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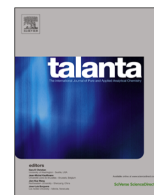

Acetylcholinesterase immobilized capillary reactors coupled to protein coated magnetic beads: A new tool for plant extract ligand screening

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ABSTRACT

The use of immobilized capillary enzyme reactors (ICERs) and enzymes coated to magnetic beads ((NT or CT)-MB) for ligand screening has been adopted as a new technique of high throughput screening (HTS). In this work the selected target was the enzyme acetylcholinesterase (AChE), which acts on the central nervous system and is a validated target for the treatment of Alzheimer's disease, as well as for new insecticides. A new approach for the screening of plant extracts was developed based on the ligand fishing experiments and zonal chromatography. For that, the magnetic beads were used for the ligand fishing experiments and capillary bioreactors for the activity assays. The latter was employed also under non-linear conditions to determine the affinity constants of known ligands, for the first time, as well as for the active fished ligand.

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1. Introduction

The enzyme acetylcholinesterase (AChE) acts in the central nervous system and rapidly hydrolyzes the active neurotransmitter acetylcholine into the inactive compounds choline and acetic acid [1]. Low levels of acetylcholine in the synaptic cleft are associated with a decrease in cholinergic function characterizing Alzheimer's disease, which is the most common cause of dementia among the elderly. Because AChE inhibitors are currently one of the few therapies approved for the treatment of Alzheimer's disease, the identification of novel ligands that could modulate AChE activity is of great therapeutic importance.

Newmann and Cragg [2] determined that more than 70% of all drugs approved from 1981 and 2006, were either derived from or structurally similar to nature based compounds. Considering the structural diversity of known AChE inhibitors, a logical extension is to screen plant extracts to identify potentially unknown modulators of AChE. In fact, a number of known inhibitors of AChE have been derived from plant extracts, including galanthamine, a drug accepted for Alzheimer's disease treatment by the FDA since 2001 that is extracted from *Galantus nivalis* species [3]; Huperzine A (HupA), an alkaloid from the extract of *Lycopodium* genus [4]; and indole alkaloids from *Ervatamia hainanensis* [5]. To date, plant extracts from *Melodinus* genus, Apocynaceae family, have not been investigated for AChE activity. Pharmacological assays using the crude extract or purified compounds of *Melodinus* species, i.e. *Melodinus tenuicaudatus*, demonstrated potent cytotoxicity against human cancer and bacterial cell lines. Although *Melodinus* genus has not been used for short term memory loss, it is known to be a good source of alkaloids, [4,6], and therefore could be a good source for an AChE inhibitor.

Currently, bioguided fractionation is most commonly used for traditional drug screens. However, this method is costly and time consuming. Dereplication is the most common approach for screening complex matrixes (plant extracts) to identify known compounds and has been approached using a variety of methods including HPLC-SPE coupled to NMR-MS for structural identification [7]. Another method that is commonly used to screen plant extracts is on-line screening with bioactive detection, which has also been previously used to identify inhibitors of AChE [8–10]. In this case, the enzymatic hydrolysis of acetylcholine is monitored and the inhibition of this activity is used to identify active

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components. These AChE bioreactors have also been used to determine the affinity, equilibrium and kinetic constants [9,11–13]. This approach uses frontal or zonal (linear and non-linear) chromatography techniques to characterize the desired protein [14–16].

While there are several advantages of bioaffinity chromatography, the process involved for screening plant extracts is nevertheless still challenging. If active compounds are not retained for a significant amount of time by the immobilized protein, binders and non-binders will be co-eluted. In order to circumvent this problem, another plausible approach is the use of protein-coated magnetic beads [17–21]. In this technique, the protein-coated beads are immersed directly into the extract. Any compound with an affinity for the immobilized protein will therefore be retained while non-binders will remain in the extract. The bound compounds can then be eluted to identify active compounds [17,18–20–22]. Previously, Moaddel and collaborators [17], have demonstrated that HSA-MB successfully 'fished' out 3 known binders from a mixture of 6 compounds. In addition, they demonstrated that the HSP-90α-MB could be used to fish out client proteins from a cellular matrix [18]. More recently, Forsberg et al. [9], reported the use of AChE reactors to determine the effect of a complex matrix on the inhibition of AChE activity, while other groups have used AChE coated magnetic beads to determine the inhibitory activity of a known compound [21,22]. However, none of these groups used these bioreactors to 'fish' out the active compound, but rather selected them by their effect on the enzymatic activity. Herein, we report for the first time the use of AChE-coated magnetic beads for the extraction and isolation of an active compound from a complex matrix. In addition, non-linear zonal bioaffinity chromatography using AChE immobilized onto silica fused capillary (AChE-ICER) was used to assess the inhibitors affinity [13] and to assess whether the bound material could inhibit AChE activity.

2. Materials and methods

2.1. Chemicals

Ammonium acetate, tris(hydroxymethyl)aminomethane, acetylcholinesterase from *Electrophorus electricus* (*eel*AChE) type VI-S, choline iodide (Ch), acetylcholine iodide (ACh), galanthamine bromide (GAL), 9-amino-1,2,3,4-tetrahydroacridine hydrochloride hydrate (tacrine –TAC) and dimethyl sulfoxide 99.9% were supplied by Sigma-Aldrich. All chemical materials used during the immobilization procedure were of analytical grade and were purchased from Sigma-Aldrich. BcMag™ amine-terminated magnetic beads were purchased from Bioclone. Methanol and acetonitrile were HPLC grade and the water used for all experiments was deionized in a Millipore Milli-Q system. The mobile phases were prepared daily. The coumarin derivative was prepared as previously described [23].

The LC–MS analyses were performed on an Agilent HP 1100 system, equipped with a vacuum degasser, autosampler, DAD detector and two binary pumps. Data collection was performed using Agilent ChemStation software.

2.2.1. AChE-ICER

The *eel*AChE enzyme was immobilized onto the internal surface of an open tubular silica capillary (100 μm i.d., 30 cm) as previously described [13]. Briefly, the silica-fused capillary was cleaned and coated using a 10% solution (v/v) of APTS in water and left overnight. Then phosphate buffer [50 mM, pH 7.0] containing 4% gluteraldehyde was run through the column followed by phosphate buffer [50 mM, pH 7.0]. An *eel*AChE solution (0.125 mg mL^{-1}) was prepared in phosphate buffer [50 mM, pH 7.0] and run through the column twice. Subsequently, the *eel*AChE was washed with Tris–HCl [100 mM, pH 8.0] and stored at 4.0 °C.

2.2.2. *eel*AChE-MB

The *eel*AChE was immobilized through the N-terminus onto the surface of magnetic beads resulting in *eel*AChE-MB, following a previously described protocol [17]. Briefly, 25 mg of BcMag was washed three times with 1.0 mL of pyridine [10 mM, pH 6.0] using the manual magnetic separator Dynal MPC-S. The supernatant was discarded and the BcMags were suspended in 1.0 mL of pyridine [10 mM, pH 6.0] containing 5% glutaraldehyde and shaken, at 4.0 °C, for 3.0 h. After magnetic separation, BcMags were washed three more times with 1.0 mL of pyridine [10 mM, pH 6.0], followed by the addition of 0.40 mg of *eel*AChE in 500 μL of pyridine [10 mM, pH 6.0]. The reaction was left for 16 h at 4.0 °C with gentle rotation. The supernatant was discarded and the beads were washed three times with 0.50 mL of Tris–HCl [100 mM, pH 8.0] at 4.0 °C.

2.3. Ligand fishing assay using *eel*AChE-MB

The prepared *eel*AChE-MB was suspended in 500 μL of an ammonium acetate buffer [15 mM, pH 8.0] containing 100 nM of the reference compounds (Fig. 1: tacrine, galanthamine, coumarin derivative, labetalol and ketamine), alone or in a mixture. The tube was mixed by vigorously shaking for 30 s, set for 90 s and placed into the magnetic separator for 120 s. The supernatant (S-1) was collected, and the *eel*AChE-MB was washed twice with 500 μL of ammonium acetate [15 mM; pH 8.0] by vigorously shaking for 10 s, placing into the magnetic separator for 120 s. The *eel*AChE-MB was then suspended in the elution buffer (500 μL of ammonium acetate [15 mM, pH 8.0] containing 20% of methanol and 1826 μM ACh) and was shaken at 300 rpm with a thermomixer model R (Eppendorf) at 25 °C for 10 min. The supernatant (S-2) was collected and the *eel*AChE-MB was washed twice. To the S-1 100 μL of methanol was added and to S-2 100 μL of ammonium acetate buffer [15 mM, pH 8.0] was added, to maintain a similar ratio of organic to aqueous. Nicotine at 20 μM was used as internal standard.

Calibration curves for all the tested compounds were prepared in serial dilution from 1.28 to 0.02 μM with 20 μM nicotine as the internal standard in ammonium acetate [15 mM, pH 8.0]: methanol (83:17 (v/v)) and 10 μL was injected in the LC–MS system. The samples were prepared in triplicate for the calibration curves that were constructed for each compound from a linear

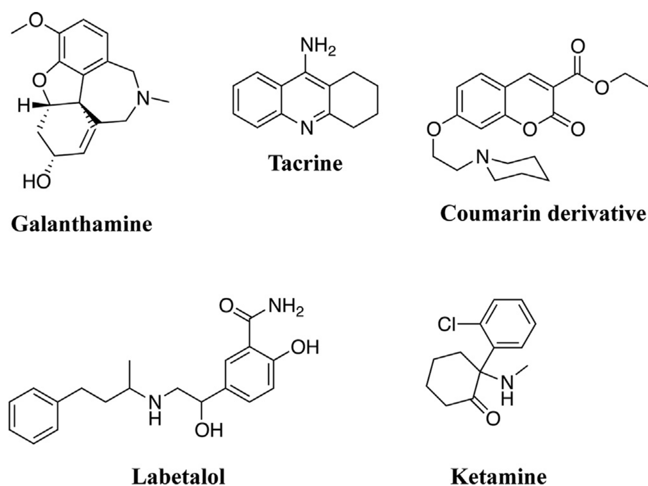

Fig. 1. Reference mixture of acetylcholinesterase ligands and non-ligands.

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