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Naphthoxazoles and heterobenzoxazoles: cholinesterase inhibition and antioxidant activity

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Abstract: Finding novel cholinesterase inhibitors that would be able to cross the blood–brain barrier, have favorable pharmacokinetic parameters, and reduce hepatotoxicity along with other side effects has been the main focus of investigations dealing with Alzheimer disease. In this study we evaluated cholinesterase inhibitory and antioxidant activity of seven oxazole derivatives. These compounds have been efficiently and sustainably prepared by photochemical electrocyclization reaction. Various naphthoxazoles have been previously investigated as potential antibacterial, antituberculosis, and anticancer agents. They have also been tested for antioxidant activity, but never for cholinesterase inhibitory activity. Among the tested oxazole derivatives, fused heterobenzoxazole compounds with pyridine and thiophene moiety, oxazolo[5,4-*h*]isoquinoline, thieno[2',3':5,6]benzo[1,2-*d*]oxazole, and thieno[3',2':5,6]benzo[1,2-*d*]oxazole, showed the greatest potential for both cholinesterase inhibitory and antioxidant activity. Among them, thieno[2',3':5,6]benzo[1,2-*d*]oxazole was found to be the best one.

Key words: Antioxidant activity, cholinesterase activity, naphthoxazoles, fused heterobenzoxazoles

1. Introduction

Aerobic metabolic processes produce reactive oxygen species and free radicals that accumulate in cells on a daily basis. These oxygen species and free radicals affect different organs and systems in our body and are usually controlled via internal antioxidants as well as antioxidant enzymes and enzyme systems.[1,2] Uncontrolled production of these species leads to attacks on various biomolecules such as lipids, proteins, and DNA, as well as on cellular machinery and membranes, thus causing oxidative stress. Oxidative stress is most commonly associated with the development of many disorders and diseases, including neurodegenerative diseases (i.e. Alzheimer and Parkinson diseases).[3]

Alzheimer disease is one of the most common neurodegenerative disorders in western society, mostly affecting the elderly population.[4,5] The most prominent symptom is a decrease in cognitive function, which in turn leads to changes in the behavioral patterns of an individual. As the reduction of neurotransmitter acetylcholine in the brain is one of the hallmarks of Alzheimer disease, acetylcholinesterase and butyrylcholinesterase inhibition represents the only pharmacotherapy able to increase the acetylcholine neurotransmitter in the brain.[6–8]

A group of compounds called naphthoxazoles have been known and studied ever since Fisher synthesized 2-methylnaphtho[1,2-*d*]oxazole and 2-methylnaphtho[2,1-*d*]oxazole in 1906.[9] In the subsequent years different

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synthetic approaches were developed and the oxazole nuclei became an important building block for the synthesis of many biologically active molecules.[10−24] Some of the fused polycyclic compounds with oxazole rings have shown antibacterial and antituberculosis activities as well as anticancer activity and some have been tested for antioxidant activity.[25−28] New efficient methodology for the preparation of oxazole derivatives provides a valuable tool to the organic chemist. The synthesis of naphthoxazoles and fused heterobenzoxazoles in one step of the reaction path and by utilizing light, as a very clean reagent, gave an easy route to this kind of polycyclic systems, which are taxing to obtain via ground state organic synthesis.[29]

In the present study, seven in-house synthesized compounds, naphthoxazoles (**1**–**3**) and fused heterobenzoxazoles (**4**–**7**, Figure), were tested for their antioxidant activity, using 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging and ferric reducing antioxidant power (FRAP) methods. They were also tested for cholinesterase inhibitory activity using Ellman's method. To the best of our knowledge this is the first time that both antioxidant and cholinesterase inhibitory activities have ever been tested for these types of compounds.

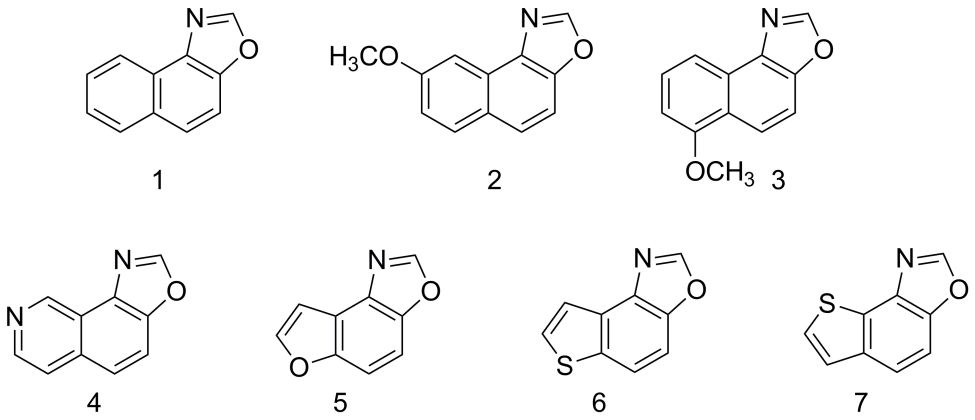

Figure. Structures of the tested naphthoxazoles and fused heterobenzoxazoles: naphtho[1,2-*d*]oxazole (**1**), 8-methoxynaphtho[1,2-*d*]oxazole (**2**), 6-methoxynaphtho[1,2-*d*]oxazole (**3**), oxazolo[5,4-*h*]isoquinoline (**4**), benzofuro[4,5-*d*]oxazole (**5**), thieno[2',3':5,6]benzo[1,2-*d*]oxazole (**6**), thieno[3',2':5,6]benzo[1,2-*d*]oxazole (**7**).

2. Results and discussion

Structures of tested naphthoxazoles **1**–**3** and fused heterobenzoxazoles **4**–**7** are given in the Figure.

2.1. Antioxidant activity

The antioxidant activity potential of naphtho[1,2-*d*]oxazole (**1**), 8-methoxynaphtho[1,2-*d*]oxazole (**2**), 6-methoxynaphtho[1,2-*d*]oxazole (**3**), oxazolo[5,4-*h*]isoquinoline (**4**), benzofuro[4,5-*d*]oxazole (**5**), thieno[2',3':5,6]benzo[1,2-*d*]oxazole (**6**), and thieno[3',2':5,6]benzo[1,2-*d*]oxazole (**7**) was determined using two methods, DPPH and FRAP, and the acquired results are shown in Table 1.

2.2. Radical scavenging activity

The half-maximal DPPH radical scavenging concentration (IC_{50}) was calculated for oxazoles **2**, **4**, **6**, and **7** having IC_{50} values of 0.400, 0.600, 0.226, and 0.229 mM, respectively. When comparing naphthoxazoles (i.e. **1**, **2**, **3**) it can be concluded that naphthoxazole **2**, having a methoxy group in position 8 of the fused moiety, shows the best activity, while compound **3**, having a methoxy group in position 6, shows rather weak activity,

Table 1. Antioxidant properties of naphthoxazoles and fused heterobenzoxazoles.

Naphthoxazole/	DPPH			FRAP	
heterobenzoxazole	IC_{50} mM	inhibition % [a]	Eqv. vit. C µM	Eqv. Fe^{2+} µM [b]	Eqv. vit. C µM
1	-	19.22 (at 2.81)	10.52	15.23 (at 1.91)	8.76
2	0.40	83.69 (at 1.20)	42.92	154.76 (at 0.81)	95.13
3	-	7.70 (at 3.59)	4.73	22.24 (at 2.43)	13.10
4	0.60	96.68 (at 1.68)	49.44	100.13 (at 1.14)	61.31
5	-	0.58 (at 1.50)	1.16	2.37 (at 1.01)	0.79
6	0.226	74.94 (at 0.54)	38.52	120.17 (at 0.37)	73.72
7	0.229	92.36 (at 2.72)	47.27	333.44 (at 1.84)	205.74

-not determined; n.d. not detected; [a] concentrations for maximal effect measured are given in parentheses in mM; [b] concentrations for maximal effect measured are given in parentheses in mM.

even lower than that of the unsubstituted naphtho[1,2-*d*]oxazole (**1**). Among the heterobenzoxazole derivatives (i.e. **4**, **5**, **6**, **7**) the best scavenging activity was shown by thienobenzoxazoles **6** and **7**. The position of the sulfur atom in these two derivatives (**6**, **7**) does not seem to affect their activity since there is practically no difference in their IC_{50} values. Oxazolo[5,4-*h*]isoquinoline (**4**), bearing the pyridine fused moiety, also showed good scavenging ability although it was about three times lower than the ability of the derivatives with the thiophene fused moiety. Benzofuroxazole **5**, on the other hand, having a furan fused ring, showed practically no activity at all. This indicates that thiophene moiety contributes highly to DPPH scavenging activity while its analogue furan does not. Also, thienobenzoxazoles have been shown to be the best DPPH scavengers among all of the tested oxazole derivatives. There is an earlier example of a hydroxyl radical scavenging study of naphthoxazole compounds, performed by Wang et al.[28] In that study the antioxidant activities of naphtho[2,1-*d*]oxazoles bearing an acetyloxymethyl group on position 2 of the oxazole ring were tested. It was also concluded that derivatives with substituents in positions 8 and 9 of the fused moiety showed the greatest hydroxyl radical scavenging properties.[28]

Oxazole derivatives **5**, **3**, and **1** were found to be poor scavengers of DPPH even at relatively high tested concentrations, having 0.58% at 1.5 mM, 7.7% at 3.59 mM, and 19.2% at 2.81 mM, respectively.

2.3. Reducing activity

Naphthoxazoles and fused heterobenzoxazoles were also tested using the FRAP assay, which is presumed to estimate the total antioxidant power. The relevant chemical reaction of the FRAP method represents the total reducing power as it involves a single electron reaction between $Fe(TPTZ)_2$(III) and any species able to reduce it to $Fe(TPTZ)_2$(II), making this species an antioxidant.[30] Oxazole derivatives **2**, **4, 6**, and **7** showed relatively high antioxidant activity when compared to vitamin C (Table 1). These results are consistent with those from DPPH scavenging, which confirms the good antioxidant activity.

2.4. Cholinesterase inhibition

To the best of our knowledge, the present paper reports the first investigation on cholinesterase (ChE) inhibitory activity of naphthoxazoles and fused heterobenzoxazoles. The inhibition of acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) was assessed for 7 compounds and the results are reported in the Table 2.

Table 2. Cholinesterase inhibition by naphthoxazoles and fused heterobenzoxazoles.

Naphthoxazole/heterobenzoxazole	AChE		BChE	
	IC_{50} mM	inhibition (%) [a]	IC_{50} mM	inhibition (%) [a]
1	-	1.80 (2.69)	0.25	83.20 (2.69)
2	-	n.d. (1.14)	0.30	99.51 (1.14)
3	-	38.00 (0.29)	0.21	66.50 (0.29)
4	0.29	71.54 (1.34)	0.52	83.67 (1.34)
5	-	15.50 (1.43)	-	n.d. (1.43)
6	0.26	80.79 (0.52)	0.40	72.06 (1.04)
7	1.09	73.89 (2.59)	0.97	98.03 (2.59)

-not determined; n.d. not detected; [a] concentration in mM is given in parentheses for maximal effect measured.

Generally, all the tested oxazole derivatives (Figure) showed both AChE and/or BChE inhibitory activity, except for derivatives **2** and **5**, which showed no inhibitory activity on AChE and BChE, respectively.

Only three oxazole derivatives having heterocyclic fused moieties, i.e. **4**, **6**, and **7**, have shown meaningful AChE inhibitory activity with IC_{50} values of 0.29, 0.26, and 1.1 mM, respectively. In this case, derivative **7**, having a different position of the sulfur atom, showed more than three times lower activity than its counterpart **6**. This may be due to the AChE selectivity towards the specific position of the sulfur atom in derivative **6** since its active site possesses a binding region called an oxyanion hole that can bind oxygen or sulfur atom-containing substrates.[31]

Heterobenzoxazole compounds having a fused pyridine ring (**4**) or thiophene ring (**6** and **7**) also showed good antioxidant activity, which is a favorable trait when considering these compounds as potential anti-Alzheimer drugs.

In contrast to AChE, all of the tested oxazole derivatives showed relatively good BChE inhibitory activity. In the case of AChE, naphthoxazoles having a benzene ring modification (**1**, **2**, and **3**) showed better activity (having IC_{50} of 0.25, 0.30, and 0.21 mM, respectively) than the fused heterobenzoxazoles (**4**, **6**, and **7**, with IC_{50} values of 0.52, 0.40, and 0.97 mM). These observations are consistent with differences between the BChE and AChE active sites. In the case of the BChE active site, certain aromatic amino acid residues are replaced with aliphatic ones, thus enabling wider spectra of substrates to enter.[32] BChE activity of naphthoxazole derivatives is affected by benzene ring functionalization as well as by the position of the methoxy group on the benzene ring.

The derivative with the unsubstituted benzene ring (**1**, with IC_{50} of 0.25 mM) showed better activity than compound **2** (having a methoxy group in position 8 of the fused moiety, with IC_{50} of 0.30 mM), while derivative **3** showed the best activity among the three naphthoxazoles (IC_{50} of 0.21 mM), which would mean that methoxy group position plays a crucial role in BChE inhibition potential and selectivity.

Heterobenzoxazoles **4**, **6**, and **7** inhibited both AChE and BChE. Pyridine heterobenzoxazole (**4**) and thiophene heterobenzoxazole (**6**) showed nearly two times better inhibitory activity on AChE than on BChE. In contrast to derivative **6**, thiophene heterobenzoxazole (**7**) showed similar activity on both enzymes.

The best inhibitory activity for AChE was shown for thiophene derivative **6** (having IC_{50} value of 0.26 mM), while the best inhibitory activity for BChE was shown by methoxy-substituted benzene derivative **3** (having IC_{50} value of 0.21 mM).

In this study we tested naphtho[1,2-*d*]oxazoles as well as fused heterobenzoxazoles for their antioxidative and anticholinesterase activity. Generally, it can be suggested that heterobenzoxazole derivatives **4**, **6**, and **7** showed the most promising potential to be further studied as anti-Alzheimer drug candidates. All three compounds showed relatively good activity in all the methods tested, which is significant since they would be able to tackle both cholinesterase inhibition and oxidative stress at the same time. Among all of the compounds tested, heterobenzoxazole derivative **6** showed the best activity in all methods tested and may very well be the best lead candidate for treatment of Alzheimer disease.

2.5. Experimental

All reagents and solvents used were of analytical grade. Acetylcholinesterase (AChE, from *Electrophorus electricus* - electric eel, type V-S), butyrylcholinesterase (BChE, from equine serum), acetylthiocholine iodide (ATChI), butyrylthiocholine iodide (BTChI), 5,5-dithiobis(2-nitrobenzoic acid) (DTNB, Ellman's reagent), 2,2-diphenyl-1-picrylhydrazyl radical (DPPH), and 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ) were purchased from Sigma-Aldrich. Absorbance measurements were performed on a Synergy HTX S1LFA multimode microplate reader (BioTek Instruments, Inc., Winooski, VT, USA). All the tested naphthoxazoles and heterobenzoxazoles were prepared as described previously. In short, the necessary starting oxazole derivatives were synthesized in one step from aryl-/heteroaryl-substituted α,β-unsaturated aldehydes using Van Leusen reagent, tosylmethyl isocyanide (TosMIC), in refluxing methanol and in the presence of potassium carbonate. After the solvent was evaporated and the crude reaction mixture was passed through a silica gel chromatographic column, the 5-substituted oxazoles were isolated in good yields (60%–98%). In the case of the synthesis of 2- and 3-thienylethenyl- and 4-pyridylethenyloxazoles, the necessary unsaturated aldehydes were prepared by a Wittig reaction from the corresponding thiophene-2(3)-carbaldehydes and pyridine-4-carbaldehyde, respectively, and formylmethylenetriphenylphosphorane. At this point the obtained 5-substituted oxazoles were converted to naphthoxazoles and heterobenzoxazoles by UV irradiation in benzene solution with the addition of iodine, using a Rayonet reactor equipped with 300-nm lamps.[29]

2.6. Antioxidant activity

Antioxidant activity assessment was carried out using two methods: scavenging ability of naphthoxazoles and fused heterobenzoxazoles towards both DPPH and FRAP.

2.7. DPPH assay

DPPH scavenging ability of the samples was measured according to a recently reported procedure.[33] The results for the free radical scavenging activities of the samples are expressed as IC_{50} values (where possible) and inhibition percentages of DPPH radical (% inhibition).

2.8. FRAP assay

The reducing potential of naphthoxazoles and heterobenzoxazoles was measured as described by Benzie and Strain.[34] In this assay, antioxidants are evaluated as reducing agents of Fe^{3+} to Fe^{2+}, which undergoes chelation by 2,4,6-Tris(2-pyridyl)-*s*-triazine (TPTZ) to form a Fe^{2+}-TPTZ complex absorbing at 593 nm.

2.9. Acetylcholinesterase/butyrylcholinesterase inhibitory activity

AChE/BChE inhibitory activity measurements were carried out by a slightly modified Ellman assay as described before for AChE/BChE inhibitory activity.[33] In this assay, cholinesterase inhibitory activity is measured indirectly via reduction of ATChI breakdown. All spectrophotometric measurements were performed at 405 nm (where the reaction of thiocholine with DTNB can easily be monitored) and at room temperature for 6 min. The results are expressed as percentage of enzyme activity inhibition.

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