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Acetylcholinesterase Immobilized Capillary Reactors−Tandem Mass Spectrometry: An On-Flow Tool for Ligand Screening

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Supporting Information

ABSTRACT: The use of immobilized capillary enzyme reactors (ICERs) for online ligand screening has been adopted as a new technique for high-throughput screening (HTS). In this work, the selected target was the enzyme acetylcholinesterase (AChE), and the AChE-ICERs produced were used in a liquid chromatograph−tandem ion-trap mass spectrometer. The activity and kinetic parameters were evaluated by monitoring the choline's precursor ion $(M + H)^+$ m/z 104.0 and its ion fragment $(C_2H_3OH) - (M + H)^+$ m/z 60.0. The assay method was validated using the reference AChE inhibitors tacrine and galanthamine. Two new ligands, out of a library of 17 coumarin derivatives, were identified, and the half-maximal inhibitory concentration (IC_{50}), inhibition constant (K_i), and the inhibition mechanism were determined. A coumarin derivative with IC_{50} similar to tacrine was highlighted.

INTRODUCTION

In the genome era, compounds with low molecular weight are gaining importance as targets in the development of new drugs.[1] As a biological target, acetylcholinesterase (EC 3.1.1.7) (AChE), a serine hydrolase enzyme, has gained significant interest, as one of the factors associated with Alzheimer disease is the low level of acetylcholine in the neural synapses. AChE acts in the central nervous system (CNS) and rapidly hydrolyzes the active neurotransmitter acetylcholine (ACh) into the inactive compounds choline (Ch) and acetic acid.[2] Inhibitors of AChE reestablish the cholinergic function in humans as a result of an increase in ACh in the CNS. While, in insects, AChE inhibitors are associated with death.[3] AChE's inhibitors (AChEIs) are currently the most recommended approved therapy for the treatment of Alzheimer's disease and for production of insecticides.[3−6]

A variety of approaches to identify AChEIs in natural or synthetic libraries have been described.[7] The vast majority of them are based on colorimetric methods using Ellman's reagent[8] or Fast Blue B salt reagent,[9,10] with solution enzyme assays. The choice of a suitable assay is, however, crucial in searching for inhibitory activity. In this context, selective affinity chromatography has been extensively explored to mimic biological actions, showing great advantages as a screening tool.[11−15] Such methods can provide rapid evaluation of thermodynamic and kinetic constants as well as screening and determination of mechanisms of action of inhibitors hits.[16] To meet this approach, AChE has been immobilized onto different supports by different immobilization procedures.[17−19] Recently, we reported[20] the efficient immobilization of AChE from *Electrophorus electricus* onto a fused silica capillary (*eel*AChE-ICER) and its use in screening assays for identifying and characterizing new ligands. For measuring the enzymatic reaction product, the Elman's detection approach[8] was used with acetylthiocholine as the enzyme substrate.[17−19] Herein, we report an inhibitor screening method by on-flow direct quantification of choline using AChE-ICER in a tandem mass spectrometer (IT-MS/MS). The developed assay was used for assessing activity and kinetics parameters of the developed AChE-ICERs. The AChEIs assay validation was carried out with two reference inhibitors (tacrine and galanthamine) followed by identification and characterization of the mechanism of action of lead ligands from a small synthetic coumarin library.

RESULTS

LC-IT-MS/MS conditions. The configuration of the liquid chromatograph−tandem ion-trap mass spectrometer (LC−IT-MS/MS) is given in Figure 1. Ammonium acetate solution (15 mM, pH 8.0) was used as mobile phase, in a flow rate of 0.05 mL min^{-1}, and the injection sample volume at the AChE-ICER was 10 μL. Methanol was used to improve ionization, which was delivered by pump B, at the same flow rate as pump A. The total analysis time was 20 min. IT-MS/MS parameters for the analysis of choline were the following: nebulizer pressure of 30 psi, drying gas flow of 7.5 L.min^{-1}, temperature of 335 °C, capillary voltage of 4500 V, and fragmentation amplitude of 0.71 V.

The activity and kinetic parameters were evaluated by multiple reaction-monitoring mode (MRM) the choline's precursor ion $(M + H)^+$ m/z 104.0 and its ion fragment $(C_2H_3OH) - (M + H)^+$ m/z 60.0. The isolation width was set at m/z 1.0. The choline precursor ion was used for quantification, while MRM

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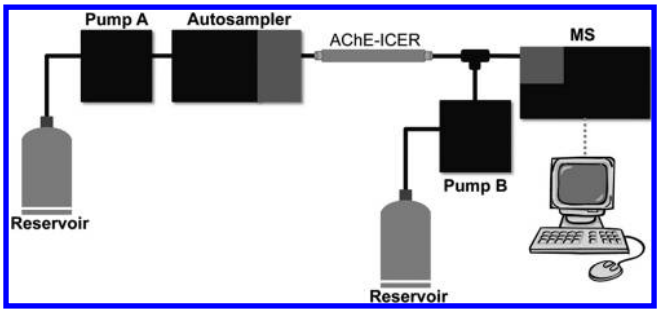

Figure 1. The schematic LC−IT-MS/MS system used. Pump A, ammonium acetate solution (15 mM; pH 8.0), and pump B, methanol, at a flow rate of 0.050 mL.min^{-1}.

ratio and the first transition were used for confirmatory purposes.[3,21] The first transition corresponds to the loss of $[M - C_2H_3OH]^+$ (m/z 60.0) (Supporting Information, Figure S1), and this is in accordance with data previously reported for this compound.[22−24]

Validation Study. The calibration curves were logarithmic in the ranges studied, with mean correlation coefficients (n = 3) of 0.99 or higher. The CV% for the replicates was below 15% and the accuracy showed a deviation below 15% of the nominal value, indicating that no carry over has happened between injections. Accuracy values between 86 and 113% for curve A and 85 and 112% for curve B. The interlot precision values with RSD in the range of 2.0−13% for curve A and 0.4−4.0% for curve B were in the range of accepted criteria, especially considering that QCs were prepared as replicates (n = 5).

Activity and Kinetics Studies of AChE-ICER. The immobilization of *eel*AChE was carried out as previously reported,[20] but this time we investigated the reduction of the formed Schiff's base for the ICER's activity. The experiments showed that the reduction procedure decreased by 84% the *eel*AChE-ICER activity.

To optimize their activities, different protein concentrations were used in the preparation of the *eel*AChE-ICERs. The experiments demonstrated that the ICERs had a decrease in activity with an increase in the amount of used protein (Table 1).

Table 1. Values of Ch Produced, by the Injection of ACh at 200 μM in the LC−IT-MS/MS System[a]

enzyme soln concn (mg mL^{-1})	K_m (μM)	Ch produced (μM)
0.125	325 ± 34.9	198 ± 15.9
0.250	268 ± 13.0	206 ± 10.1
0.500	734 ± 68.4	10.0 ± 0.100

[a] K_m values for *eel*AChE-ICERs prepared with 0.5, 0.25, and 0.125 mg mL^{-1} of enzyme solution.

The kinetics parameters for *eel*AChE-ICERs (0.125 mg mL^{-1}) and *hu*AChE-ICER (0.125 mg mL^{-1}) were obtained by varying the substrate concentration while measuring the choline formation. For *hu*AChE-ICER the K_m value was 38.5 μM.

The results obtained (Table 1) showed that the immobilized enzyme retained its activity toward its natural substrate, ACh. The ICERs showed high stability and they kept their activity for almost 1 year.

Validation of Screening Assay. For validation of the inhibition screening assays, the *eel*AChE was selected due to its prevalence in HTS besides its lower cost and similarity with *hu*AChE.

Tacrine and galanthamine were selected as reference inhibitors. The dose−response curve plots of inhibition percentage (IC_{50}) for these two known AChEIs and the Lineweaver−Burk reciprocal plots for the Ch produced demonstrated that the screening assay herein reported is capable of identifying and characterizing AChE inhibitors (Figure 2).

The Lineweaver−Burk plots showed different values of V_{MAX} for tacrine and no change in V_{MAX} for galanthamine, characterizing them as noncompetitive and competitive inhibitors, respectively (Figure 2). These results are in accordance with published results for these well-known AChE inhibitors.[25,26]

Screening of a Coumarin Library. A small library composed by 17 coumarin derivatives (Figure 3) was screened for inhibitors identification through hydrolysis of ACh by the *eel*AChE and *hu*AChE-ICERs.

Two hits were identified, coumarin derivatives **16** and **17**, with an inhibition percentage above 80.0 for the *eel*AChE-ICER (Figure 4). The IC_{50}, K_i, and inhibition mechanisms were determined and are given in Table 2 (Figure 5).

DISCUSSION

In the classical soluble AChE assays,[7] the enzymatic reaction products are measured by spectrophotometric means. The false positive results, the inability of enzyme reuse, and, thus, high costs are the main drawbacks of these methods. A variety of approaches using the more versatile immobilized enzyme model have been explored to try to overcome these shortcomings.[17,18,22,27] Acetylthiocholine, instead of the natural substrate acetylcholine, has been used, however, as the substrate in a number of assays approaches, even when MS/MS has been used to monitor immobilized AChE-catalyzed hydrolysis.[19] The method herein described has a number of benefits over previously published procedures, the main one being that the natural substrate is used and its product is directly quantified by an on-flow LC−IT-MS/MS system. Thus, there is no need of evaluation of false positives results. The gain in stability achieved by AChE immobilization in an open tubular silica capillary allowed the screening results, here described, to be obtained from only one *eel*AChE-ICER. The importance of protein concentration for the ICERs preparations was fully investigated. The results demonstrate that higher protein concentration was detrimental to the ICERs activities (Table 1). This may be explained by the effects caused by immobilization as changes on the quaternary structure, flexibility of dynamical domains, and accessibility to the binding sites of the substrate. These effects might well be potentiated at high protein concentration. It is noteworthy, however, that the structural determinants for molecular recognition were not affected. The validation of the screening assay demonstrated that the reference AChEIs, tacrine and galanthamine, were recognized and characterized as noncompetitive and competitive inhibitors, respectively. The automation gained by working with the ICERs on-flow with the LC-IT-MS/MS system made the determination of kinetics parameters, potency inhibition assay, and determination of IC_{50} (μM) and K_i (μM), using either *eel*AChE-ICER or *hu*AChE-ICER, easily performed. The screening assay for hit AChEIs using a coumarin library identified two compounds (Figure 4). These coumarin derivatives arise as hit ligands, with similar or better K_i values than the one determined for tacrine. Coumarin derivatives **16** and **17** (Figures 5B,D) were demonstrated to follow a competitive action mechanism in a similar mode as galanthamine (Figure 2B).

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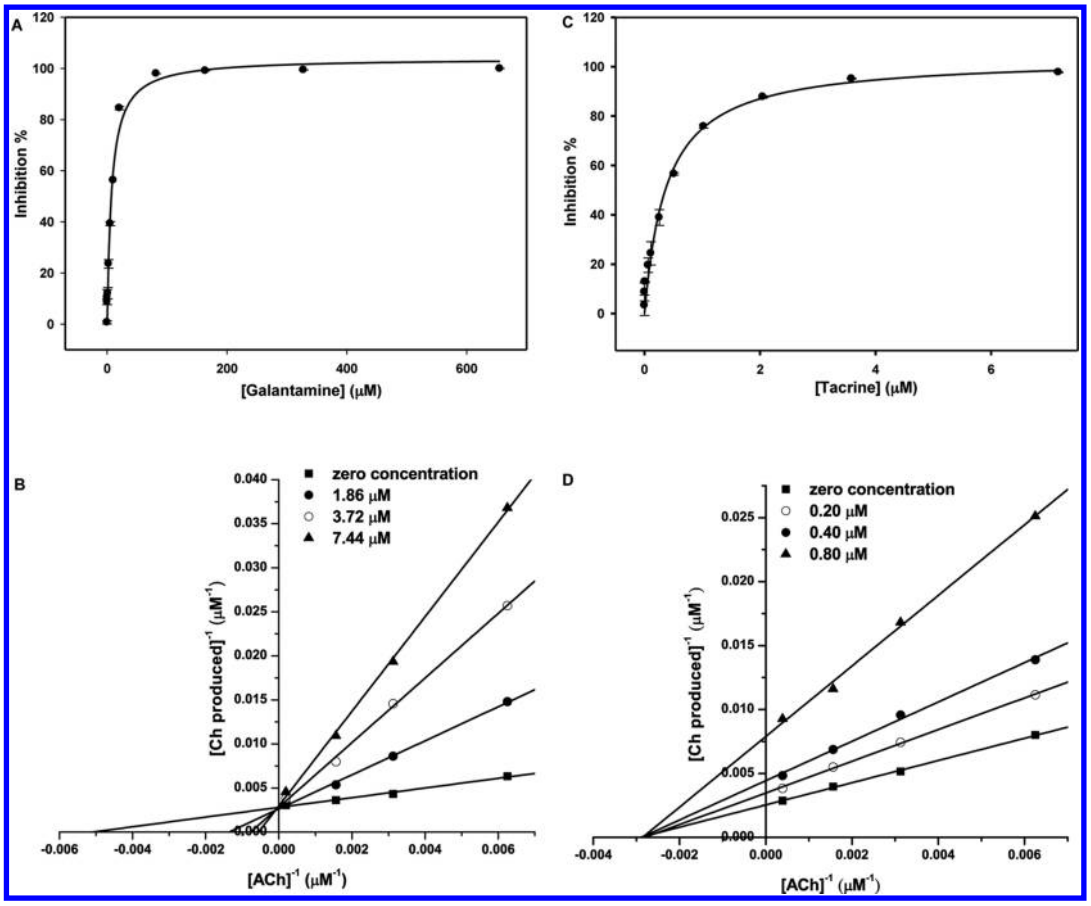

Figure 2. Dose–response curve plots of inhibition percentage for galanthamine (A) and tacrine (C). Lineweaver–Burk reciprocal plots for galanthamine (B) and tacrine (D).

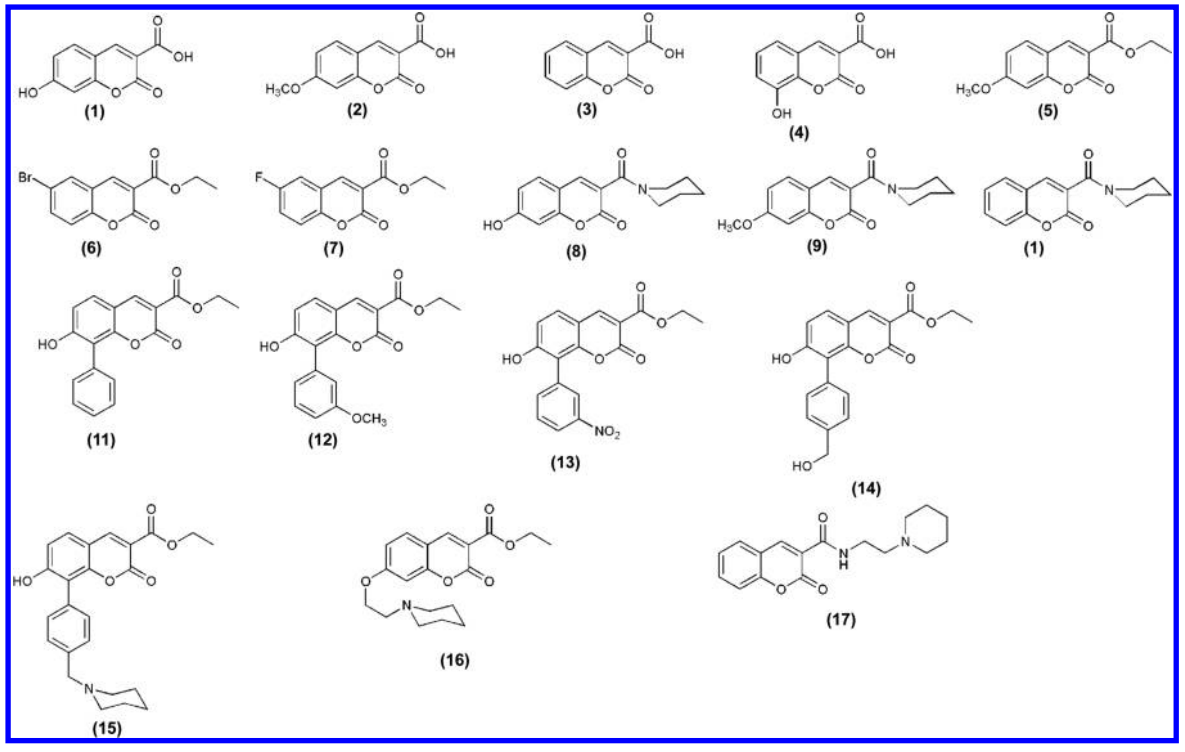

Figure 3. Coumarin library.

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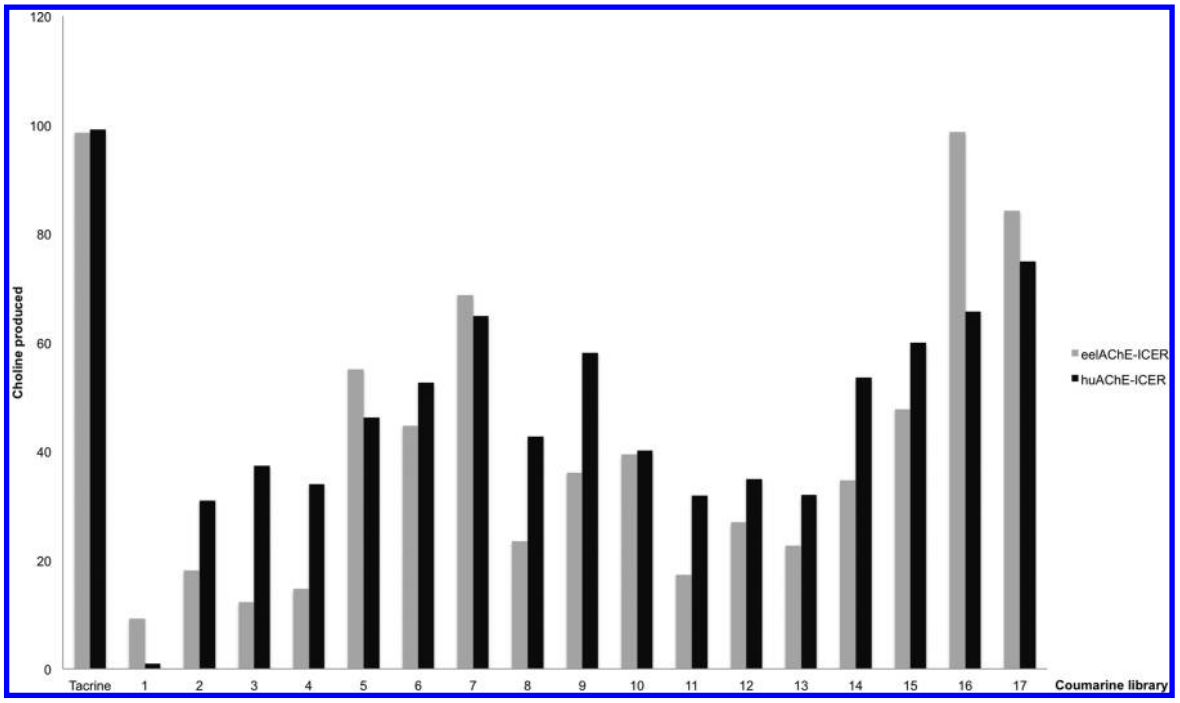

Figure 4. Inhibition percentage of AChE hydrolysis of acetylcholine (ACh at 200 μM) by the *eel*AChE- and *hu*AChE-ICERs.

Finally, differences in specificity between *eel*- and *hu*AChE were assessed by the screening assay carried out with the coumarin library, with both ICERs (Figure 4).

CONCLUSIONS

The validated method for direct quantification of choline produced by the AChE-ICERs in a LC−IT-MS/MS system provides an easy and reliable tool for the identification and characterization of hit inhibitors. The AChE-ICERs were efficiently used for kinetic studies by directly quantifying the choline formation. The validated inhibitor screening assay was applied to a small library of synthetic coumarins and it was able to identify a hit coumarin with IC_{50} similar to tacrine in the *eel*AChE-ICER. The validated assay was used for assessing the mechanisms of action of newly identified inhibitors. The herein described assay is an innovative and valuable approach for ligand studies. In addition to the specificity, selectivity, and sensibility of the method, due to the short analysis time and automation by the autosampler of the LC system, over 100 samples can be screened daily.

EXPERIMENTAL SECTION

Preparation of AChE-ICER. The immobilization of *eel*- and *hu*AChE was carried out on the basis of a previously reported procedure[20] (Supporting Information). Different concentrations of protein were investigated for the ICERs preparation, and the ones selected for the screening assays were prepared with 0.125 mg mL^{-1} of protein.

Chromatography System and Conditions. The LC system (Shimadzu, Kyoto, Japan) used consisted of two LC-20AD pumps, a SIL 20A autosampler with a 50 μL loop, a DGU-20A5 degasser, and a CBM-20A interface. The LC system was coupled to an Esquire 6000 IT mass spectrometer (BrukerDaltonics, GmbH, Bremen, Germany) equipped with an ESI source, operating in a positive mode. Data acquisition was carried out using the BrukerDaltonics data analysis software. All LC analyses were performed at room temperature (±20 °C).

The AChE-ICERs were interfaced to the IT-MS/MS as a biochromatography column, and the acetylcholine hydrolysis product, Ch ($[M + H]^+$ m/z 104), was analyzed in positive ion mode (ESI+), while multiple reaction-monitoring (MRM) was carried out for the acquisition.

Method Validation. The method validation was accomplished in accordance with internationally accepted criteria[21] (Supporting Information).

Kinetics Studies. The kinetics parameters were obtained for the series of ICERs prepared with different protein concentration. For the 0.125 mg mL^{-1} of protein *eel*AChE- and*hu*AChE-ICERs, a 10 μL ACh solution at the concentration range of 0.3125−5120 μM was used. For the 0.5 and 0.25 mg mL^{-1} of protein *eel*AChE-ICERs, the concentration range used was 2.5−5120 and 0.25−512 μM, respectively. All samples were prepared and analyzed in duplicate. The results are given as the average of the duplicate analysis.

Sigma Plot software version 10.0 was used to obtain Michaelis−Menten plots by nonlinear regression analysis. Hyperbolic saturation curves[28] were fitted to eq 1 at varying concentrations of substrate

$$U = \frac{VA}{K_a + A} \tag{1}$$

where U is the measured reaction velocity, V is the maximal velocity, A is the substrate concentration (ACh), and K_a is the substrate's Michaelis constant.

Screening Assays. Tacrine and galanthamine were used as reference AChEIs. A coumarin library composed of 17 derivatives[29] was used in the AChEIs screening assay. To this end, a 1.00 mM methanol stock solution was prepared for each tested compound. The 100 μL assay samples were prepared with ammonium acetate solution (15.0 mM, pH 8.0) containing 25.0 μL of 1960 μM ACh aqueous solution and 20.0 μL of the tested compound methanol stock solution. Analyses were carried out by aliquot injection of 10.0 μL of each tested AChEIs, at a fixed concentration of 200 μM with ACh at 490 μM.

For each tested AChEIs sample, a negative control (absence of ACh) and a positive control sample (ACh and absence of ligand) were analyzed.

The percent inhibition for each compound was calculated using eq 2.

$$100 - \left(\frac{P_i}{P_0} \times 100\right) \tag{2}$$

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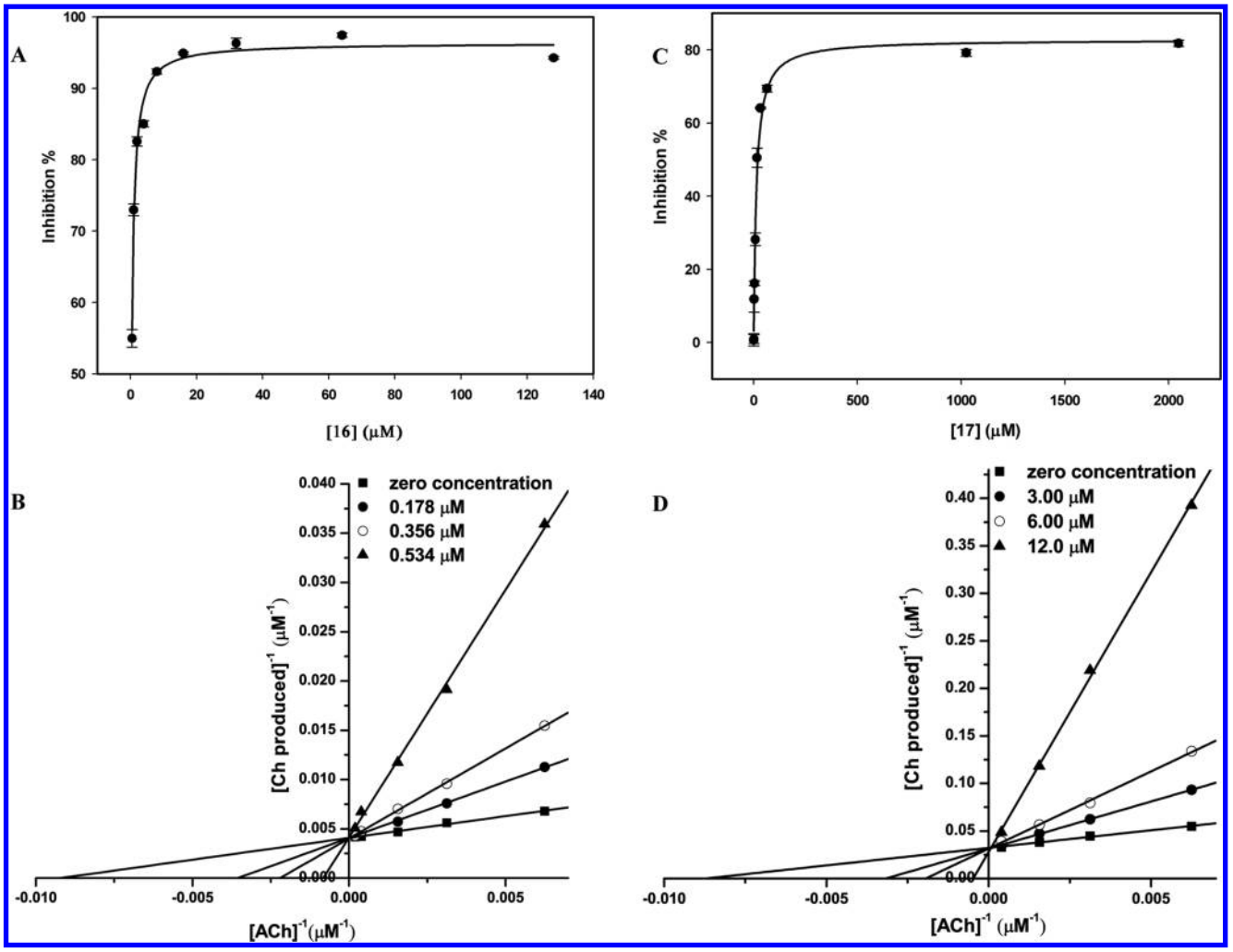

Figure 5. Dose−response plots of inhibition percentage for the coumarins **16** (A) and **17** (C). Lineweaver−Burk reciprocal for coumarins **16** (B) and **17** (D).

Table 2. Values of IC_{50} and K_i Calculated for AChEIs in *eel*AChE-ICER

ligands	IC_{50} (μM)	K_i (μM)
coumarin **16**	0.356 ± 0.010	0.031 ± 0.010
coumarin **17**	12.6 ± 1.07	13.8 ± 1.10
tacrine	0.386 ± 0.044	0.340 ± 0.097
galanthamine	7.44 ± 0.663	1.06 ± 0.274

In eq 2 P_i is the Ch production quantified from the hydrolysis of acetylcholine in the presence of tested inhibitors and P_0 is the Ch production as in the positive control experiment.

Determination of Inhibitory Potency (IC_{50}). The IC_{50} values were calculated by quantifying the Ch production in the hydrolysis of ACh at different concentrations of the tested AChEIs.

Stock solutions of the reference inhibitors galanthamine (0.05, 1.6, 51.2, and 1638.4 μM) and tacrine (0.005, 0.160, 5.12, and 10.2 μM) were prepared in water. Samples of the assay solutions [galanthamine, 0.0025−655.4 μM; tacrine, (0.025×10^{-3})−7.17 μM] were prepared by diluting the stock solutions in water together with the substrate acetylcholine at a 490 μM fixed concentration.

For the coumarin derivatives assay, aliquots (10 μL) of the solutions containing acetylcholine at 490 μM and increasing concentrations either of coumarin **16** (from 0.5000 to 128.0 μM) or **17** (0.5000−2048 μM) were injected in to the chromatography system. All analyses were carried out in duplicate.

As positive control, aliquots of 10 μL of a 490 μM acetylcholine aqueous solution were injected, and the production of Ch was quantitatively measured. These results were set as 100% of activity and compared with the concentration of choline obtained in the hydrolysis of acetylcholine in the presence of tested inhibitors. Thus, the percentages of inhibitions were calculated in accordance with eq 2.

The inhibition curve was obtained using the Sigma Plot software (version 10.0) by nonlinear regression analysis, for each tested inhibitor, as the percentage of inhibition versus their concentration.

Determination of Steady-State Inhibition Constant (K_i) and Mechanism of Action. To verify the inhibition mechanism, reciprocal plots of 1/[Ch] versus 1/[ACh] were constructed. To this, ACh solutions (160−5120 μM) containing a fixed AChEIs concentration (Table 3) were prepared in duplicate and injected into the chromatographic system, to quantify the Ch production. Lineweaver−Burk plots were used to determine the action mechanism for each ligand.[30]

Table 3. AChEIs Concentration Ranges Used at the Inhibition Mechanism Assays

ligands	concn range (μM)
tacrine	0.200−0.800
galanthamine	1.86−7.44
coumarin **16**	0.178−0.534
coumarin **17**	3.00−12.0

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OriginPro software version 8.0 was used for data analysis of the inhibition mechanisms analysis and for the K_i plots. The data obtained were properly fitted to eqs 3 and 4, which describe a competitive and noncompetitive inhibition mechanism.

$$U = \frac{VA}{K_a\left(1 + \frac{I}{K_{is}}\right) + A} \quad (3)$$

$$U = \frac{VA}{K_a\left(1 + \frac{I}{K_{is}}\right) + A\left(1 + \frac{I}{K_{is}}\right)} \quad (4)$$

In the eqs 3 and 4, I is the inhibitor concentration and K_{is} is the slope inhibition constant.

The K_i values for fortacrine, galanthamine, coumarin **16**, and coumarin **17** were determined individually by plotting K_m/V_{MAX} (calculated to each ligand concentration) versus the ligand concentration. A linear fit was traced and the quotient of division between linear/angular coefficients provided the K_i value.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Chemicals, validation method data, and immobilization procedures for *eel*- and *hu*AChE. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

K.L.V., L.C.C.V., A.G.C., and C.L.C. contributed equally to this work.

Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

AChE, acetylcholinesterase; *eel*AChE, acetylcholinesterase from *E. electricus*; *hu*AChE, human acetylcholinesterase; AD, Alzheimer disease; CNS, central nervous system; ACh, acetylcholine; Ch, choline; AChEIs, acetylcholinesterase inhibitors; AChE-ICER, acetylcholinesterase immobilized onto a fused silica capillary; K_i, inhibition constant; K_m, Michaelis−Menten constant; IC_{50}, half-maximal inhibitory concentration; HTS, high-throughput screening; IT-MS/MS, LC−tandem ion-trap mass spectrometer.

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