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Acetylcholinesterase activity of electric eel is increased or decreased by selected monoterpenoids and phenylpropanoids in a concentration-dependent manner

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ABSTRACT

The profitable insecticidal action of monoterpenoids prompted us to test their efficiency against stored-grain beetle species, via inhibition of acetylcholinesterase (AChE). For this, we first studied the ability of the monoterpenoids geraniol, linalool, camphor, fenchone, carvone and γ-terpinene, besides the phenylpropanoids *trans*-anethole and estragole to inhibit *Electrophorus* AChE. The results indicated that while AChE activity increased (15–35%) with 40 μM geraniol, camphor, γ-terpinene and linalool, the activity decreased (60–40%) with 5 mM carvone, γ-terpinene, and fenchone. The Km for AChE was 0.52 ± 0.02 mM in control assays, which fell to 0.28 ± 0.01 mM or 0.32 ± 0.01 mM in assays with 20 μM linalool or γ-terpinene added. In the millimolar range, the terpenoids behaved as weak inhibitors. Unexpectedly, AChE inhibition by camphor, carvone, γ-terpinene, and fenchone gave Hill numbers ranging 2.04–1.57, supporting the idea that AChE was able to lodge more than one monoterpenoid molecule. The plots of $1/v$ vs. $1/S$ at varying monoterpenoid provided straight lines, fenchone and γ-terpinene acting as competitive inhibitors and carvone and camphor as non-competitive inhibitors. Moreover, the secondary plots of the slope K_M^{app}/V_{max}^{app} vs. [I] and of $1/V_{max}^{app}$ vs. [I] gave parabolic curves, which lent support to the proposed capacity of AChE to bind more than one monoterpenoid molecule. The fitting of the curves to a second-order polynomial equation allowed us to calculate the inhibition constants for the interaction of AChE with fenchone, γ-terpinene, carvone and camphor. The previously unnoticed increase in AChE activity with monoterpenoids should be considered as a reminder when advising the use of essential oils of plants or their constituents as anti-AChE agents to attenuate pathological signs of Alzheimer's disease.

1. Introduction

Among the great variety of natural products, essential oils from aromatic plants and their components are attracting much interest for the diversity of biological activities that they exhibit [1–3]. Apart from the well known antimicrobial and antioxidant actions of essential oils [4], the last decade has seen particular attention paid to them for their ability to inhibit acetylcholinesterase (AChE) [5]. Plant oils accumulate mono- and sesqui-terpenoids as well as phenylpropanoids [1–3], and, despite their great structural diversity, many terpenoids and phenylpropanoids are able to inhibit to a variable extent AChE, which makes it that AChE inhibition by essential oils should be explained more specifically with consideration of the variety of chemical components in a given plant oil.

Since AChE is the principal protein involved in the hydrolysis of the neurotransmitter acetylcholine (ACh) [6], the capacity of some plant components to inhibit AChE has raised expectations concerning their possible use as pharmaceuticals and pesticides [1,7]. In the former case, the goal is to increase the level of ACh in the human brain and by this means alleviate the cholinergic deficit and memory impairment of Alzheimer's disease (AD) [8,9]. In support of this are some reports showing the *in vitro* and *in vivo* inhibition of AChE by essential oils of plants that have traditionally been administered to enhance memory [9–13]. In the second case, the fact that invertebrate AChE activity can be substantially decreased by certain plant components raises the possibility of their use as a profitable alternative to synthetic insecticides [14].

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At this point, it is worth mentioning the fact that the natural product class that has been investigated more extensively and, which, in general, inhibited AChE more potently, are alkaloids, for instance the quinolizidine alkaloid huperzine-A and the isoquinoline alkaloid galanthamine [5,15].

Site-directed mutagenesis and crystallographic studies have revealed a deep narrow crevice in AChE that penetrates halfway into the protein and contains the catalytic triad at 4 Å from its base. The AChE gorge is lined by the aromatic residues which form part of the various subsites involved in the fitting of ACh. While the catalytic triad Glu-His-Ser allows rapid withdrawal of ACh by AChE [16–18], the catalytic parameters may be greatly altered by a large number of structurally different compounds that interfere with the AChE acetylation or deacetylation processes, via occupation or distortion of the range of ACh binding sites. This range includes the peripheral site, the anionic subsite, the acyl-binding pocket and the oxyanion hole. Molecular docking studies show that the inhibitory potency of terpenoids on AChE depends on the strength of hydrogen bonding and hydrophobic interactions with the amino acids that make the ACh binding sites [19–21].

Despite the abundant information on the monoterpenoids' potency to inhibit mammalian AChE, less attention has been paid to their actions on insect AChE. However, AChE inhibition may be responsible for the mortality of stored-product pest-inducer arthropods (Coleoptera) that we have observed after exposure to the monoterpenoids geraniol, linalool, camphor, fenchone, carvone, and γ-terpinene, and the phenylpropanoids *trans*-anethole, and estragole [22]. Within the overall goal of our current research programme is to test the effectiveness of using monoterpenoids for pest control management through inhibition of AChE in the stored-grain beetle species *Cryptolestes pusillus* and *Sitophilus oryzae*. The well studied *Electrophorus electricus* AChE (from now on Ee-AChE) was used as the reference enzyme. In the course of the work, we observed that in the sub-millimolar range some monoterpenoids were able to increase Ee-AChE activity and in the millimolar range to inhibit it. The heretofore unreported dual ability of monoterpenoids to increase and decrease AChE activity in a concentration dependent manner prompted us to study this phenomenon.

2. Materials and methods

2.1. Chemicals

The monoterpenoids, (±) linalool, (−) L-camphor, γ-terpinene, geraniol, (−) fenchone and (+) carvone, and the phenylpropanoids, *trans*-anethole and estragole, were purchased from Acros Organics BVBA/SPRL. Their structures are shown in Fig. 1. *E. electricus* AChE, acetylthiocholine iodide (ATCh) and 5,5′-dithio-bis(2-nitrobenzoic) acid (DTNB) were all supplied by Sigma–Aldrich Chemical Co. (UK).

2.2. AChE assay

Stock monoterpenoids and phenylpropanoids were made by dissolving suitable amounts in 5 ml ethanol before bringing them to 100 ml with 0.1 M phosphate buffer, pH 7.5 (PB). For the stock DTNB (10 mM), 0.16 g was dissolved in 40 ml PB containing 0.6 g of $NaHCO_3$. AChE was assayed at 25 °C by the Ellman method [23], the reaction mixture consisting of 2.6 ml PB, 0.2 ml DTNB (10 mM), 50 µl enzyme (5 units of Ee-AChE per ml water) and 0.2 ml ATCh (15 mM, 1 mM in the mixture). A blank made to subtract non-enzymic hydrolysis of ATCh was included in the double beam spectrophotometer. In assays made with monoterpenoids and phenylpropanoids (0.02–10 mM), 1 ml of appropriate stock in ethanol-PB replaced 1 ml PB in the reaction mixture. Ethanol

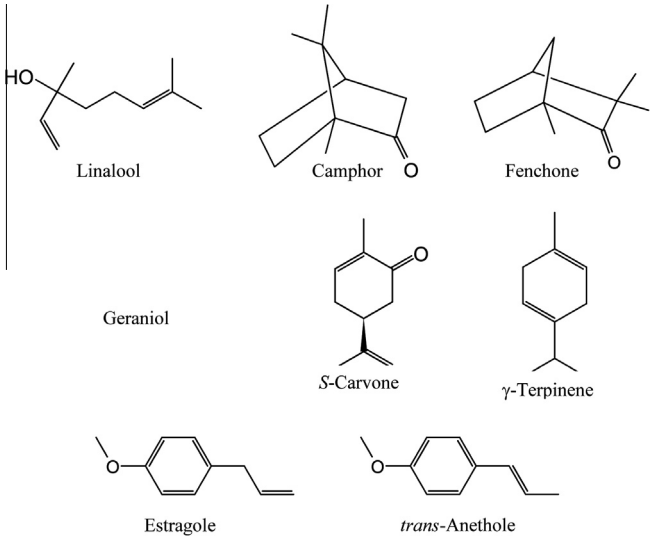

Fig. 1. Chemical structures of the monoterpenoids and phenylpropanoids used in this study.

was kept below 1%. AChE activity was measured from the increase in absorbance at 412 nm in a Helios-Zeta double beam spectrophotometer (Thermo Scientific). AChE activity is given in micromolar of ATCh hydrolyzed per min (U/min). Activity values are given as mean ± SD of 3–4 separate experiments. The extent of AChE activation or inhibition by monoterpenoids and phenylpropanoids was calculated from the activity change in assays without (control) and with the compounds.

The reported ability of thiocholine (TCh) to react with carvone and with other Michael systems containing a keto group [24–26] makes it possible a capture by carvone of liberated TCh, which would prevent it from reacting with DTNB. For testing this, a suitable TCh solution was prepared [27], using 2 ml ATCh (50 mM in PB) and 10 U Ee-AChE. After assessing the conversion of ATCh into TCh, AChE was removed using Amicon Ultracell 10,000 filters. The equal absorbance at 412 nm of a control TCh solution (0.05 mM TCh plus 0.6 mM DTNB) and the corresponding terpenoid-added mixtures showed that no terpenoid, carvone included, was able to react with a significant part of the freshly formed TCh during the enzymic reaction time (5–10 min). Nevertheless, the interaction carvone–TCh should always be tested before using the Ellman assay for measuring AChE in samples whose low activity makes it necessary a longer reaction time.

In the search for the molecular basis of AChE inhibition, the IC50 values for the selected monoterpenoids were calculated and compared with the values given by other authors (Table 1). Looking for a possible relationship between the AChE inhibitory potency of monoterpenoids and their lipid solubility, the logarithm of the IC50 value of each monoterpenoid was plotted against the logarithm of its octanol/water partition coefficient (Supplementary Fig. S1). Finally, a possible cooperativity in the monoterpenoid–AChE interaction was tested by plotting in logarithmic scale the fraction of inhibited AChE, i.e. the extent of AChE inhibition at each monoterpenoid concentration with respect to the maximum inhibition level attained, against the monoterpenoid concentration. When this kind of plot is made, the resulting straight line displays a positive slope, whose value (Hill number) is higher than one in case of positive cooperative in the protein–ligand interaction. The unexpected finding of Hill numbers ranging 2.04–1.57 supported the idea that AChE of *Electrophorus* at least can bind more than one monoterpenoid molecule, which made necessary kinetic analysis for assessing whether one or more inhibitor molecules bind to the enzyme.