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Artigo

A Comparative Evaluation of Acetylcholinesterase Inhibition by AChE-ICER and *in vitro* Ellman's Modified Method of Simplified Analogs 3-*O*-Acetyl-*N*-Benzyl-Piperidine of Donepezil

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Avaliação comparativa da inibição de acetilcolinesterase por AChE-ICER e pelo método *in vitro* de Ellman modificado de análogos simplificados 3-*O*-acetil-*N*-benzil-piperidínicos do Donepezil

Resumo: Inibidores de Acetilcolinesterase (AChEI) ainda são os melhores fármacos usados para a terapêutica da doença de Alzheirmer (DA). Dentre os IAChEs disponíveis, o donepezil apresenta a maior meia-vida, os efeitos colaterais mais brandos, além de segurança e tolerância satisfatórias; portanto, este tem sido utilizado como medicamento de primeira escolha no tratamento de DA leve a moderada. A triagem bem sucedida de novos AChEI depende de métodos rápidos e eficientes. Os ensaios empregados mais frequentemente são baseados no uso do reagente de Ellman (ensaio colorimétrico) ou do reagente *Fast Blue B*, além de métodos radioquímicos, espectrométricos ou cromatográficos. Uma abordagem mais recente utiliza a enzima imobilizada em reator capilar, acoplada a um sistema cromatográfico, que é conhecido como ICER (do inglês *immobilized capillary enzyme reactor*). Como parte de um projeto que visa à identificação de novos IAChEs, este artigo descreve a avaliação comparativa da atividade inibitória da AChE de uma série de derivados 3-*O*-acetil-*N*-benzilpiperidínicos substituídos por duas diferentes abordagens: método de Ellman modificado *in vitro* e uma metodologia de ICER. Embora nenhum dos compostos da série tenha apresentado alta atividade inibitória, todos os ensaios revelaram resultados consistentes, demostrando que é possível aplicar diferentes metodologias confiáveis para a busca e idendificação de novos inibidores de AChE.

Palavras-chave: Química Medicinal; análogos *N*-benzil-piperidinícos; inibidores de acetilcolinesterase; doença de Alzheimer; enzima imobilizada; enzima imobilizada em reator capilar.

Abstract

Acetylcholinesterase inhibitors (AChEI) remain the best pharmacotherapy for Alzheimer´s disease (AD). Among the currently available AChEI drugs, Donepezil has longer half-life, fewer clinical side interactions, and satisfactory safety and tolerability; therefore, it is the first choice medicine to treat mild to moderate AD. Successful screening for new AChEIs relies on effective and fast assays. The most frequently employed assays are based on the use of Ellman's reagent (colorimetric method) or the Fast Blue B salt reagent, despite of other radiochemical, spectrometric, or chromatographic methods. A more recent and interesting approach is to immobilize the target enzyme in a chromatographic system, which is known as immobilized capillary enzyme reactors (ICERs). As part of a current project aiming to search for novel AChEIs, in this paper we have reported a comparative evaluation of AChE inhibitory activity of a series of substituted 3-*O*-acetyl-*N*-benzylpiperidine derivatives by two different approaches: *in vitro* Ellman's modified method and an ICER-based methodology. Although none of the compounds has shown high activity, all different assays revealed consistent results, showing that is possible to apply different reliable methodologies for the screening of new AChEIs.

Keywords: *N*-benzyl-piperidine analogs; Acetylcholinesterase inhibition; Alzheimer's Disease; enzyme immobilization; immobilized capillary enzyme reactor.

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A Comparative Evaluation of Acetylcholinesterase Inhibition by AChE-ICER and *in vitro* Ellman's Modified Method of Simplified Analogs 3-*O*-Acetyl-*N*-Benzyl-Piperidine of Donepezil

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1. Introduction

Alzheimer's Disease (AD) is an incurable neurodegenerative condition. It is the most common type of dementia among the elderly and affects over 36 million people worldwide.[1] Three main hypotheses, the so-called cholinergic, amyloid, and the tau hypothesis, have been proposed to explain the pathophysiology of AD.[2,3] The main factors associated with AD is the low level of acetylcholine in the neural synapses, deposits of senile plaques and neurofibrillary tangles in the barin tissue, a severe neuroinflammatory process and neuronal death.[2-6] Deficits in the brain cholinergic system contribute to cognitive dysfunction in AD.[4-6]

Due to the multifactorial characteristics this disease, many researchers have conducted studies on natural products and synthetic new compounds searching for more effective and secure drug prototypes, on the

basis of the cholinergic and amyloid hypotheses and, more recently, antioxidant, anti-inflammatory and/or neuroprotective therapies.[7-9] This new way of "thinking" bioactive compounds that act on different target simultaneously is based on the modern concept of multi-target directed ligands (MTDLs).[10,11] In particular, cognitive deficits have led to the development of cholinergic strategies, aiming the improvement of cholinergic function and this approach led to most of the medicines used today.

Acetylcholinesterase (AChE) is a serine hydrolase enzyme that catalyzes the hydrolysis of the neurotransmitter acetylcholine. The main medications that treat AD symptoms belong to the class of cholinesterase inhibitors (ChEIs). Indeed, among the five commercially available drugs to treat AD, Tacrine, Donepezil, Rivastigmine, and Galanthamine, are AChE inhibitors (AChEI), whereas memantine is an NMDA receptor antagonist. Unfortunately, all of these medicines are only palliative, bringing on unwanted side effects, and their effectiveness diminishes after prolonged treatment.[4] Donepezil, approved by the FDA in 1996, is a reversible and highly selective AChEI. It is well-tolerated and displays a side effect profile typical of cholinergic agents, with minimal influence on the hepatic and renal impairment that is common in the elderly population. Moreover, Donepezil has a long half-life, which allows the use of lower doses, less frequent and smaller variation of drug concentrations in plasma. Furthermore, donepezil has few clinical side interactions, and adequate safety and tolerability. Thus, it is usually the first choice medicine to treat mild to moderate AD.[10-12]

As part of an ongoing project on the search for novel anti-Alzheimer drug candidate prototypes, we have reported the design and synthesis of new series of AChEIs based on molecular simplification of donepezil [13], preserving the common pharmacophoric substituted piperidine moiety present in donepezil (1) and the AChE inhibitor (−)-3-O-acetyl-spectaline hydrochloride (LASSBio-767 (**2**)), a semisynthetic derivative of the natural (-)-spectaline[14,15] taken as structural models (A and B, Figure 1). Considering that choosing appropriate assay is crucial when assessing the inhibitory activity of a given compound, we decide to use these series of simple small molecules for a comparative study, using the most common and fast methods describe in the literature for AChE activity identification.

In the past years, several methods to determine AChE activity has been described.[16] The most frequently employed solution enzyme assays rely on a colorimetric method using Ellman´s reagent[17] or the Fast Blue B salt reagent, and also on radiochemical, spectrometric, and chromatographic methods[16,18] and capillary electrophoresis.[19-20] Other methods involve an interesting approach: the immobilization of the target enzyme and its inclusion in a chromatographic system. Such methods can provide a shorten analysis and more reliable results, besides furnishing information about the affinity of the tested molecule for the target protein. Therefore, they could allow for the online determination of a compound inhibitory potency as well as help getting information about the possible enzyme inhibition mechanism.[21,22] In this context, various authors have immobilized AChE onto different supports, by different immobilization methods.[22-31] Our group has contributed a previous paper on the development and application of ChEIs screening methodologies using ICER-AChE and UV detection,[22] and also reported a direct method using AChE-ICER and LC/IT-MS/MS.[31]

In the present work, we have used a series of substituted 3-*O*-acetyl-*N*-benzylpiperidine derivatives (**3**, Figure 1)[13] for evaluation of their activity by an ICER- based screening methodology[22] in comparison to inhibitory data displayed on the *in vitro* Ellman's modified method,[14,17] and even by the classical qualitative TLC assays.[16,33,34]

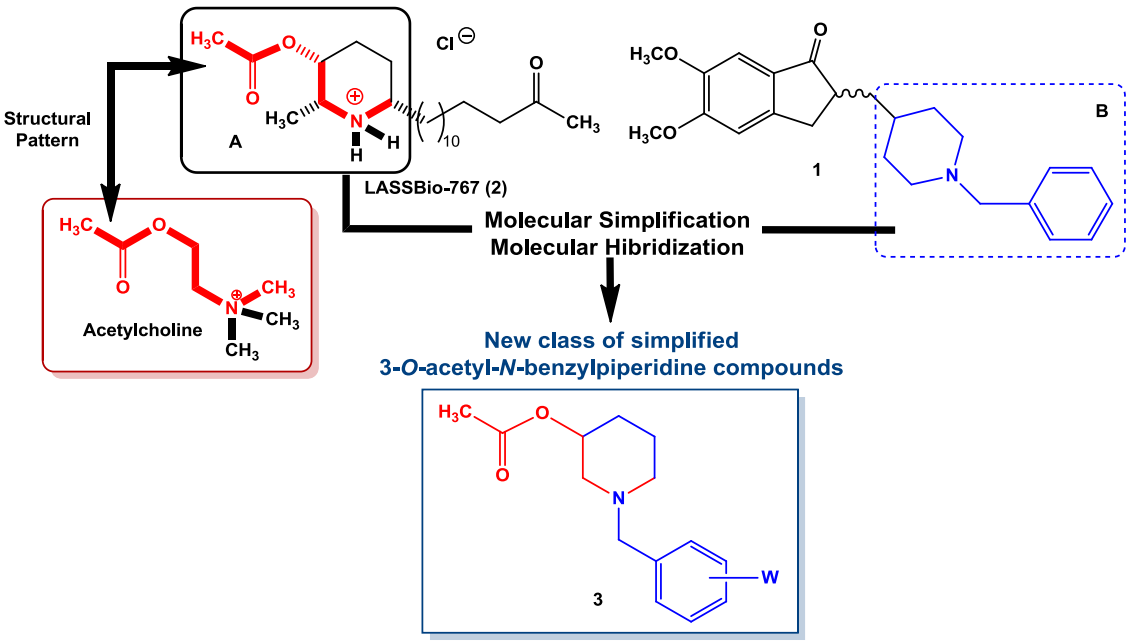

Figure 1. Design of a series of 3-*O*-acetyl-*N*-benzylpiperidine derivatives (**3**) based on the structures of Donepezil (**1**) and the selective AChEI LASSBio-767 (**2**)

2. Experimental

2.1. Chemistry

^{1}H and ^{13}C NMR spectra were determined in chloroform-d3 and methanol-d4 with a Varian INOVA 500 spectrometer at 500 and 125 MHz, respectively. IR spectra were obtained with a Nicolet-1400 FTIR spectrometer using KBr pellets. Prior to concentration under reduced pressure, all the organic extracts were dried over anhydrous magnesium sulfate powder. The progress of all the reactions was monitored by TLC performed on aluminum sheets pre-coated with silica gel (F-254, Merck) and viewed by spraying with iodochloroplatinate reagent (Merck). Purifications by column chromatography were performed with silica gel (70–230 mesh, Merck). Solvents used in the reactions were dried, redistilled prior to use, and stored over 3-4 A˚ molecular sieves.

Preparation of 3-O-acetyl-piperidine

To a solution containing 406.0 mg (3.96 mmol) of 3-hydroxypiperidine in hexane (6.5 mL), 24.6 mg (0.202 mmol) of 4-DMAP and 0.37 mL (4.04 mmol) of acetic anhydride were added. The mixture was vigorously stirred at room temperature for 72 h until TLC analysis indicated that reaction was complete. Then, the solvent was evaporated under vaccum, and the crude product was purified by silica gel column chromatography eluted with CH_2Cl_2 : MeOH 9:1, to furnish 441.3 mg of a pale yellow oil (85% yield). The structure of the 3-*O*-acetyl-piperidine was confirmed by IR spectrometry.

*General procedure for preparation of compounds **3a-r** and **3x***

To a solution of 3-*O*-acetylpiperidine (1.05 mmol) in anhydrous methanol (5 mL), 1.05 mmol of the substituted aldehyde was added. Then, the pH was adjusted to 6 with acetic acid, and the reaction was vigorously stirred at room temperature, for 48 h. After this time, 1 mmol of the starting amine was added and the reaction mixture was stirred overnight. After 72 h, $NaBH_3CN$ (6 mmol) was

added, and the reaction was stirred overnight at room temperature. After the reaction was completed as judged from (TLC), a 10% sodium phosphate solution (5 mL) and water (5 mL) were added, and the crude product was extracted with CH_2Cl_2 (3 x 10 mL). The organic phases were combined, washed with saturated NaCl solution, and dried over $MgSO_4$. The solvent was removed, and the crude product was purified by silica gel column chromatography eluted with (CH_2Cl_2: MeOH 9:1), to furnish the desired 3-*O*-acetylpiperidinylbenzyl derivatives in 60-90% yield. To prepare derivative **3x**, compound **3k** was reacted with anhydrous HCl in AcOEt, to furnish the desired chloridrate in quantitative yield. The structures of all the compounds were confirmed by ^{1}H and ^{13}C NMR and compared with literature data.

2.2. Biological Assays

Reagents and Materials

Acetylcholinesterase (AChE, EC 3.1.1.7, from Electric eel, 333 units/MG, and from human Erythrocytes) lyophilized powder and its substrate (S)-acetylthiolcholine iodide (ATCh); 5,5'-Dithiobis (2-nitrobenzoic acid) (Ellman's reagent or DTNB); tacrine (9-amino-1,2,3,4-tetrahydroacridine hydrochloride hydrate), Fast Blue B salt (*O*-dianisidine-bis(diazotized) hydrochloride zinc double salt), β-naphthyl acetate, and glutaraldehyde were supplied by Sigma-Aldrich. Buffer components, and all the chemical materials used during the immobilization procedure were of analytical grade and were purchased from Sigma, Merck (Darmstdt, Germany), Synth (São Paulo, Brazil), or Acros (Geel, Belgica). Water employed in all the experiments had been purified in a Millipore Milli-Q system (Millipore, São Paulo, Brazil). Fused silica capillary (0.375 mm × 0.10 mm) was acquired from Polymicro Technologies (Phoenix, AZ, USA). The entire buffer solutions were filtered through cellulose nitrate membrane filters (0.45 µm) provided by Phenomenex. Stock solutions (10 mM) of the evaluated inhibitors were prepared in water/methanol [(50% (v/v)] and diluted with water, to give concentrations in the range of 10 – 1000 µM. Silica gel plates (silica gel 60 F_{245}) aluminum sheets were provided by Merck (Darmstadt, Germany).

Apparatus

Enzyme immobilization was carried out by using a syringe-pump 341B (Sage instruments, Boston, USA). The AChE-ICER was placed in a Shimadzu HPLC system (Shimadzu, Kyoto, Japan) consisting of two LC 20AD pumps. One of the pumps contained an FCV-20AL valve for low-pressure gradient, a UV-Vis detector (SPD-M20AV), an autosampler equipment (SIL-20A), and a six-port and three-way switching sample valve Valco. Data acquisition was accomplished on a Shimadzu CBM-20A system interfaced with a computer, using the Shimadzu-LC Solutions (LC Solution 2.1) software (Shimadzu, Kyoto, Japan). A microplate reader system (Elisa readers) Versa Max-Molecular Device (Silicon Valley, CA, USA) was employed in the assays involving the free enzyme in solution.

Buffer

Buffer 1: 0.1 M Tris-HCl (pH 8.0) 1.26×10^{-4} M Ellman's reagent designated working buffer and used as the mobile phase for all the chromatographic systems, in all the experiments employing the AChE-IMER.

AChE columns preparation

Columns were prepared by a protocol described in our previous reports[22] by using a solution containing 0.5 mg of AChE (approximately 150 U) in 50 mM phosphate buffer (pH 8.0) and fused-silica capillary tube (100 µm I.D × 0.375 mm × 30 cm).

Screening studies by using AChE-ICER assay

All compounds **3a-3x** were evaluated using the method described in our previous report.[22] To this goal, a 1 mM stock solution (MeOH/ H_2O 1:1 v/v) was prepared for each inhibitor. For analytical purposes, a sample with a final volume of 100 µL was obtained by using 20 µL of the inhibitor stock solution, 20 µL of ATCh solutions (5 mM), and 60 µL of Buffer 1, which resulted in a concentration of 200 µM for the candidate compound and 1 mM for ATCh in Buffer 1. The samples were prepared in duplicate, and 10-µL aliquots were injected into the chromatographic system using the following conditions: mobile phase consisting of Buffer 1, flow rate = 0.05 $mL.min^{-1}$, and UV-Vis detection at 412 nm. The percent inhibition obtained for each compound was calculated by comparing the attained peak areas with the area achieved for the YA (yellow anion) when a sample containing water and substrate ATCh (1 mM) was injected into the chromatographic system under the same operating conditions. The percentage inhibition for each inhibitor concentration was calculated by comparing the peak areas with those obtained in the absence of the inhibitor. To this end, the following expression was employed: $100-(Ai/A_0 \times 100)$, where A_i is the peak area calculated in the presence of the inhibitors, and A_0 is the peak area calculated in the absence of the inhibitors.

TLC and microplate assay using Ellman´s method

The inhibitory activity of compounds **3a-3x** was determined by using Ellman´s reagent either via a TLC assay or a microplate assay, as previously described.[17,34]

TLC assay using Fast Blue B salt as reagent

The inhibitory activity of compounds **3a-3x** was also evaluated with a TLC assay using Fast Blue B salt as reagent as previously described.[18]

False positive assay (Chemical reaction of thiocholine and DTNB)

To verify whether the positive results shown by compounds in the TLC or microplate assay was not due to inhibition of the chemical reaction between DTNB and the product of the enzyme reaction (thiocholine), a false positive assay was performed as previously described.[34]

3. Results and discussion

The target compounds **3a-x** were prepared in two steps from commercial 3-hydroxypiperidine (**4**) by reaction with Ac_2O/4-DMAP in hexane, to produce the corresponding piperidine acetate **5**. In a second step, compound **5** was submitted to reductive amination reactions with several substituted benzaldehydes in the presence of $NaBH_3CN$ to furnish the desired substituted 3-*O*-acetyl-*N*-benzylpiperidines (**3a-v**) in 60-89% yields (Figure 2, Table 1). Compound **3x** was obtained by reaction of the amino derivative **3k** with anhydrous HCl in a quantitative yield (Figure 3, Table 1).

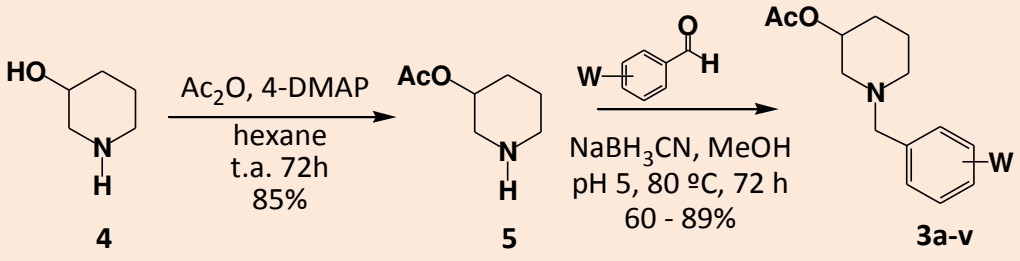

Figure 2. General synthetic route for the preparation of the substituted 3-*O*-acetyl-*N*-benzylpiperidine derivatives **3a-v**

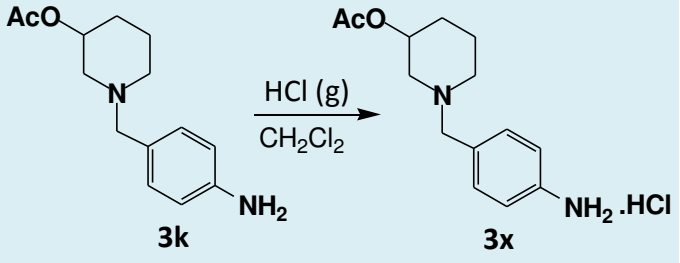

Figure 3. General synthetic route for the preparation of the substituted 3-*O*-acetyl-*N*-benzylpiperidine derivatives **3x**

Table 1. Structural and chemical data for compounds **3a-x**

Compound	Code	W	Yield (%)
3a	**LFQM-03**	3,4-di-OH	63
3b	**LFQM-04**	4-Cl	65
3c	**LFQM-05**	4-F	64
3d	**LFQM-06**	4-OH	60
3e	**LFQM-07**	4-Br	76
3f	**LFQM-08**	3,4-di-OCH_3	70
3g	**LFQM-09**	3-OCH_3, 4-OH	56
3i	**LFQM-11**	3-OCH_3	66
3j	**LFQM-12**	4-NO_2	80
3k	**LFQM-13**	4-NH_2	64
3l	**LFQM-14**	4-CF_3	78
3m	**LFQM-15**	4-$N(CH_3)_2$	61
3n	**LFQM-16**	3,4-OCH_2O-	60
3o	**LFQM-17**	H	90
3p	**LFQM-28**	3-OH, 4-OCH_3	58
3q	**LFQM-37**	3-OH	60
3r	**LFQM-38**	4-OCH_3	72
3s	**LFQM-40**	4-(1-morpholyl)	89
3t	**LFQM-41**	4-(1-piperidinyl)	81
3u	**LFQM-42**	4-(1-pirrolidinyl)	75
3v	**LFQM-43**	4-SCH_3	65
3x	**LFQM-70**	4-NH_2. HCl	100

Choosing an appropriate screening assay to evaluate AChE inhibition or other biological activity is crucial. Indeed, the results of biological activities obtained by different assays and/or by different research groups can vary considerably.[35-37] Here, we evaluated the inhibitory activities of the 22 compounds of the series **3** against AChE by two different methods aiming to establish a qualitative comparison. To this goal, we used the classical *in vitro* methodology based on the modified Ellman's test, which data are displayed on Figure 4, and a method based on AChE–ICER bioreactor prepared by immobilization of AChE onto fused silica capillaries[22] as LC column for online studies, with UV-Vis detection (Table 2).

The results achieved for the majority of the compounds were consistent in both methods, revealing a comparable same order of AChE inhibitory activity for most of compounds. For some, the numeric values varied depending on the evaluation method. Furthermore, both methodologies also serve to evidence that the absence of the 3,4-dimethoxyindanone subunit of donepezil, is not vital for an AChE inhibitory profile. In fact, the singular structural pattern of donepezil attribute good drug ability properties, but the preservation of the substituted piperidine moiety seems to be sufficient to achieve a significant inhibitory profile, as observed for the piperidine alkaloid LASSBio-767 (**2**). Considering literature data, these findings were not surprising, because AChE recognizes donepezil by interactions including the middle gorge of the active site of the enzyme, which involves three subunits: the benzyl moiety, the nitrogen atom on the piperidine ring, and the dimethoxy-indanone portion. These interactions involve direct contacts mediated by water molecules, which seem to be crucial for binding and specificity. Some studies with other donepezil analogs evidenced that the presence of two aromatic functional groups in the structure of the inhibitor is also important. These aromatic subunits probably play an essential role in π-π stacking interactions with the aromatic residues Trp84 and Trp279 located in the peripheral regions of the AChE gorge.[38]

Thus, it is possible to explore simplified donepezil analogs with preserved *N*-benzylpiperidine pharmacophoric subunit for structure-activity studies, and to establish rational comprehensive contributions of the different substituents on the aromatic ring to the potency and selectivity of AChE inhibitors. Here, the *in vitro* evaluation revealed that compounds LFQM-06 (**3d**), LFQM-08 (**3f**), LFQM-12 (**3j**), and LFQM-38 (**3r**) were the most active, exhibiting AChE inhibitory effects of 27.88, 22.79, 21.32 and 21.53%, respectively, in comparison to the prototype LASSBio-767 (**2**) at a dose of 100 µM (Figure 4).

According to Table 2, the method *in vitro* Ellman's test was capable to identified compounds **3d**, **3e**, **3f**, 3p and **3r** as the most active of the series. In order to evaluate the selectivity of the target compounds toward AChE of different origins, we prepared two AChE-ICERs by using AChE from *Electrophohus electricus* (*ee*-AChE-ICER-) and AChE from human erythrocytes (*hu*-AChE-ICER). Again, the results depicted in Table 2 demonstrated weaker inhibitory activity for all compounds, in comparison to LASSBio-767, but in this test compound LFQM-70 (**3x**) afforded the highest inhibitory activity (56%). Considering the selectivity toward *hu*-AChE and *ee*-AChE, compounds **3a** (32.4 %), **3b** (28.7 %) and **3x** (56.0 %) were more selective for *hu*-AChE-ICER, whereas compound **3u** showed higher selectivity for *ee*-AChEI-CER.

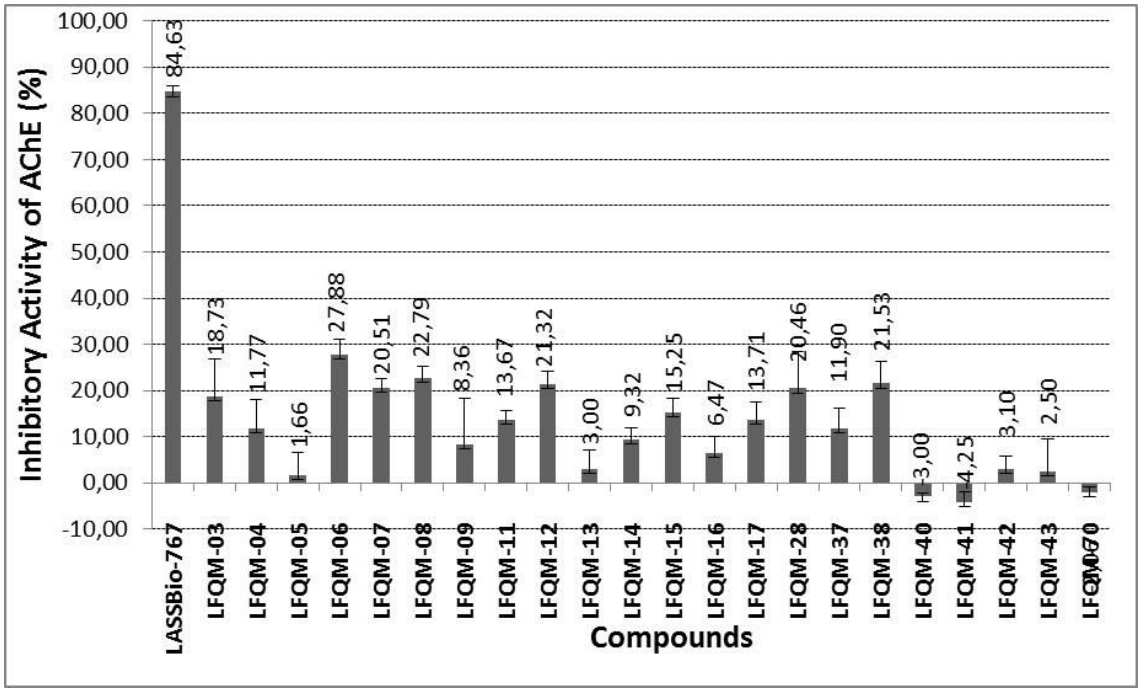

Figure 4. Inhibitory activity of AChE data obtained by the modified Ellman's test

Table 2. Comparative AChE inhibitory data of compounds **3a-3x** in different screening tests

Compound		TLC assays			Microplate assay (200 µM)	*ee*-AChE ICER (200 µM)	*hu*AChE ICER (200 µM)	*In vitro Ellman´s method* (100 µM)
		Ellman´s method[a]	*False positive*[b]	*Fast Blue B salt as reagent*[c]	*Ellman´s method* (%)	*(%)*	*(%)*	*(%)*
Tacrine[*]	**Tacrine**[*]	**+++**	**-**	**+++**	**90.0**	**93.1**	**90.0**	**----**
3a	**LFQM-03**	+	-	+	11.2	16.4	32.40	18.73
3b	**LFQM-04**	+	-	+	11.2	16.3	28.72	11.77
3c	**LFQM-05**	+	-	+	14.8	0	3.51	1.66
3d	**LFQM-06**	+	-	+	11.4	14.5	4.01	27.88
3e	**LFQM-07**	+	-	+	7.1	26.2	3.37	20.51
3f	**LFQM-08**	+	-	+	23.0	23.7	14.55	27.79
3g	**LFQM-09**	+	-	+	31.5	24.5	13.33	8.36
3i	**LFQM-11**	+	-	+	21.8	0	4.06	13.6
3j	**LFQM-12**	+	-	+	31.6	12.6	0.7	21.3

3k	**LFQM-13**	+	-	+	19.3	0	3.79	3.0
3l	**LFQM-14**	+	-	+	22.8	21.5	2.25	9.32
3m	**LFQM-15**	+	-	+	NT	0	7.76	15.25
3n	**LFQM-16**	+	-	+	25.9	24.1	1.7	6.47
3o	**LFQM-17**	+	-	+	NT	0.20	0.20	13.71
3p	**LFQM-28**	+	-	+	32.8	16.2	10.5	20.46
3q	**LFQM-37**	+	-	+	38.6	22.3	6.6	11.90
3r	**LFQM-38**	+	-	+	NT	21.46	1.2	21.53
3s	**LFQM-40**	+	-	+	0	0	0	3.0
3t	**LFQM-41**	+	-	+	45.8	18.6	2.6	4.25
3u	**LFQM-42**	+	-	+	NT	45.1	2.6	3.10
3v	**LFQM-43**	+	-	+	0	0	0	2.5
3x	**LFQM-70**	+	-	+	49.1	41.7	56.0	0

4. Conclusion

Screening assays on the AChE inhibitory activity are suitable to identify new potential drugs. The AChE-ICER assay is appropriate and a fast alternative method for screening libraries of compounds and enables automation and easy handling of large amounts of samples. Although the collection assessed here afforded only weak AChEIs that was prepared as chemical intermediates, structure-activity information from the biological assays could be useful for guiding the design and synthesis of more efficient donepezil-based AChEIs with innovative structural patterns and also help researchers to choose the most adequate method for biological screening.

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