl	H	CHOH	0	>100
5cg	8-Cl	H	CHOH	0	0.45
5dg	7-Cl	H	CHOH	0	>100
5eg	6-Cl	H	CHOH	0	0.32
5fg	7-OCH_3	H	CHOH	0	>100
6ag	H	H	CHOH	1	2.56
6bgI[d]	9-Cl	H	CHOH	1	>100
6bgII[e]	9-Cl	H	CHOH	1	>100
6cgI[d]	8-Cl	H	CHOH	1	49.6
6cgII[e]	8-Cl	H	CHOH	1	20
7ag	H	H	CNOH	0	>100
Velnacrine					3.27
THA					0.46

[a]The activity was obtained using a slightly modified Ellman's method; see Experimental.
[b]A 1:1 mixture of two diastereoisomers; see Experimental.
[c]Pure diastereoisomer; see Experimental.
[d]First eluted diastereoisomer; see Experimental.
[e]Second eluted diastereoisomer; see Experimental.

with the aromatic side chains of Trp-84 and Phe-330; two hydrogen bonds, one between the protonated endocyclic nitrogen and the main-chain carbonyl oxygen of His-440, and another between the exocyclic amino group and the oxygen from crystal water. In contrast, the C-7 and C-9 chlorine derivatives, **5dg** and **5bg**, do not allow a similarly favorable interaction and the calculation results indicate that these derivatives cannot assume the same binding orientation as THA, which explains their inactivity.

The results of the GRID calculations seem to explain the effects of having chlorine in the different positions on the inhibitory activity. The C-6 and C-8 chlorine derivatives allow the halogen atom to be positioned in a region that favours an interaction with the enzyme as shown by the GRID maps in Figure 3b. These results are in agreement with previously reported data which showed an increased binding for 6-chloro THA derivative compared to THA.[19]

The C-6 chloro derivative **5eg**, together with prototype **5ag**, were assayed in a mouse passive avoidance model for their ability to reverse scopolamine and cyclohexamide-induced amnesia. Both compounds were seen to reverse the experimentally induced amnesia, even though complete reversal was not observed.

The results of the acute toxicity studies showed that the most prominent effects, the onset of which were at lower doses than the lethal ones, were associated with an over activation of the cholinergic system. The mortality observed was also due to over stimulation of the cholinergic system. Indeed pre-treatment with atropine, a muscarinic receptor antagonist, prevented death. In particular, **5eg** had an LD_{50} of 87.6 mg/kg following oral administration, while that of **5ag** was 120 mg/kg compared to 65 mg/kg for velnacrine. It is interesting to note that **5eg** was less toxic even though it was 10 times more active on the enzyme, AChE.

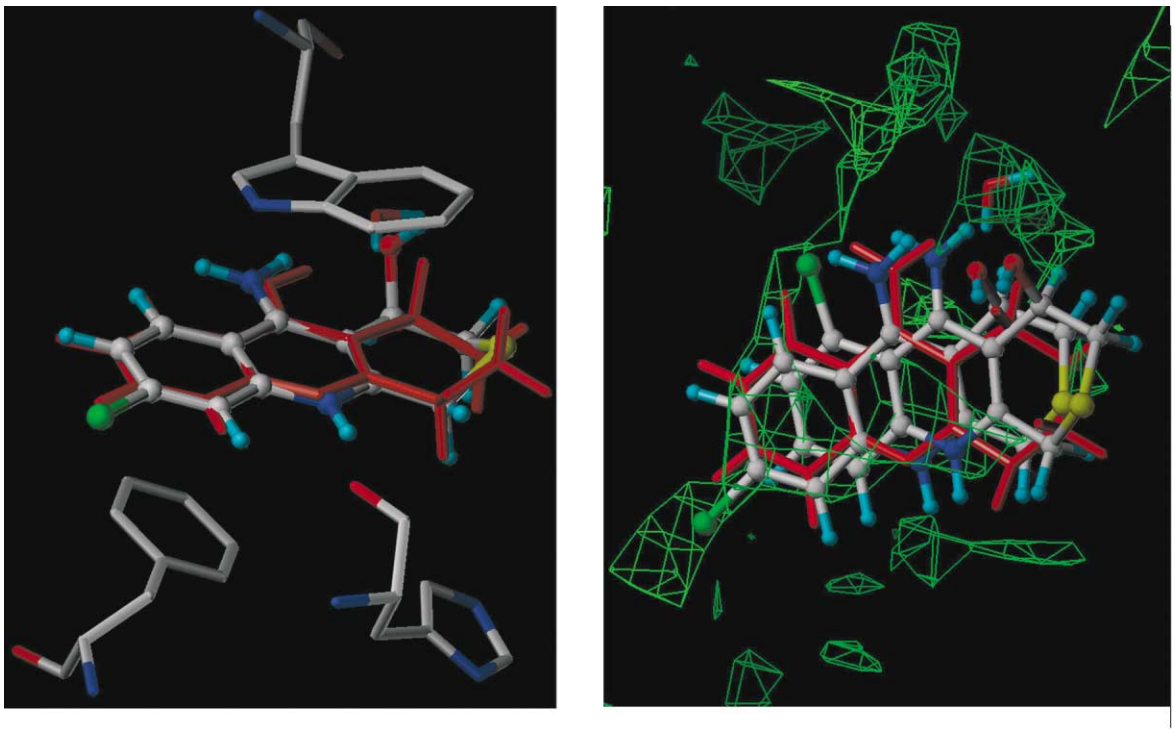


Figure 3. (a) Structure of 6-chlorothiopyranoquinoline **5eg** superimposed to tacrine; (b) GRID maps of the interaction between the enzyme and chlorine atom probes shown for compounds **5cg**, **5eg**, and tacrine.

Experimental

Thin-layer chromatography (TLC) was performed on pre-coated sheets of silica gel $60F_{254}$ (Merck) and visualized by using UV. Column chromatography separations were carried out on Merck silica gel 40 (mesh 70–230). Melting points were determined in capillary tubes (Büchi melting point apparatus) and are uncorrected. Elemental analyses were performed on a Carlo Erba elemental analyzer, Model 1106, and the data for C, H and N are within ±0.4% of the theoretical values. ^{1}H NMR and ^{13}C NMR spectra were recorded at 200 MHz (Bruker AC-200) with Me_4Si as internal standard and chemical shifts are given in ppm (δ). The spectral data are consistent with the assigned structures. Reagents and solvents were purchased from common commercial suppliers and were used as received. Organic solutions were dried over anhydrous Na_2SO_4 and concentrated with a Büchi rotary evaporator at low pressure. Yields were of purified product and were not optimized. All starting materials were commercially available unless otherwise indicated.

2-[(5-Oxo-5,6-dihydro-2*H*-3-thiopyranyl)amino]benzonitrile (3ag). A suspension of commercial anthranilonitrile **1a** (2.40 g, 0.02 mol), tetrahydro-2*H*-3,5-thiopyrandiones (**2g**)[13] (2.60 g, 0.02 mol) and *p*-toluenesulphonic acid monohydrate (0.11 g, 0.67 mmol) in dry benzene (15 mL) was refluxed for 2 h, and the water was collected in a Dean-Stark water separator. The reaction mixture was then chilled to 0 °C and the product was filtered off. Washed with cold benzene followed by cold hexane, and dried. After crystallization by Et_2O, 2.320 g (60%) of **3ag** were obtained: mp 198–200 °C; IR ($CHCl_3$): 3380, 2210, 1590, 1340 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 3.10–3.40 and 3.55–3.80 (each 2H, m), 4.90 (1H, s), 7.35–7.50 (2H, m), 7.70–7.95 (2H, m), 9.30 (1H, s).

The other compounds **3** were prepared from the appropriately substituted anthranilonitriles **1**[12] and diketones **2**[13] in a similar manner. The physical properties of compounds **3** are reported in Table 1.

5-Amino-3,4-dihydro-1*H*-thiopyran[3,4-*b*]quinoline-4-one (4ag). K_2CO_3 (0.2 g, 2.2 mmol) and CuCl (1.0 g, 10.4 mmol) were added to a solution of **3ag** (2.40 g, 10.4 mmol) in dry DMF (5 mL) and the mixture was heated at 80–90 °C for 3–4 h. After cooling, the reaction mixture was poured into ice-water containing sodium tartrate. The precipitate obtained was filtered and extracted several times with a mixture of 50% MeOH/EtOAc to eliminate the insoluble inorganic materials. The resulting solid was finally purified by column chromatography eluting with CH_2Cl_2/MeOH, (9:1) to give 1.43 g (60%) of **4ag**: mp 198–200 °C (dec); IR ($CHCl_3$): 3490, 3280, 1620, 1340 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 3.70 (2H, s), 4.03 (2H, s), 7.40–7.60 (1H, m), 7.65–7.85 (2H, m), 8.30–8.45 (1H, m), 8.55 (2H, bs). Anal. calcd for $C_{12}H_{10}N_2OS$: C, 62.59; H, 4.38; N, 12.16. Found: C, 42.60; H, 4.36; N, 12.05.

The other thiopyranoquinolinones **4** were prepared from the analogous enamino ketones **3** in a similar manner. The physical properties of compounds **4** are reported in Table 2.

5-Amino-3,4-dihydro-1*H*-thiopyran[3,4-*b*]quinoline-4-ol (5ag). A solution of $LiAlH_4$ in Et_2O (1.8 mL of 1.0 M, 1.8 mmol) was added dropwise to a solution of thiopyranoquinolinones **4ag** (0.4 g, 1.8 mmol) in dry THF (20 mL) maintained at 0 °C. After 1 h at room temperature, the reaction was quenched by adding 10% HCl, then made basic with 30% NaOH and extracted with EtOAc. The combined organic layers were dried and evaporated to dryness giving a residue which was purified by column chromatography eluting with CH_2Cl_2/MeOH (9:1) to give 0.376 g (90%) of **5ag**: mp 204–205 °C; ^{1}H NMR (DMSO-d_6) δ 2.90–3.25 (2H, m), 3.70–4.00 (2H, m), 4.95–5.10 (1H, m), 5.50 (1H, bs), 6.60–6.90 (2H, s), 7.30–7.45 (1H, m), 7.50–7.80 (2H, m), 8.15–8.30 (1H, m). ^{13}C NMR (DMSO-d_6) δ 154.6, 150.9, 145.9, 129.1, 127.9, 123.4, 122.1, 118.0, 110.9, 63.1, 35.1, 33.7. Anal. calcd for $C_{12}H_{12}N_2OS$: C, 62.05; H, 5.21; N, 12.06. Found: C, 62.25; H, 5.15; N, 12.05.

The other hydroxy compounds **5ah**, **5bg**, **5cg**, **5dg**, **5eg**, **5fg**, and **5ai**, were prepared from the analogous thiopyranoquinolinones **4** in a similar fashion. Starting from compound **4ah** two diastereoisomers were obtained. After column chromatography purification, one of two diastereoisomers, referred to as **5ahI**, was obtained as pure compound in a small amount (15%), together with a mixture (1:1) of both diastereoisomers referred to as **5ah** (50%).

The reduction of ketone **4ai** only gave one diastereoisomer, **5aiI**.

The physical properties of the target thiopyranoquinolone-4-ols are summarized in Table 3, while their spectral data are enumerated below.

5ah. ^{1}H NMR ($CDCl_3$/DMSO-d_6) δ 1.50 and 1.48 (each 3H, d, J = 7.2 Hz), 3.30 (2H, bs) 3.10–3.28 (2H, m), 3.75, 3.80, 4.08, and 4.15 (each 1H, AB system), 4.60 (1H, d, J = 7 Hz), 4.65–4.72 (1H, m), 6.41 (2H, bs), 6.00 (2H, bs), 7.82–7.96, 7.53–7.68, 7.73–7.84, and 8.00–8.12 (each 2H, m). Anal. calcd for $C_{13}H_{14}N_2OS$: C, 63.39; H, 5.73; N, 11.37. Found: C, 63.50; H, 6.00; N, 11.20.

5ahI. ^{1}H NMR ($CDCl_3$/DMSO-d_6) δ 1.48 (3H, d, J = 7.2 Hz), 3.18 (1H, dq, J = 3 and 7.2 Hz), 3.75 (1H, AB system), 4.15 (1H, AB system), 4.48 (1H, bs), 4.62–4.72 (1H, m), 6.22 (2H, bs), 7.48 (1H, dt, J = 1.5 and 7.8 Hz), 7.58 (1 H, dt, J = 1.5 and 7.8 Hz), 7.75 (1H, dd, J = 1.5 and 7.8 Hz), 8.04 (1 H, dd, J = 1.5 and 7.8 Hz). Anal. calcd for $C_{13}H_{14}N_2OS$: C, 63.39; H, 5.73; N, 11.37. Found: C, 63.42; H, 5.70; N, 11.35.

5bg. ^{1}H NMR (DMSO-d_6) δ 2.95 (1H, dd, J = 6 and 13 Hz), 3.12 (1 H, dd, J = 5.5 and 13 Hz), 3.76 (1H, AB system), 3.88 (1H, AB system), 4.90–5.05 (1H, m), 5.48 (1H, d, J = 7 Hz), 6.84 (2H, bs), 7.32 (1H, t, J = 7.5 Hz), 7.75 (1H, dd, J = 1.5 and 7.5 Hz), 8.18 (1H, dd, J = 1.5 and 7.5 Hz). Anal. calcd for $C_{12}H_{11}ClN_2OS$: C, 54.03; H, 4.16; N, 10.50. Found: C, 54.03; H, 4.30; N, 10.45.

5cg. ^{1}H NMR (DMSO-d_6) δ 2.90 (1H, dd, J = 7.4 and 16 Hz), 3.10 (1H, dd, J = 5.5 and 16 Hz), 3.70–3.90 (2H, m), 4.90–5.05 (1H, m), 5.40 (1H, d, J = 8 Hz),), 6.84 (2H, bs), 7.40 (1H, dd, J = 1.4 and 7.8 Hz), 7.69 (1H, d, J = 1.4 Hz), 8.30 (1H, d, J = 7.8 Hz). Anal. calcd for $C_{12}H_{11}ClN_2OS$: C, 54.03; H, 4.16; N, 10.50. Found: C, 54.30; H, 4.25; N, 10.50.

5dg. ^{1}H NMR (DMSO-d_6) δ 2.95 (1H, dd, J = 5 and 13.5 Hz), 3.10 (1H, dd, J = 4 and 13.5 Hz), 3.65–3.92 (2H, m), 5.48 (1H, d, J = 7.8 Hz), 6.80 (2H, bs), 7.55 (1H, dd, J = 2 and 8 Hz), 7.68 (1H, d, J = 8 Hz), 8.35 (1H, J = 2 Hz). Anal. calcd for $C_{12}H_{11}ClN_2OS$: C, 54.03; H, 4.16; N, 10.50. Found: C, 54.27; H, 4.40; N, 10.35.

5eg. ^{1}H NMR (DMSO-d_6) δ 2.88–3.24 (2H, m), 3.80 (2H, s), 4.75–4.96 (1H, m), 5.58–5.78 (1H, m), 7.00 (2H, s), 7.38 (1H, dd, J = 1.5 and 8.2 Hz), 7.50 (1H, t, J = 8.2 Hz), 7.65 (1H, dd, J = 1.5 and 8.2 Hz). Anal. calcd for $C_{12}H_{11}ClN_2OS$: C, 54.03; H, 4.16; N, 10.50. Found: C, 54.10; H, 4.25; N, 10.43.

5fg. ^{1}H NMR (DMSO-d_6) δ 2.88–3.20 (2H, m), 3.90 (3H, s), 3.70–3.90 (2H, m), 4.90–5.05 (1H, m), 5.50 (1H, bs), 6.78 (2H, bs), 7.25 (1H, dd, J = 2.6 and 8.5 Hz), 7.54–7.70 (2H, m). Anal. calcd for $C_{13}H_{14}N_2O_2S$: C, 59.52; H, 5.38; N, 10.68. Found: C, 59.70; H, 5.40; N, 10.42.

5aiI. ^{1}H NMR (DMSO-d_6) δ 2.86–3.05 (1H, m), 3.15–3.35 (2H, m), 3.72 (1H, AB system), 4.04 (1H, AB system), 4.65–4.78 (1H, m), 5.00 (1H, d, J = 7 Hz), 6.40 (2H, s), 7.16–7.40 (6H, m), 7.56 (1H, dt, J = 1.4 and 7.8 Hz), 7.70 (1H, dd, 1.4 and 7.8 Hz), 8.12 (1H, dd, 1.4 and 7.8 Hz). Anal. calcd for $C_{19}H_{18}N_2OS$: C, 70.78; H, 5.63; N, 8.69. Found: C, 70.90; H, 5.75; N, 8.68.

5-Amino-4-hydroxy-3,4-dihydro-1*H*-thiopyran[3,4-*b*]quinoline-2-one (6ag). 70% MCPBA (0.250 g, 1 mmol) was added portionwise to a stirred solution of **5ag** (0.232 g, 1 mmol) in MeOH (10 mL) maintained at 0 °C. After 10 min a saturated solution of $NaHCO_3$ was added and the reaction mixture was extracted with EtOAc. The organic layers were dried, evaporated to dryness and the residue was purified by column chromatography eluting with CH_2Cl_2/MeOH (9:1) to give 0.160 g (70%) of **6ag** as a mixture of diastereoisomers: mp 244–247 °C; ^{1}H NMR (DMSO-d_6) δ 2.80–3.40 (6H, m), 4.00–4.35 (4H, m), 4.90 (2H, bs), 6.75 (4H, bs), 7.25–7.65 (4H, m), 7.76–7.80 (1H, m), 7.82–8.08 (2H, m), 8.18–8.30 (1H, m). Anal. calcd for $C_{12}H_{12}N_2O_2S$: C, 58.05; H, 4.87; N, 11.28. Found: C, 58.15; H, 4.72; N, 11.20.

The other sulphoxide derivatives **6bg** and **6cg** were prepared from the analogous sulfphur **5bg** and **5cg** in a similar fashion. Two diastereoisomers were separated by column chromatography eluting with a gradient of CH_2Cl_2/MeOH (98:2) to CH_2Cl_2/MeOH (90:10). Their physical properties are reported in Table 3, while their spectral data are enumerated below:

6bgI (first eluted diastereoisomer). ^{1}H NMR (DMSO-d_6) δ 3.25–3.50 (2H, m), 4.20 (1H, AB system), 4.40 (1H, AB system), 5.20–5.45 (1H, m), 5.60 (1H, m), 7.00 (2H, bs), 7.40 (1H, dd, J = 8.2 Hz), 7.55 (1H, t, J = 8.2 Hz),

7.65 (1H, dd, $J=1.5$ and 8.2 Hz).[13] C NMR (DMSO-d_6) δ 57.4, 60.7, 63.5, 112, 118.9, 125, 125.6, 127.9, 138.3, 145.5, 147.1, 150.8. Anal. calcd for $C_{12}H_{11}ClN_2OS$: C, 50.98; H, 3.92; N, 9.91. Found: C, 50.90; H, 3.58; N, 9.80.

6bgII (second eluted diastereoisomer). ^{1}H NMR (DMSO-d_6) δ 3.20–3.50 (2H, m), 4.10 (1H, AB system), 4.40 (1H, AB system), 5.10–5.40 (1H, m), 5.70 (1H, bs), 7.10 (2H, bs), 7.40 (1H, dd, $J=8.2$ Hz), 7.50 (1H, t, $J=8.2$ Hz), 7.65 (1H, dd, $J=1.5$ and 8.2 Hz). ^{13}C NMR (DMSO-d_6) δ 55.3, 58.9, 62, 110.8, 118.2, 125.2, 127, 137.9, 145, 146.5, 151.5. Anal. calcd for $C_{12}H_{11}ClN_2OS$: C, 50.98; H, 3.92; N, 9.91. Found: C, 51.08; H, 4.20; N, 9.85.

6cgI (first eluted diastereoisomer). ^{1}H NMR (DMSO-d_6) δ 3.25–3.50 (2H, m), 4.20 (1H, AB system), 4.35 (1H, AB system), 5.20–5.30 (1H, m), 5.35 (1H, d, $J=7.5$ Hz), 7.00 (2H, bs), 7.45 (1H, dd, $J=2$ and 8 Hz), 7.75 (1H, d, $J=2$ Hz), 8.30 (1H, d, $J=8$ Hz). ^{13}C NMR (DMSO-d_6) δ 52.7, 54.6, 63, 110.6, 117.2, 124.7, 125.7, 127, 134.6, 148.6, 148.7, 150. Anal. calcd for $C_{12}H_{11}ClN_2OS$: C, 50.98; H, 3.92; N, 9.91. Found: C, 50.75; H, 3.92; N, 9.91.

6cgII (second eluted diastereoisomer). ^{1}H NMR (DMSO-d_6) δ 3.20–3.50 (2H, m), 4.05 (1H, AB system), 4.35 (1H, AB system), 5.37–5.45 (1H, m), 5.70 (1H, bs), 7.00 (2H, bs), 7.45 (1H, dd, $J=1.5$ and 7.0 Hz), 7.72 (1H, d, $J=1.5$ Hz), 8.30 (1H, d, $J=7$ Hz).^{13}C NMR (DMSO-d_6) δ 55.2, 55.4, 61.9, 110.5, 117.3, 124.6, 125.2, 127.2, 128.2, 134.4, 148.7, 150.7, 151. Anal. calcd for $C_{12}H_{11}ClN_2OS$: C, 50.98; H, 3.92; N, 9.91. Found: C, 51.30; H, 4.25; N, 9.86.

5-Amino-4-hydroxyimino-3,4-dihydro-1*H*-thiopyran[3,4-*b*]quinoline (7ag). Hydroxylamine hydrochloride (0.30 g, 4 mmol) was added to a solution of compound **5ag** (0.23 g, 1 mmol) in EtOH (3 mL) and pyridine (0.5 mL), and the solution was refluxed for 1 h. The mixture was evaporated to dryness and the residue was treated with water which then gave a solid which was filtered, washed with EtOAc and dried, to give 0.180 g (79%) of **7ag**: mp >340 °C; ^{1}H NMR (DMSO-d_6) δ 3.80 (2H, s), 4.40 (2H, s), 7.58–7.78 (1H, m), 7.80–8.10 (3H, m), 8.55–8.75 (1H, m), 10.50 (1H, bs), 11.0 (1H, bs). Anal. calcd for $C_{12}H_{11}N_3OS$: C, 58.76; H, 4.52; N, 17.13. Found: C, 58.72; H, 4.50; N, 17.45.

Acetylcholinesterase inhibition

This inhibition was determined using acetylcholinesterase derived from human blood cells (AChE hRBCs) in a slightly modified Ellman's method.[15] ACh (Boehringer Mannheim) was used as substrate, at a concentration of 5.0 mM. Determinations were made at four different concentrations and an IC_{50} was calculated with log-probit analyses.

Briefly, the method used was as follows: AChE hRBCs are incubated for 10 min at 25 °C with both the test compound (four different concentrations) and heptylphysostigmine (0.5 μM), a concentration that completely inhibits the enzyme. Heptylphysostigmine is a slow tight-binding pseudo-irreversible AChEI, and is not removed by washing. The test compounds are reversible inhibitors and hence, the complex formed (AChE-inhibitor) is easily dissociated by simple dilution. These compounds, however, bind the enzyme very quickly. After the 10 min incubation period, the inhibitor-complex (40 μL) was diluted 1:466 by adding distilled water. The residual inhibition, with respect to the control hRBC (no inhibitor), was measured using a specific chromogenic substrate, 5,5′-dithio-bis-2-nitrobenzoic acid. After careful mixing, acethylthiocholine iodide was also added. Absorbance at 405 nm was then measured after 0, 30, 60 and 90 s. The percentage inhibition observed was that due the presence of heptylphysostigmine. Hence, to determine the inhibitor effect of the compounds one has to detract the residual percentage inhibition from 100% (inhibition observed with only 0.5 μM heptylphysostigmine). The calculated IC_{50} value can be seen in Table 4.

Acute toxicity test

Acute toxicity studies were conducted in young male albino mice (CD-1 strain), weighing 30 g. The compounds were administered orally. The principal signs of toxicity were tremors, salivation, fasciculation, lacrimation, miosis, straub tail, exophthalmos, respiratory distress, and/or diarrhea. These signs are consistent with the excessive cholinergic activity induced by cholinesterase inhibition. The cholinergic antagonist atropine was shown to block the acute toxicity of these products in mice, confirming that these effects were related to the pharmacological activity of the drug (data not shown). The mortality rate was determined after 14 days, and the DL_{50} values were calculated by the method of Litchfield and Wilcoxon.[20]

Passive avoidance

In the passive avoidance test, rats (Sprague–Dawley) are conditioned to go against their natural behaviour, that is to enter and explore dark areas. An electric shock (0.5 mA for 5 s) was given to the animals in order to reinforce this behavioural change. The animals were placed in a two-chambered compartment, one white and well lit, the other black and dark. The rats were placed in the white chamber and allowed to move freely into the dark chamber. Thereupon they received an electric shock (training session). After 24 h, the animals were again placed into the white chamber (trial session), however, this time the control animals manifested a long step through latency (STL), this is the time required for the animal to pass from the white chamber into the dark ones.

The effect of velnacrine derivatives on passive avoidance learning was examined in rats. Untreated rats crossed rapidly to the dark side of the apparatus before the electric shock, but after the shock they were reluctant to cross over at either 1 or 24 h. Amnesia was induced in these animals using scopolamine (1.0 mg/kg, sc)[21] and cyclohexamide (1.0 mg/kg, sc).[22] In fact, animals

receiving either scopolamine or cyclohexamide would step freely into the dark chamber and receive an electric shock. The STL was considerably shorter. An amnesia state was then induced. A dose-dependent inhibition of the scopolamine and cyclohexamide-induced deficits were reversed by oral or intraperitoneal administration of compounds under investigation, 2 hs prior to the trial session.

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