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Article

Aminonaphthoquinone Mannich Bases Derived from Lawsone and Their Copper(II) Complex Derivatives: Synthesis and Potential Cholinesterase Inhibitors as Identified by On-flow Assay

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A new series of Mannich bases derived from 2-hydroxy-1,4-naphthoquinone (lawsone), substituted benzaldehydes and two primary amines, and their Cu^{2+} complexes were synthesized and evaluated for their potential as selective cholinesterase inhibitors (ChEIs). Immobilized capillary enzyme reactors (ICERs) bearing butyrylcholinesterase (BChE) and acetylcholinesterase (AChE) were used not only for the on-flow screening assay but also for determining the inhibitory potency and equilibrium binding constants of the lead inhibitors. Eight copper complexes were identified and characterized as potent reversible and selective ChEIs with inhibitory potencies (IC_{50}) and constants of inhibition (K_i) ranging from 1.24 to 11.5 μmol L^{-1}. One of the compounds was particularly promising, showing IC_{50} and K_i values of 1.24 ± 0.01 and 1.06 ± 0.01 μmol L^{-1}, respectively, for huAChE. These values were lower than those for the standard inhibitor galanthamine (IC_{50} = 206 ± 30.0 and K_i = 126 ± 18.0 μmol L^{-1}). Even though, it is showing noncompetitive inhibition of huAChE and linear mixed-type inhibition of eeAChE. These complexes showed a promising cholinesterase inhibitory activity and can be used as model inhibitors.

Keywords: aminonaphthoquinones, lawsone copper complexes, cholinesterase inhibitors, on-flow assay, biometal-chelating agents

Introduction

Natural and synthetic naphthoquinones present a wide range of biological activities, including anti-cancer,[1-3] antimalarial,[4,5] leishmanicidal,[6] tripanocidal,[7] and molluscicidal effects.[8] Quinones can potentially bind to metal ions in three different oxidations states: (*i*) quinone, (*ii*) its one-electron reduced form, semiquinone, and (*iii*) catechol, the two-electron reduced form. The binding ability of quinones in different oxidations states allows them to play an important role in biological systems.[9] For a representative active group of 1,4-naphthoquinones, which are widely distributed in nature, there are only a few reported examples of metal complexes. Oramas-Royo *et al.*[9] published the synthesis, characterization and potential cytotoxicity in the mouse macrophage leukaemic RAW 264.7 cell line of five metallic complexes of Co^{II}, Ni^{II}, Cu^{II}, Mn^{II} and Zn^{II} with the 1,4-naphthoquinone lawsone. In this sense, the synthesis and biological activities of lanthanide (III)-plumbagin complexes[10] and lapachol complexes[11] had been reported.

The enzymes acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) are ubiquitous cholinesterases (ChEs) among animals. These enzymes play important roles in central and peripheral cholinergic neurotransmission; they also participate in choline ester hydrolysis and xenobiotic detoxification.[12-14] In particular, BChE displays interesting toxicological and pharmacological properties and has been used as a prophylactic and therapeutic drug against some toxic chemicals.[14]

The mode of action of many synthetic chemical pesticides, including organophosphates (OPs) and carbamates, is inhibition of AChE enzymes. Both OPs and carbamates are known to bind and inhibit AChE enzymes,

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causing overstimulation of neurons, which leads to rapid twitching of the muscles, convulsions and insect death.[15]

Although the Mannich bases 2-hydroxy-3-alkylamine-1,4-naphthoquinones and/or their metal complexes have demonstrated a series of important biological effects, such as antimicrobial,[16] antimalarial[17] and molluscicidal activities,[8] the cholinesterase inhibitory activity of these compounds have not yet been reported. Herein, we describe the preparation of a novel series of Mannich bases (**1-10**) derived from 2-hydroxy-1,4-naphthoquinone (lawsone), substituted benzaldehydes and primary amines, and their Cu^{2+} complexes (**11-20**).

The characterization by spectroscopic methods is also given. Furthermore, we report the results of cholinesterase inhibitory activity screening assays for all synthesized compounds. The screening assays, as well as the evaluation of the inhibition mechanism, were carried out on-flow using the appropriate immobilized capillary enzyme reactor (ICER).[18,19]

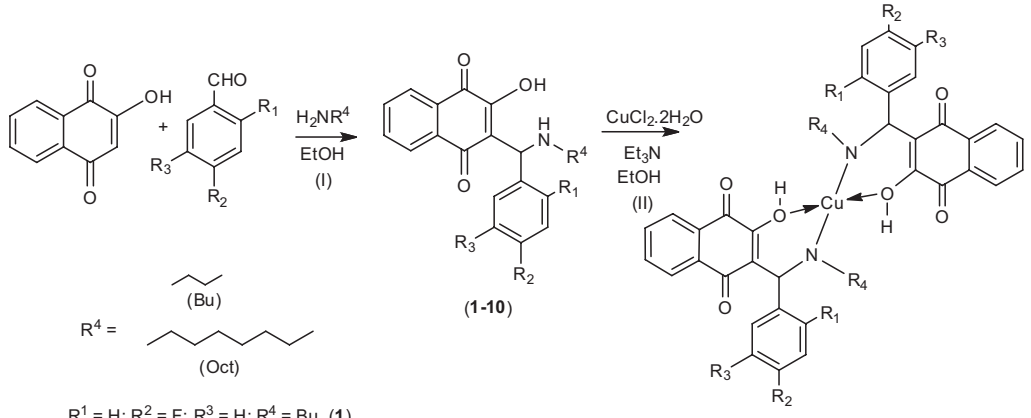
Results and Discussion

Syntheses of the compounds

The Mannich reactions used for the preparation of a series of lawsone derivatives (**1-10**) (Figure 1) were based on the work of Dalgliesh[4] and Neves *et al.*[20] Improvements in the methodology of this reaction were also described.[21-23]

At the first stage (Figure 1), 2-hydroxy-1,4-naphthoquinone undergo an amino alkylation with butylamine (**1**, **2**, **3**, **7**, **9**) or octylamine (**4**, **5**, **6**, **8**, **10**) and benzaldehydes, which led to lawsone derivatives in high yields 70-95% (Figure 1).

Complexes **11**-**20** (Figure 1) were obtained by addition of triethylamine to an ethanolic suspension of compounds (**1**-**10**) and $CuCl_2.2H_2O$ under stirring at room temperature for 8 h, with yields varying from 45 to 93%. All compounds and complexes were characterized by ^1H nuclear magnetic resonance (NMR), ^{13}C NMR (**1-10**), infrared (IR), UV-Vis, and mass spectrometry (MS). For the complexes, elemental analysis confirmed the proposed molecular formula and, as they were not obtained as mono crystals, X-ray data was not obtained. The ^1H NMR of compounds (**1**-**10**) displayed signals of naphthoquinone aromatic hydrogens, H5-H8 which appeared in the δ 7.57-7.90 ppm region as dd or ddd. The hydrogen H11 at δ 5.40 ppm region appeared as a singlet, while the alkyl hydrogens, H19-H22 (butyl) and H19-H26 (octyl) at δ 2.80-0.82 ppm region as multiplets and triplets (H22 and H26). The hydrogens H13, H14, H15, H16 and H17 are aromatic and are in accordance with their substitution pattern for benzyl, 4-fluoro, 4-chloro, 4-trifluoromethoxy or methylenedioxy.



$R^1 = H; R^2 = F; R^3 = H; R^4 = Bu$, (**1**)
$R^1 = H; R^2 = R^3 = OCH_2O; R^4 = Bu$, (**2**)
$R^1 = R^2 = R^3 = H; R^4 = Bu$, (**3**)
$R^1 = H; R^2 = F; R^3 = H; R^4 = Oct$, (**4**)
$R^1 = H; R^2 = R^3 = OCH_2O; R^4 = Oct$, (**5**)
$R^1 = R^2 = R^3 = H; R^4 = Oct$, (**6**)
$R^1 = R^2 = OCF_3; R^3 = H; R^4 = Bu$, (**7**)
$R^1 = R^2 = OCF_3; R^3 = H; R^4 = Oct$, (**8**)
$R^1 = R^2 = Cl; R^3 = H; R^4 = Bu$, (**9**)
$R^1 = R^2 = Cl; R^3 = H; R^4 = Oct$, (**10**)

$R^1 = H; R^2 = F; R^3 = H; R^4 = Bu$, (**11**)
$R^1 = H; R^2 = R^3 = OCH_2O; R^4 = Bu$, (**12**)
$R^1 = R^2 = R^3 = H; R^4 = Bu$, (**13**)
$R^1 = H; R^2 = F; R^3 = H; R^4 = Oct$, (**14**)
$R^1 = H; R^2 = R^3 = OCH_2O; R^4 = Oct$, (**15**)
$R^1 = R^2 = R^3 = H; R^4 = Oct$, (**16**)
$R^1 = R^2 = OCF_3; R^3 = H; R^4 = Bu$, (**17**)
$R^1 = R^2 = OCF_3; R^3 = H; R^4 = Oct$, (**18**)
$R^1 = R^2 = Cl; R^3 = H; R^4 = Bu$, (**19**)
$R^1 = R^2 = Cl; R^3 = H; R^4 = Oct$, (**20**)

Figure 1. Synthesis of Mannich bases **1**-**10** and of complexes **11**-**20**. Reagents and conditions: (I) EtOH, aliphatic amine (butylamine or octylamine, 1.1 equiv.), benzaldehydes (1.1 equiv.), stirred for 12 h, room temperature; (II) EtOH, TEA, $CuCl_2.2H_2O$ (0.5 equiv.), stirred 8 h, room temperature.

The Fourier transform infrared (FTIR) spectra of compounds (**1**-**10**) showed a broad band ranging from 3448 to 3423 cm^{-1} which can be assigned to νO–H and νN–H. The complexes (**11**-**20**) showed two new broad bands ranging from 3546 to 3274 cm^{-1} and 3953 to 3276 cm^{-1}which can be assigned to νN–H. This shift in νN–H frequency infers the complexation of the ligands to the Cu^{2+} center.[16] Two small bands around 430 and 380 cm^{-1} can be assigned to Cu–O and to Cu–N, respectively. These bands were also not observed in the spectra of compounds (**1**-**10**). The FTIR showed a series of band that can be attributed to the aromatic moiety such as 3100-3200 cm^{-1} νC–H and alkyl (butyl) or (octyl) ranging from 2960 to 2800 cm^{-1} νC–H.

Electrospray ionization Fourier transform ion cyclotron resonance mass spectrometry (ESI-FT-ICR MS) spectra showed fragmentation patterns compatible with the proposed molecular structures as depicted in Figure 2.

Biological activity

The on-flow cholinesterase ICER assays[18] were successfully employed to probe whether the synthesized compounds (Figure 1) acted as inhibitors towards AChE and BChE.

According to the literature, ChEs are sensitive to metals, and in the presence of high metal concentrations, the activity of free ChEs diminishes significantly.[24] Indeed, the results reported herein disclosed that the Cu^{2+} complexes possessed higher inhibitory potency than did the corresponding monomer (Figure 3).

Complexes (**11**-**20**) displayed significant and selective inhibitory activity (> 70%) against AChE from human erytrocites (huAChE) and eeAChE (from electric eel) at 200 μmol L^{-1} (Figure 3). None of the tested compound showed activity towards huBChE (from human serum). For the best AChE inhibitors, the IC_{50} values and inhibition constants K_i were determined. The results presented in Table 1 revealed a number of AChE inhibitors with IC_{50} and K_i values of the same order of magnitude as those of galanthamine.

Using the appropriate equations, the mechanism type for each active compound was determined.[25,26]

Inhibition mechanism studies conducted on the tested compounds showed that the Lineweaver-Burk double reciprocal plots obtained in the absence (control) and in the presence of each compound intersected above or below the 1/[ATChI]-axis, suggesting that the inhibition was hyperbolic or linear mixed-type.[25,26] The constant K_i can be

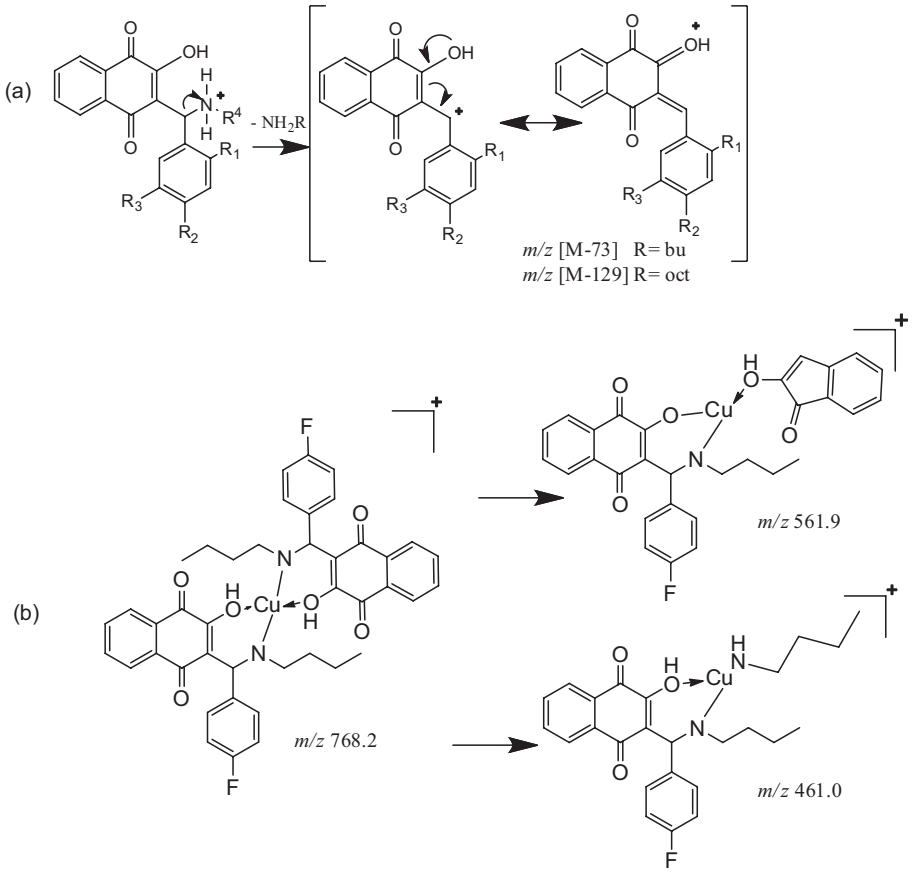


Figure 2. Proposed formation of ions fragments $[M - 73]^+$ and $[M - 129]^+$ (a) and fragmentation pathway for compound **11** (b).

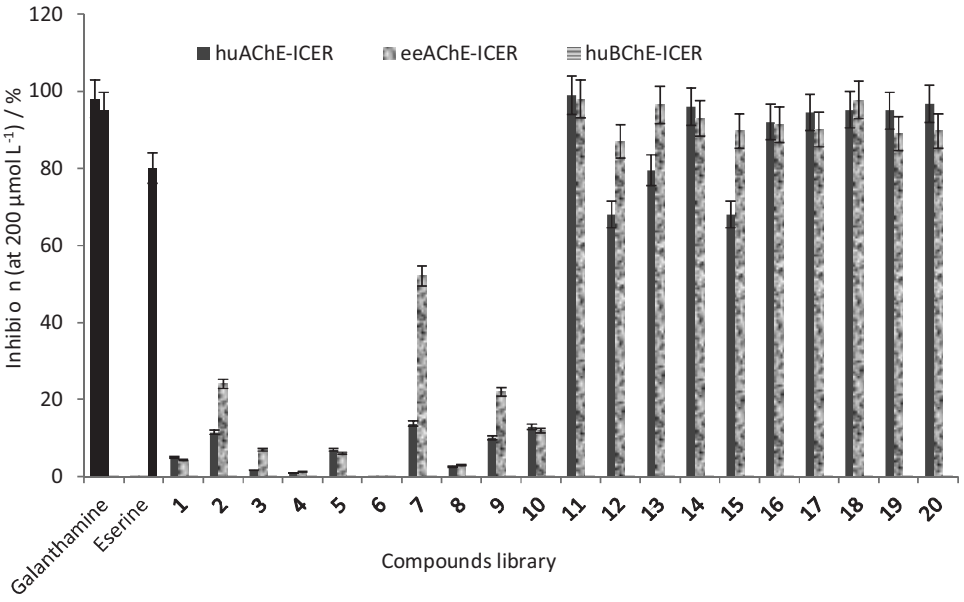


Figure 3. Inhibition of eeAChE, huAChE and huBChE activity by standard inhibitors and compounds **1**-**20** at 200 µmol L^{-1}.

Table 1. IC_{50} and K_i values calculated for selected compounds against eeAChE and huAChE-ICER

Compound	huAChE-ICER			eeAChE-ICER		
	IC_{50} ± SEM / (µmol L^{-1})	K_i ± SEM / (µmol L^{-1})	Mechanism type	IC_{50} ± SEM / (µmol L^{-1})	K_i ± SEM / (µmol L^{-1})	Mechanism type
Galanthamine[a]	206 ± 30	126 ± 18	competitive	12.8 ± 2[b]	11.9 ± 4[b]	competitive
11	1.75 ± 0.4	0.74 ± 0.1	linear mixed-type	10.0 ± 1	0.32 ± 0.09	linear mixed-type
13	11.5 ± 1	1.42 ± 0.2	hyperbolic mixed-type	2.05 ± 0.4	0.16 ± 0.09	hyperbolic mixed-type
14	5.94 ± 0.7	1.35 ± 0.3	hyperbolic mixed-type	1.31 ± 0.1	0.17 ± 0.01	noncompetitive
16	8.19 ± 0.7	0.49 ± 0.1	hyperbolic mixed-type	2.9 ± 0.3	2.88 ± 0.3	linear mixed-type
17	1.24 ± 0.1	1.06 ± 0.1	noncompetitive	0.74 ± 0.1	0.28 ± 0.07	linear mixed-type
18	2.37 ± 0.7	0.65 ± 0.03	noncompetitive	2.78 ± 0.7	0.94 ± 0.03	noncompetitive
19	3.05 ± 0.4	2.14 ± 0.4	hyperbolic mixed-type	0.88 ± 0.15	2.09 ± 0.5	linear mixed-type
20	1.65 ± 0.14	0.8 ± 0.1	noncompetitive	0.12 ± 0.02	0.27 ± 0.09	noncompetitive

IC_{50}: inhibitory potency; K_i: inhibition constant; SEM: standard error of the mean; [a]standard inhibitor; [b]obtained from the publication da Silva *et al*.[18]

determined from the replots of primary reciprocal plot data. The slope and 1/v-axis intercept of each can be replotted *versus* the corresponding inhibitor concentration. When the slope *versus* [I] and/or the 1/v-axis intercept *versus* [I] replots are linear, the result indicates linear mixed-type inhibition, and when the replots are hyperbolics, the result indicates hyperbolic mixed-type inhibition.[26]

Figure 4 illustrates some examples of the inhibition mechanisms observed for the tested compounds. Plots that intersected the X-axis indicated a non-competitive mechanism. In contrast to galanthamine, a competitive AChEI, we found that compound **11** constituted a linear mixed-type inhibitor of both eeAChE and huAChE enzymes, and compound **13** exerted inhibition of the hyperbolic mixed-type for both ee and huAChE enzymes (Figure 4).

Compounds **18** and **20** followed noncompetitive mechanism for both ee and huAChE (Figures S32-36b, S33-37b, respectively in the Supplementary Information (SI) section). An interesting observation for compound **14**, that displayed inhibition pattern of the hyperbolic mixed-type for huAChE and noncompetitive for eeAChE, and for compound **17** that showed higher activity towards both enzymes and even showed noncompetitive mechanism for huAChE but linear mixed-type for eeAChE (Figure 5). Although, compound **19** exerted inhibition mixed-type it followed linear and hyperbolic mixed-type to eeAChE and huAChE, respectively (Figures S34-35b, respectively).

False-positive effects on BChE and AChE inhibition

All active inhibitors were screened for false positive results.[27] None of the compounds fell into this category.

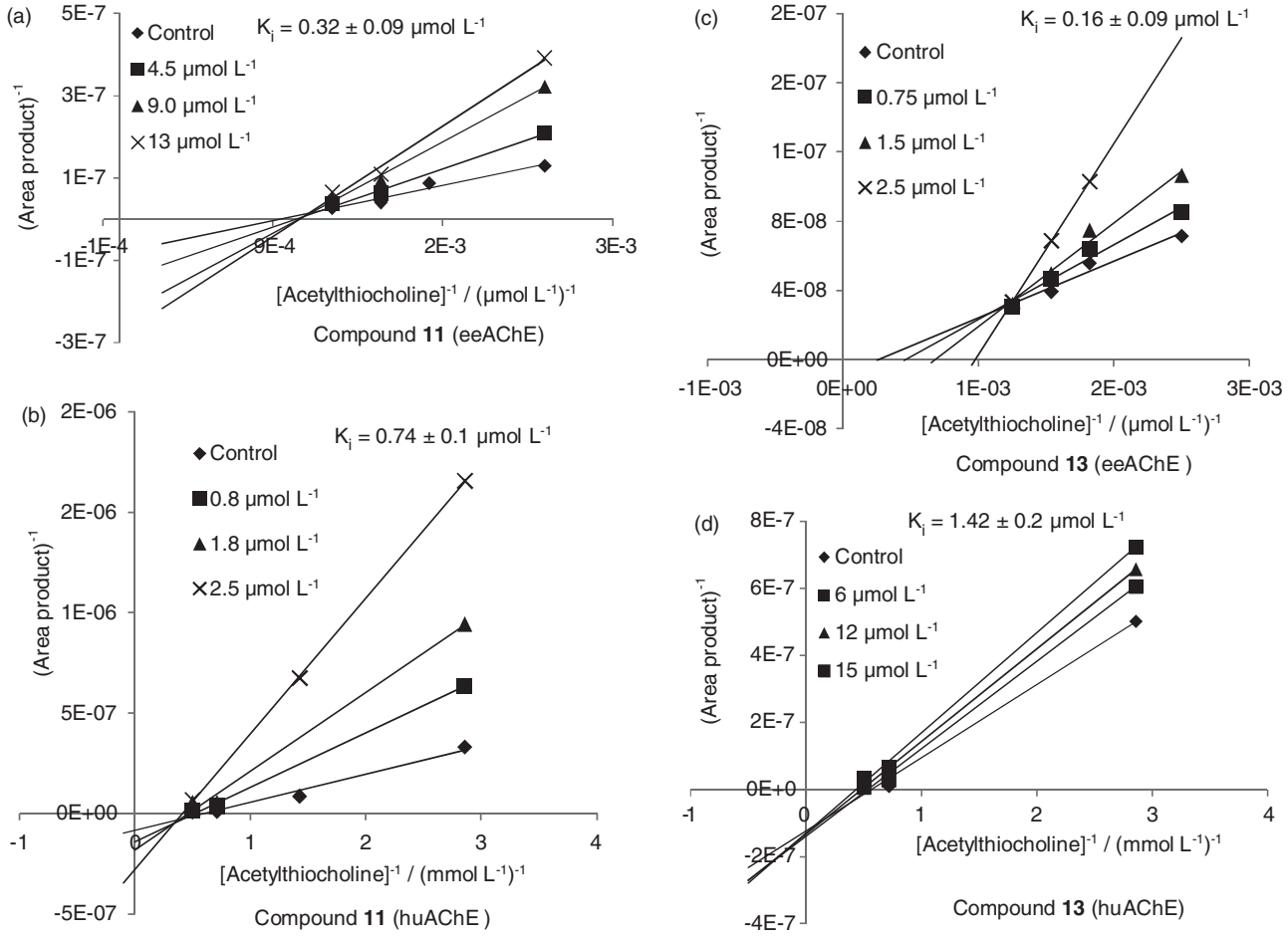


Figure 4. Inhibition mechanisms. Lineweaver-Burk graph for compound (**11**) (a) towards eeAChE-ICER and (b) towards huAChE-ICER. For compound **13** (c) line Lineweaver-Burk graph towards eeAChE-ICER and (d) towards huAChE-ICER.

Conclusions

A series of 20 aminonaphthoquinone Mannich bases and their Cu^{2+} complexes was synthesized and screened as cholinesterase (AChE and BChE) inhibitors. The Cu^{2+} complexes showed higher activity when compared to the free Mannich bases against AChE. None of the tested compounds were active against BChE. The AChE-ICERs were used not only for the inhibition screening assays but also for determining the IC_{50}, K_i, and the mechanism of action modalities for the Cu^{2+} complexes in this series that were 'hits'. The results reported herein disclose lawsone metal derivatives as templates for ChEIs.

Experimental

General

The enzymes acetylcholinesterase (AChE, EC 3.1.1.7, from electric eel, 426 units mg^{-1} and from human erythrocytes, 2419 units mg^{-1}) and butyrylcholinesterase (BChE EC 3.1.1.8, from human serum, 50 units mg^{-1}) as lyophilized powder, their substrates acetylthiocholine iodide (ACThI) and butyrylthiocholine iodide (BTChI), 5,5'-dithiobis(2-nitrobenzoic acid) (Ellman's reagent or DTNB); galanthamine (galanthamine hydrobromide), eserine and all the reagents used in the synthesis were obtained from Sigma-Aldrich (St. Louis, MO, USA). Glutaraldehyde, buffer components, and all the chemicals used during the immobilization procedure were analytical grade and were supplied by Sigma, Merck (Darmstadt, Germany), Synth (São Paulo, Brazil), or Acros (Geel, Belgium). The water used in all the preparations had been purified using a Millipore Milli-Q® system (Millipore, São Paulo, Brazil). The fused silica capillary (0.375 mm × 0.1 mm i.d.) was acquired from Polymicro Technologies (Phoenix, AZ, USA). All buffer solutions were filtered through nylon membrane filters (0.45 µm) provided by Millipore (São Paulo, Brazil). Stock solutions (1 mmol L^{-1}) of the evaluated inhibitors were prepared in methanol/water

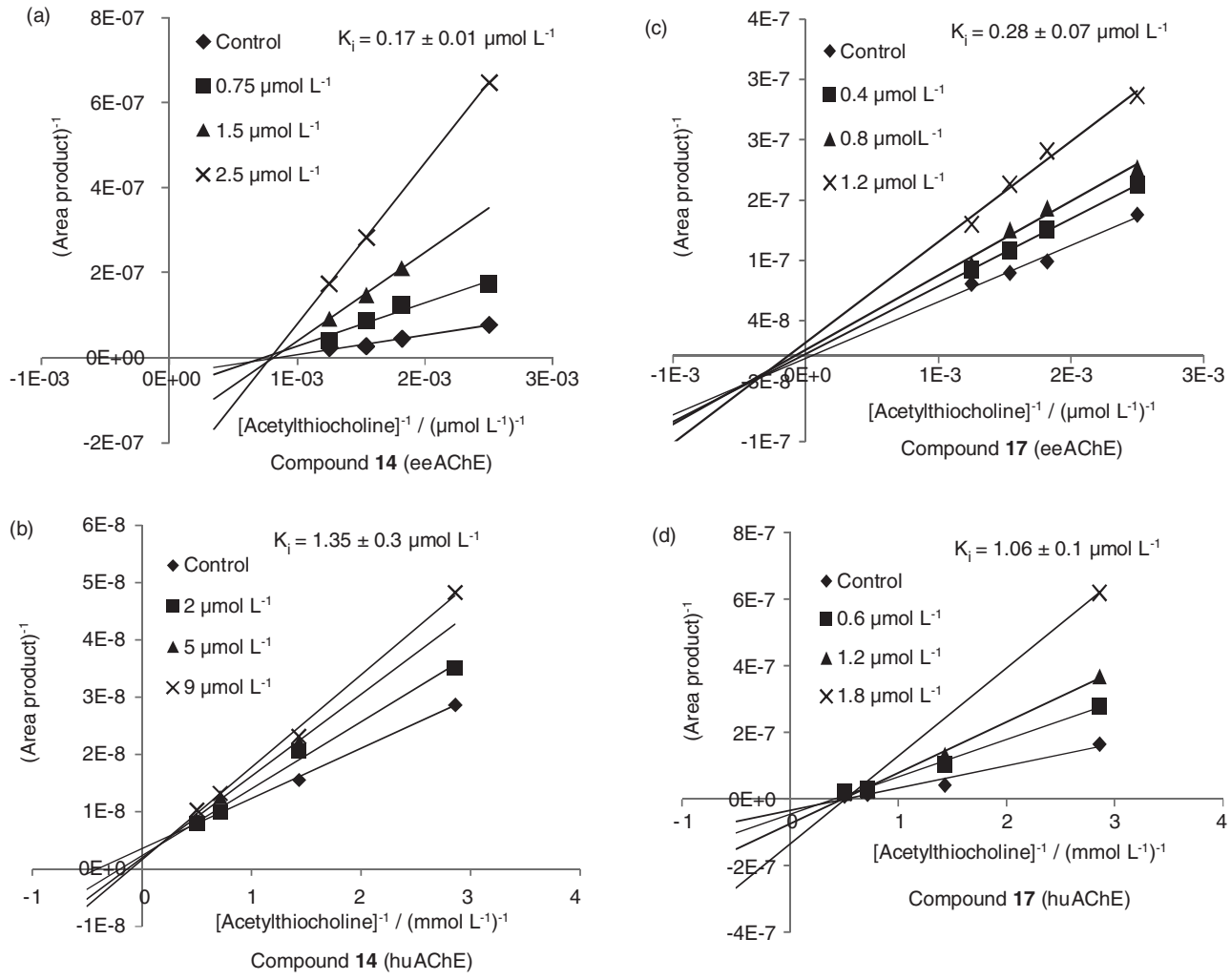


Figure 5. Inhibition mechanism studies: Lineweaver-Burk graphs for compound (**14**) (a) towards eeAChE-ICER and (b) towards huAChE-ICER; the Lineweaver-Burk graphs for compound **17** (c); towards eeAChE-ICER and (d) towards huAChE-ICER.

(50% v:v) and diluted with methanol/water (50% v:v) to give the desired concentration range.

Apparatus

The eeAChE (AChE from Electric eel), huAChE (from human erythrocytes) and huBChE (from the huma serum) ICERs were prepared according to previously reported protocols.[18,19] The enzyme immobilization was carried out using a 341B syringe pump (Sage instruments, Boston, USA). The ICERs were placed in a Shimadzu HPLC system (Shimadzu, Kyoto, Japan) consisting of two LC 20AD pumps. One of the pumps had an FCV-20AL valve for a low-pressure gradient, a UV-Vis detector (SPD-M20AV), and an auto-sampler (SIL-20A). Data were acquired on a Shimadzu CBM-20A system interfaced with a computer using the Shimadzu-LC Solutions (LC Solution 2.1) software (Shimadzu, Kyoto, Japan). All the liquid chromatography (LC) analyses were performed at room temperature (ca. 20 °C).

Chromatographic conditions

Mobile phase: Tris (pH 8.0) 0.1 mol L^{-1} and Ellman's reagent 1.26 × 10^{-4} µmol L^{-1} were designated as working buffer and used as the mobile phase for all the chromatographic experiments employing the ICERs. Chromatographic analyses using eeAChE, huAChE, and BChE-ICER were conducted as previously reported.[18,19]

General procedure of synthesis

All the melting points (mp) were determined in open capillaries on a Buchi 535 apparatus. Proton (^{1}H) and carbon (^{13}C) nuclear magnetic resonance (NMR) spectra were recorded on a Bruker DRX-400, ARX 200, Avance III

400 nano spectrometer using deuterated solvents. Optical rotation was determined on a Perkin Elmer 241 polarimeter. Mass spectra were acquired on a Shimadzu GCMS-QP5000 Micromass Quattro LC spectrometer coupled with the liquid chromatography Waters Alliance 2695. Elemental analyses were accomplished on a Fisons EA 1108 CHNS-O. UV-Vis and infrared spectra were obtained on an 8453 UV-Visible Spectrophotometer, G1103A, Agilent Technologies and on a BOMEM spectrophotometer, (BM-Aridzone series) using KBr plates, respectively.

Synthesis of the Mannich bases **1-10**

Compounds **1-10** (Figure 1) were synthesized according to Dalgliesh[4] and Neves *et al.*[20] with modifications.[28,29] Briefly, a suspension of lawsone (1 mmol) in a round-bottom flask (20 mL) in 10 mL of ethanol was stirred at room temperature under a nitrogen atmosphere until complete dissolution. Then, 1.1 mmol of primary amine (butylamine or octylamine, 1.1 equiv.) was added under constant stirring. This reaction immediately afforded an intense red solution. After 20 min, 1.1 mmol of benzaldehyde (benzaldehyde, 4-fluoro-benzaldehyde, 4-chloro-benzaldehyde, piperonal or 4-trifluoromethoxy-benzaldehyde) was added, and the mixture was left stirring at room temperature for 12 h in the dark. The orange or red solid precipitate was filtered and washed with cold ethanol and ethyl ether and dried under a vacuum.

3-[*N*-(*n*-Butyl)4-fluoro-aminobenzyl]-2-hydroxy-1,4-naphthoquinone (**1**)

Dark orange powder; yield 93%; UV-Vis (methanol) λ_{max} / nm 205, 272, 450; IR (KBr) ν_{max} / cm^{-1} 3441 (O–H and N–H), 3130 (C–H aromatic), 2958, 2930, 2868 (C–H, aliphatic), 1682 (C=O), 1589, 1520 (C=C, aromatic), 1280 (C–N), 1228 (C–F aromatic); ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.90 (dd, 1H, *J* 8.0, 2.0 Hz, H5), 7.82 (dd, 1H, *J* 8.0, 2.0 Hz, H8), 7.70 (dt, 1H, *J* 8.0, 2.0 Hz, H7), 7.64 (ddd, 2H, H13, H17, H13-F, H17-F), 7.58 (dt, 1H, *J* 8.0, 2.0 Hz, H6), 7.18 (bt, 2H, H14, H16, F-H14, F-H16), 5.49 (s, 1H, H11), 2.85 (brt, 2H, *J* 8.0 Hz, H19), 1.59 (m, 2H, H20), 1.28 (m, 2H, H21), 0.83 (t, 3H, *J* 8.0 Hz, H22); ^{13}C NMR (100 MHz, DMSO-d_6) δ 184.2, 178.3, 170.6, 163.0, 134.6, 133.7, 131.5, 130.8, 130.0, 125.3, 125.0, 115.2, 115.0, 111.0, 58.1, 45.4, 27.6, 19.3, 13.5; anal. calcd. for $C_{21}H_{20}FNO_3$: C, 71.37; H, 5.70; N, 3.96%; found: C, 70.80; H, 5.50; N, 3.91%; MS *m/z* 354.1 $[M + H]^+$, 281.1 $[M - 73]^+$, 263.1.

3-[*N*-(*n*-Butyl)aminopiperonyl]-2-hydroxy-1,4-naphthoquinone (**2**)

Dark orange powder; yield 89%; UV-Vis (methanol) λ_{max} / nm 205, 273, 453; IR (KBr) ν_{max} / cm^{-1} 3446 (O–H and N–H), 3066 (C–H aromatic), 2962, 2935, 2873 (C–H, aliphatic), 2773 (-CH_2–methylenedioxy), 1678 (C=O), 1591, 1523 (C=C aromatic), 1274 (C–N); ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.90 (dd, 1H, *J* 8.0, 2.0 Hz, H5), 7.81 (dd, 1H, *J* 8.0, 2.0 Hz, H8), 7.70 (dt, 1H, *J* 8.0, 2.0 Hz, H7), 7.57 (dt, 1H, *J* 8.0 Hz, H6), 7.22 (d, 1H, *J* 2.00 Hz, H16), 7.03 (dd, 1H, *J* 2.0, 8.0 Hz, H17), 6.87 (dd, 1H, *J* 8.0 Hz, H13), 5.98 (dd, 2H, *J* 8.0, 2.0 Hz -CH_2–methylenedioxy), 5.40 (s, 1H, H11), 2.82 (brt, 2H, *J* 8.0 Hz, H19), 1.57 (m, 2H, H20), 1.28 (m, 2H, H21), 0.83 (t, 3H, *J* 8.0 Hz, H22); ^{13}C NMR (100 MHz, DMSO-d_6) δ 184.2, 178.3, 170.4, 147.0, 146.8, 134.5, 133.6, 132.2, 130.7, 125.2, 125.0, 121.6, 111.2, 108.2, 107.8, 100.9, 58.7, 45.2, 27.5, 19.1, 13.4; anal. calcd. for $C_{22}H_{21}NO_5$: C, 69.64; H, 5.58; N, 3.69%; found: C, 69.76; H, 5.61; N, 3.71%; MS *m/z* 380.1 $[M + H]^+$, 307.2 $[M - 73]^+$, 289.1.

3-[*N*-(*n*-Butyl)aminobenzyl]-2-hydroxy-1,4-naphthoquinone (**3**)

Dark orange powder; yield 86%; UV-Vis (methanol) λ_{max} / nm 207, 272, 453; IR (KBr) ν_{max} / cm^{-1} 3448 (O–H and N–H), 3060, 3032 (C–H, aromatic), 2960, 2933, 2866 (C–H, aliphatic), 1679 (C=O), 1589, 1529 (C=C, aromatic), 1276 (C–O), 1234 (C–N); ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.90 (dd, 1H, *J* 8.0, 2.0 Hz, H5), 7.81 (dd, 1H, *J* 8.0, 2.0 Hz, H8), 7.70 (td, 1H, *J* 8.0, 2.0 Hz, H7), 7.60 (m, 1H, H6), 7.59 (m, 2H, H-Ph), 7.35 (m, 2H, H-Ph), 7.29 (m, 1H, H-Ph), 5.49 (s, 1H, H11), 2.86 (brt, 2H, *J* 8.0 Hz, H19), 1.58 (m, 2H, H20), 1.28 (m, 2H, H21), 0.83 (t, *J* 8.0, 2.2 Hz, 3H, H22); ^{13}C NMR (100 MHz, DMSO-d_6) δ 184.3, 178.4, 170.6, 138.7, 134.8, 133.7, 131.5, 130.8, 128.6, 128.3, 127.9, 125.3, 125.0, 111.1, 58.83, 45.4, 27.6, 19.3, 13.5; anal. calcd. for $C_{21}H_{21}NO_3$: C, 75.20; H, 6.31; N, 4.18%; found: C, 75.3; H, 6.10; N, 4.19%; MS *m/z* 336.2 $[M + H]^+$, 263.1 $[M - 73]^+$, 245.1, 217.1.

3-[*N*-(*n*-Octyl)4-fluoro-aminobenzyl]-2-hydroxy-1,4-naphthoquinone (**4**)

Dark orange powder; yield 80%; UV-Vis (methanol) λ_{max} / nm 204, 272, 444; IR (KBr) ν_{max} / cm^{-1} 3438 (O–H and N–H), 3074 (C–H, aromatic), 2956, 2922, 2854 (C–H, aliphatic), 1679 (C=O), 1589, 1533 (C=C, aromatic), 1274 (C–N), 1224 (C–F); ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.90 (brd, 1H, *J* 8.0 Hz, H5), 7.80 (brd, 1H, *J* 8.0 Hz, H8), 7.70 (brt, 1H, *J* 8.0 Hz, H7), 7.63 (ddd, 2H, H13, H17, H13-F, H17-F), 7.58 (brt, 1H, *J* 8.0 Hz, H6), 7.18 (bt, 2H, H14, H16, F-H14, F-H16), 5.49 (s, 1H, H11), 2.85 (brt, 2H, *J* 8.0 Hz, H19), 1.59 (m, 2H, H20), 1.19 (brs, 12H, H21-H25), 0.82 (t, 3H, *J* 8.0 Hz, H26); ^{13}C NMR (100 MHz, DMSO-d_6) δ 184.2, 178.3, 170.6, 134.6, 133.7,

131.5, 130.0, 125.3, 125.0, 115.2, 115.0, 58.0, 45.6, 31.1, 28.3, 25.8, 25.4, 22.0, 13.9; anal. calcd. for $C_{25}H_{28}FNO_3$: C, 73.33; H, 6.89; N, 3.42%; found: C, 72.04; H, 6.68; N, 3.37%; MS *m/z* 410.2 $[M + H]^+$, $[M - 129]^+$, 263.2.

3-[*N*-(*n*-Octyl)aminopiperonyl]-2-hydroxy-1,4-naphthoquinone (**5**)

Red powder; yield 87%; UV-Vis (methanol) λ_{max} / nm 205, 273, 451; IR (KBr) ν_{max} / cm^{-1} 3423 (O–H and N–H), 3070 (C–H, aromatic), 2954, 2931, 2852 (C–H, aliphatic), 2705 (-CH_2–methylenedioxy), 1672 (C=O), 1581, 1523 (C=C, aromatic), 1276 (C–N); ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.90 (brd, 1H, *J* 8.0 Hz, H5), 7.82 (brd, 1H, *J* 8.0 Hz, H8), 7.70 (brt, 1H, *J* 8.0 Hz, H7), 7.58 (brt, 1H, *J* 8.0 Hz, H6), 7.22 (brs, 1H, H16), 7.04 (brd, 1H, *J* 8.0 Hz, H17), 6.87 (brd, 1H, *J* 8.0 Hz, H13), 5.98 (brd, 2H, *J* 8.0, 2.0 Hz, -CH_2–methylenedioxy), 5.42 (s, 1H, H11), 2.83 (brt, 2H, *J* 8.0 Hz, H19), 1.59 (m, 2H, H20), 1.19 (brs, 14H, H21-H25), 0.82 (t, 3H, *J* 8.0 Hz, H26); ^{13}C NMR (100 MHz, DMSO-d_6) δ 184.3, 178.4, 170.5, 147.1, 146.9, 134.6, 133.7, 132.3, 131.5, 130.8, 125.3, 125.0, 121.6, 111.3, 108.3, 107.9, 101.0, 58.7, 45.5, 31.1, 28.4, 25.8, 25.4, 22.0, 13.9; anal. calcd. for $C_{26}H_{29}NO_5$: C, 71.70; H, 6.71; N, 3.22%; found: C, 67.87; H, 6.58; N, 3.19%; MS *m/z* 436.2 $[M + H]^+$, 307.1 [M – 129], 289.0.

3-[*N*-(*n*-Octyl)aminobenzyl]-2-hydroxy-1,4-naphthoquinone (**6**)

Dark orange powder; yield 89%; UV-Vis (methanol) λ_{max} / nm 205, 272, 451; IR (KBr) ν_{max} / cm^{-1} 3434 (O–H and N–H), 3062 (C–H, aromatic), 2956, 2921, 2854 (C–H, aliphatic), 1679 (C=O), 1589, 1535 (C=C, aromatic), 1274 (C–N); ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.91 (dd, 1H, *J* 8.0, 1.70 Hz, H5), 7.82 (dd, 1H, *J* 8.0, 2.0 Hz, H8), 7.70 (dt, 1H, *J* 8.0, 2.0 Hz, H7), 7.60 (m, 1H, H6), 7.58 (m, 2H, H-Ph), 7.36 (m, 3H, H-Ph), 5.51 (s, 1H, H11), 2.87 (brt, 2H, *J* 8.0 Hz, H19), 1.62 (m, 2H, H20), 1.59 (brs, 12H, H21-H25), 0.83 (t, 3H, *J* 8.0 Hz, H26); ^{13}C NMR (100 MHz, DMSO-d_6) δ 184.3, 178.4, 170.6, 138.7, 134.6, 133.7, 131.5, 130.8, 128.3, 127.7, 125.3, 125.0, 111.0, 58.7, 45.6, 31.1, 28.3, 25.8, 25.4, 22.0, 13.9; anal. calcd. for $C_{25}H_{29}NO_3$: C, 76.70; H, 7.47; N, 3.58%; found: C, 74.00; H, 6.83; N, 3.65%; MS *m/z* 392.2 $[M + H]^+$, 263.1 $[M - 129]^+$, 245.1, 217.1, 130.3.

3-[*N*-(*n*-Butyl)4-trifluoromethoxy-aminobenzyl]-2-hydroxy-1,4-naphthoquinone (**7**)

Dark orange powder; 95%; UV-Vis (methanol) λ_{max} / nm 205, 272, 448; IR (KBr) ν_{max} / cm^{-1} 3438 (O–H and N–H), 3068 (C–H, aromatic), 2952, 2935, 2875 (C–H, aliphatic), 1676 (C=O), 1591, 1525 (C=C, aromatic), 1263, 1105 (C–O–C), 1218 (C–F), 1166 (C–N); ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.91 (brd, 1H, *J* 8.0 Hz, H5), 7.83 (brd, 1H, *J* 8.0 Hz, H8), 7.74 (m, 1H, H6), 7.59 (brt, 1H, *J* 4.0 Hz, H7) , 7.37 (d, 2H, *J* 8.0 Hz, H13 and 17), 7.72 (m, 2H, H14 and 16), 5.55 (s, 1H, H11), 2.89 (brt, 2H, *J* 8.0 Hz, H19), 1.60 (m, 2H, H20), 1.29 (m, 2H, H21) 0.83 (t, 3H, *J* 8.0 Hz, H24); ^{13}C NMR (100MHz, DMSO-d_6) δ 181.5, 175.7, 168.0, 145.1, 135.5, 131.9, 131.1, 128.9, 128.2, 127.0, 122.7, 122.4, 118.3, 108.0, 55.4, 42.8, 25.0, 16.6, 10.8; anal. calcd. for $C_{22}H_{20}F_3NO_4$: C, 65.50; H, 5.00; N, 3.47%; found: C, 66.30; H, 5.10; N, 3.60%; MS *m/z* 420.2 $[M + H]^+$, 347.1 $[M - 73]^+$, 329.1, 319.1, 310.1.

3-[*N*-(*n*-Octyl)4-trifluoromethoxy-aminobenzyl]-2-hydroxy-1,4-naphthoquinone (**8**)

Dark orange powder; yield 70%; UV-Vis (methanol) λ_{max} / nm 209, 272, 449; IR (KBr) ν_{max} / cm^{-1} 3442 (O–H and N–H), 3068 (C–H, aromatic), 2956, 2931, 2856 (C–H, aliphatic), 1674 (C=O), 1591, 1533 (C=C, aromatic), 1265, 1162 (C–O–C), 1220 (C–F), 1109 (C–N); ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.91 (dd, 1H, *J* 8.0, 1.7 Hz, H5), 7.83 (dd, 1H, *J* 8.0, 2.0 Hz, H8), 7.70 (dt, 1H, *J* 8.0, 2.0 Hz, H6), 7.58 (dt, 1H, *J* 8.0, 2.0 Hz, H7), 7.23 (m, 2H, H13 and H17), 7.35 (d, *J* 8.0 Hz, 2H, H14 and H16), 5.56 (s, 1H, H11), 2.88 (brt, 2H, *J* 8.0 Hz, H19), 1.60 (m, 2H, H20), 1.19 (m, 12H, H21-H25), 0.83 (t, 3H, *J* 8.0 Hz, H26); ^{13}C NMR (100 MHz, DMSO-d_6) δ 184.2, 178.4, 170.6, 147.8, 138.1, 134.6, 133.7, 131.5, 130.9, 129.7, 125.4, 125.1, 120.9, 110.6, 58.0, 45.7, 31.1, 28.3, 25.8, 25.4, 22.0, 13.9; anal. calcd. for $C_{26}H_{28}F_3NO_4$: C, 65.67; H, 5.94; N, 2.95%; found: C, 72.00; H, 6.13; N, 3.18%; MS *m/z* 476.3 $[M + H]^+$, 347.1 $[M - 129]^+$, 329.1, 301.1, 130.2.

3-[*N*-(*n*-Butyl)4-chloro-aminobenzyl]-2-hydroxy-1,4-naphthoquinone (**9**)

Dark orange powder; yield 78%; UV-Vis (methanol) λ_{max} / nm 219, 272, 448; IR (KBr) ν_{max} / cm^{-1} 3429 (O–H and N–H), 3115 (C–H, aromatic), 2960, 2933 (C–H, aliphatic), 1679 (C=O), 1581, 1523 (C=C, aromatic), 1228 (C–N), 1274 (C–O), 1095, 738 (C–Cl); ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.90 (d, 1H, *J* 8.0 Hz, H5), 7.81 (d, 1H, *J* 8.0 Hz, H8), 7.70 (t, 1H, *J* 8.0, 2.0, H7), 7.57 (brd, 1H, *J* 8.0 Hz, H6), 7.41 (brd, 2H, H14 and H16), 7.61 (brd, 2H, H13, H17), 5.49 (s, 1H, H11), 2.85 (brt, 2H, *J* 8.0 Hz, H19), 1.62 (m, 2H, H20), 1.33 (m, 2H, H21), 0.83 (t, 3H, *J* 8.0 Hz, H22); ^{13}C NMR (100 MHz, DMSO-d_6) δ 184.4, 178.3, 170.7, 134.6, 133.7, 132.4, 131.5, 130.8, 129.6, 128.2, 125.3, 125.1, 117.0, 113.4, 110.9, 58.0, 45.4, 27.7, 19.3, 13.5; anal. calc. for $C_{21}H_{20}ClNO_3$: C, 68.20; H, 5.45; N, 3.79%; found: C, 69.00; H, 5.60; N, 4.00%; MS *m/z* 370.1 $[M + H]^+$, 297.1 $[M - 73]^+$, 279.0, 251.1.

3-[*N*-(*n*-Octyl)4-chloro-aminobenzyl]-2-hydroxy-1,4-naphthoquinone (**10**)

Orange powder; yield 75%; UV-Vis (methanol) λ_{max} / nm 219, 273, 451; IR (KBr) ν_{max} / cm^{-1} 3442 (O–H and N–H), 3062 (C–H, aromatic), 2956, 2925, 2852 (C–H, aliphatic), 1672 (C=O), 1591, 1525 (C=C, aromatic), 1274 (C–O), 1089, 732 (C–Cl); ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.90 (brd, 1H, *J* 8.0 Hz, H5), 7.82 (brd, 1H, *J* 8.0 Hz, H8), 7.70 (brt, 1H, *J* 8.0, 2.0 Hz, H7), 7.58 (bd, 1H, *J* 8.0 Hz, H6), 7.41 (brd, 2H, H14 and H16), 7.61 (brd, 2H, H13 and H17), 5.50 (s, 1H, H11), 2.86 (brt, 2H, *J* 8.0 Hz, H19), 1.60 (m, 2H, H20), 1.19 (brs, 12H, H21-H25), 0.83 (t, 3H, *J* 8.0 Hz, H26); ^{13}C NMR (100 MHz, DMSO-d_6) δ 184.2, 178.3, 170.6, 137.6, 134.6, 133.7, 132.4, 130.8, 129.6, 128.2, 125.3, 125.0, 110.7, 58.0, 45.6, 31.1, 28.3, 25.8, 25.4, 22.0, 13.9; anal. calcd. for $C_{25}H_{28}ClNO_3$: C, 70.49; H, 6.63; N, 3.29%; found: C, 68.62; H, 6.26; N, 3.38%; MS *m/z* 426.3 $[M + H]^+$, 297.0 $[M - 129]^+$, 279.1, 130.3.

Synthesis of complexes $[Cu(L)_2]$ **11-20** from compounds **1-10**

Compounds **11**-**20** (Figure 2) were synthesized according to Neves *et al.*[20] with modifications. Suspension of ligands (2 mmol) in a round-bottom flask (20 mL) in 10 mL of ethanol was stirred at room temperature, and after 10 min, triethylamine (50 µL) was added. Then, $CuCl_2.2H_2O$ (1 mmol) in 5 mL of ethanol was added. The reaction was left under constant stirring for 8 h at room temperature and protected from light. The brown solid precipitate was filtered and washed with cold ethanol and acetone and dried under a vacuum.

Cu(3-[*N*-(*n*-Butyl)4-fluoro-aminobenzyl]-2-hydroxy-1,4-naphthoquinone)$_2$ (**11**)

Brown powder; yield 48.3%; UV-Vis (methanol) λ_{max} / nm 205, 264, 427; IR (KBr) ν_{max} / cm^{-1} 3512, 3438 (O–H and N–H), 3168 (C–H, aromatic), 2958, 2929, 2871 (C–H, aliphatic), 1672 (C=O), 1591, 1544 (C=C, aromatic), 1276 (C–N), 1234 (C–F), 430 (Cu–O), 385 (Cu–N); anal. calcd. for $C_{42}H_{38}CuF_2N_2O_6$: C, 65.66; H, 4.99; N, 3.65%; found: C, 60.00; H, 5.05; N, 3.45%; MS *m/z* 768.2 $[M]^+$, 707.2, 561.9, 461.0.

Cu(3-[*N*-(*n*-Butyl)aminopiperonyl]-2-hydroxy-1,4-naphthoquinone)$_2$ (**12**)

Dark yellow powder; yield 90%; UV-Vis (methanol) λ_{max} / nm 204, 267, 448; IR (KBr) ν_{max} / cm^{-1} 3525, 3460 (O–H and N–H) 2964, 2937, 2875 (C–H, aliphatic), 1676 (C=O), 1591, 1542 (C=C, aromatic), 1274 (C–N), 432 (Cu–O), 379 (Cu–N); anal. calcd. for $C_{44}H_{40}N_2O_{10}Cu$: C, 64.42; H, 4.91; N, 3.41%; found: C, 60.00; H, 4.60; N, 3.25%; MS *m/z* 759.2 $[M - 61]^+$, 686.0.

Cu(3-[*N*-(*n*-Butyl)aminobenzyl]-2-hydroxy-1,4-naphthoquinone)$_2$ (**13**)

Dark yellow powder; yield 57%, UV-Vis (methanol) λ_{max} / nm 204, 267, 448; IR (KBr) ν_{max} / cm^{-1} 3460, 3292, 3278 (O–H and N–H) 3064 (C–H, aromatic), 2956, 2927, 2872 (C–H, aliphatic), 1674 (C=O), 1589, 1541 (C=C, aromatic), 1272 (C–N), 437 (Cu–O), 379 (Cu–N); anal. calcd. for $C_{42}H_{40}N_2O_6Cu$: C, 68.88; H, 5.51; N, 3.83%; found: C, 68.11; H, 5.33; N, 3.73%; MS *m/z* 732.2 $[M]^+$, 671.2 $[M - 61]^+$.

Cu(3-[*N*-(*n*-Octyl)4-fluoro-aminobenzyl]-2-hydroxy-1,4-naphthoquinone)$_2$ (**14**)

Brown powder; yield 49%; UV-Vis (methanol) λ_{max} / nm 203, 270, 443; IR (KBr) ν_{max} / cm^{-1} 3458, 3274 (O–H and N–H), 3070 (C–H, aromatic), 2954, 2927, 2852 (C–H, aliphatic), 1674 (C=O), 1591, 1541 (C=C, aromatic), 1274 (C–N), 1224 (C–F), 428 (Cu–O), 378 (Cu–N); anal. calcd. for $C_{50}H_{54}F_2N_2O_6Cu$: C, 68.20; H, 6.18; N, 3.18%; found: C, 63.50; H, 6.00; N, 3.14%; MS *m/z* 880.2 $[M]^+$, 819.4 $[M - 61]^+$, 690.0.

Cu(3-[*N*-(*n*-Octyl)aminopiperonyl]-2-hydroxy-1,4-naphthoquinone$_2$ (**15**)

Brown powder; yield 87%; UV-Vis (methanol) λ_{max} / nm 205, 270, 442; IR (KBr) ν_{max} / cm^{-1} 3446, 3354 (O–H and N–H), 3182 (C–H, aromatic), 2952, 2925, 2854 (C–H, aliphatic), 1674 (C=O), 1591, 1548 (C=C, aromatic), 1276 (C–N), 430 (Cu–O), 383 (Cu–N); anal. calcd. for $C_{52}H_{56}N_2O_{10}Cu$: C, 66.97; H, 6.05; N, 3.00%; found: C, 60.00; H, 6.90; N, 2.70%; MS *m/z* 932.3 $[M + H]^+$, 871.3 $[M - 61]^+$, 742.1, 542.9.

Cu(3-[*N*-(*n*-Octyl)aminobenzyl]-2-hydroxy-1,4-naphthoquinone)$_2$ (**16**)

Pale brown powder; yield 78%; UV-Vis (methanol) λ_{max} / nm 206, 266, 434; IR (KBr) ν_{max} / cm^{-1} 3462, 3415 (O–H and N–H), 3157 (C–H, aromatic), 2952, 2925, 2852 (C–H, aliphatic), 1670 (C=O), 1591, 1552 (C=C, aromatic), 1276 (C–N), 383 (Cu–O), 302 (Cu–N); anal. calcd. for $C_{52}H_{56}N_2O_6Cu$: C, 71.11; H, 6.68; N, 3.32%; found: C, 54.27; H, 5.10; N, 2.70%; MS *m/z* 844.5 $[M]^+$, 783.1 $[M - 61]^+$.

Cu(3-[*N*-(*n*-Butyl)4-trifluoromethoxy-aminobenzyl]-2-hydroxy-1,4-naphthoquinone)$_2$ (**17**)

Brown powder; yield 76%; UV-Vis (methanol) λ_{max} / nm 209, 264, 423; IR (KBr) ν_{max} / cm^{-1} 3460, 3276 (O–H and

N–H), 3182 (C–H, aromatic), 2958, 2931, 2875 (C–H, aliphatic), 1676 (C=O), 1591, 1548 (C=C, aromatic), 1272 (C–N), 1216 (C–F), 1166 (C–O–C), 430 (Cu–O), 383 (Cu–N); anal. calcd. for $C_{44}H_{38}CuF_6N_2O_8$: C, 58.70; H, 4.25; N, 3.11%; found: C, 62.30; H, 5.00; N, 3.20%; MS *m/z* 900.0 $[M + H]^+$, 839.0 $[M - 61]^+$, 827.1.

Cu(3-[*N*-(*n*-Octyl)4-trifluoromethoxy-aminobenzyl]-2-hydroxy-1,4-naphthoquinone)$_2$ (**18**)

Brown powder; yield 54%; UV-Vis (methanol) λ_{max} / nm 208, 263, 425; IR (KBr) ν_{max} / cm^{-1} 3546, 3462 (O–H and N–H), 3274 (C–H, aromatic), 2948, 2927, 2927 (C–H, aliphatic), 1674 (C=O), 1591, 1542 (C=C, aromatic), 1269 (C–N), 1218 (C–F), 1164 (C–O–C), 430.9 (Cu–O), 383 (Cu–N); anal. calcd. for $C_{52}H_{54}CuF_6N_2O_8$: C, 61.70; H, 5.38; N, 2.77%; found: C, 59.80; H, 6.00; N, 2.50%; MS *m/z* 1012 $[M + H]^+$, 951.2 $[M - 61]^+$, 822.1, 506.9.

Cu(3-[*N*-(*n*-Butyl)4-chloro-aminobenzyl]-2-hydroxy-1,4-naphthoquinone)$_2$ (**19**)

Pale brown powder; yield 77%; UV-Vis (methanol) λ_{max} / nm 219, 268, 432; IR (KBr) ν_{max} / cm^{-1} 3446, 3953 (O–H and N–H), 3116 (C–H, aromatic), 2958, 2931, 2871 (C–H, aliphatic), 1670 (C=O), 1589, 1548 (C=C, aromatic), 1230 (C–N), 1093, 733 (C–Cl), 430 (Cu–O), 380 (Cu–N); anal. calcd. for $C_{42}H_{38}Cl_2N_2O_6Cu$: C, 57.84; H, 4.39; N, 3.21%; found: C, 51.06; H, 4.13; N, 3.20%; MS *m/z* 801.9 $[M + H]^+$, 800.1 $[M + H, Cl]^+$, 741.3, 738.9 $[M - 63]^+$.

Cu(3-[*N*-(*n*-Octyl)4-chloro-aminobenzyl]-2-hydroxy-1,4-naphthoquinone)$_2$ (**20**)

Dark yellow powder; yield 59%; UV-Vis (methanol) λ_{max} / nm 218, 264, 430; IR (KBr) ν_{max} / cm^{-1} 3458 (O–H and N–H), 3168 (C–H, aromatic), 2952, 2925, 2854 (C–H, aliphatic), 1676 (C=O), 1591, 1548 (C=C, aromatic), 1276 (C–N), 1095, 734 (C–Cl), 430 (Cu–O), 383 (Cu–N); anal. calcd. for $C_{50}H_{54}Cl_2N_2O_6Cu$: C, 65.75; H, 5.96; N, 3.07%; found: C, 59.82; H, 5.68; N, 3.29%; MS *m/z* 912.0 $[M + H]^+$, 914 $[M + H, Cl]^+$, 851.1 $[M - 61]^+$.

Preparation of ICERs

The immobilization of ee- and huAChE, and huBChE was carried out on the basis of a previously reported procedure.[14,15]

Screening studies

Eserine and galanthamine were used as standard inhibitors towards BChE and AChE, respectively.

Compounds at 200 μmol L^{-1} were screened using eeAChE, huAChE- and huBChE-ICER with detection by UV using Elman's reagent.[19] Ten-microlitre aliquots of a sample containing BTChI at 50 mmol L^{-1} for BChE and ATChI at 1 mmol L^{-1} for AChE were injected. For this purpose, a 1 mmol L^{-1} (MeOH/H_2O 1:1 v:v) stock solution of each compound was prepared. For analytical purposes, a sample with a final volume of 100 μL was obtained using 20 μL of a stock solution of the compound, 20 μL of BTChI (250 mmol L^{-1}) solution for BChE and 20 μL of ATChI (5 mmol L^{-1}) solution for AChE, as well as 60 μL of the working buffer, which resulted in a concentration of 200 μmol L^{-1}. The samples were prepared in duplicate; aliquots (10 μL) were injected into the chromatographic system using the following conditions: mobile phase = working buffer, flow rate = 0.05 mL min^{-1}, UV-Vis detection at 412 nm. Eserine and galanthamine were used as standard inhibitors.

The percentage of inhibition was calculated for each compound by comparing the peak areas of the 5-thio-2-nitro-benzoic acid [yellow anion (YA)] in the absence of the ligand (A_o) (sample containing water and substrate, BTChI or ACThI) and in the presence of the compound (A_i), under the same operating conditions, according to the following expression: $\%I = 100 - (A_i/A_0 \times 100)$. Compounds with $\%I \geq 70\%$ were used to determine the inhibitory potency (IC_{50}).

Inhibitory potency (IC_{50}) of the compounds (**11**, **13-14**, **16-20**)

Aliquots (10 μL) of the solutions containing a fixed concentration (1 mmol L^{-1}) of the substrate and increasing concentrations of the inhibitor (0.001-500 μmol L^{-1}) were injected into the chromatographic system in duplicate. An inhibition curve for each compound was constructed by plotting the % inhibition *versus* the inhibitor concentration. IC_{50} values were independently determined by performing rate measurements for at least six concentrations of the target inhibitor. The nonlinear regression parameters were calculated, and the IC_{50} was extrapolated.

Steady-state inhibition constant (K_i) and mechanism of the action

Reciprocal plots of 1/area product *versus* 1/[S] were constructed. For AChE-ICERs, ATChI solutions (0.40, 0.55, 0.65, and 0.80 mmol L^{-1} for eeAChE and 0.35, 0.70, 1.4, and 2.0 mmol L^{-1} for huAChE) containing fixed ligand concentrations ranging from 0.001 to 20 μmol L^{-1}

were injected in duplicate, also using the chromatographic conditions reported above.

False-positive effects on BChE and AChE inhibition in the thin layer chromatography (TLC) assay based on Ellman's method

Each compound sample (2.5 µL) was eluted on a chromatographic silica gel 60 plate using $CHCl_3$:MeOH:H_2O 65:30:5 (v:v) as the mobile phase. After drying, the plates were sprayed with a solution containing BChE (0.704 mg), BSA (0.025 g), and BCThI (0.00723 g) in Tris (19 mol L^{-1}, pH 8, adjusted with HCl 10% v:v), previously incubated at 37 °C for 15 min, followed by addition of Ellman's reagent prepared in Tris (19 mol L^{-1}, pH 8, adjusted with HCl 10% v:v) for the assays with BChE. As for the assays with AChE, AChE itself and its substrate ACThI were employed at the same concentrations listed above. This assay was carried out as described in the literature.[28]

Data analysis

The IC_{50} parameters were extrapolated using the program Sigma Plot 12.0 operating in the nonlinear regression function. The inhibition mechanisms, K_i values, and their respective standard errors were calculated by fitting the data using the appropriate equations[26] and the OriginPro software version 8.0.

Supplementary Information

Supplementary information (data of compounds and figures of the inhibitory potency (IC_{50}) and mechanism action the most active compound) are available free of charge at http://jbcs.sbq.org.br as PDF file.

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