Phytochem Rev
https://doi.org/10.1007/s11101-021-09790-0

The Amaryllidaceae alkaloids: an untapped source of acetylcholinesterase inhibitors

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Received: 10 June 2021 / Accepted: 4 November 2021

Abstract The AChE inhibitory activity of alkaloid extracts and compounds has been in the focus of research on the plants of Amaryllidoideae subfamily since the approval of galanthamine in 2001 by FDA for treatment of mild to moderate Alzheimer's disease. A small fraction of the huge biodiversity of the plants producing Amaryllidaceae alkaloids has been studied as a source of AChE inhibitors. Less than 20% of the known Amaryllidaceae alkaloids have been tested in vitro for their ability to inhibit AChE. Galanthamine, lycorine and haemanthamine are scaffolds for semi-synthesis of potent AChE inhibitors. In the last years, galanthamine has been studied in silico for drug optimization and synthesis of potent dual-binding AChE inhibitors. In silico studies of other Amaryllidaceae alkaloids have also provoked the interest of researchers in the last years. The aim of this article is to provide a comprehensive review on studies of the AChE inhibitory activity of extracts and compounds from the plants of Amaryllidoideae subfamily covering the literature from the last two decades.

Keywords Amaryllidaceae alkaloids · AChE inhibitory activity · Galanthamine · Drug design · Molecular docking · Plant extracts

Abbreviations

Aβ	Amyloid-β
ACh	Acetylcholine
AChE	Acetylcholinesterase
AD	Alzheimer's disease
ADME	Absorption, distribution, metabolism, and excretion
ATCI	Acetylthiocholine iodide
BBB	Blood-brain barrier
BSA	Bovine serum albumin
BS	Binding site
CAS	Catalytic active site
Ch	Choline
DTNB	Dithiobisnitrobenzoic acid
eeAChE	*Electrophorus electrus* AChE
ESI	Electrospray ionisation
GC	Gas chromatography
hAChE	Human AChE

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s11101-021-09790-0.

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Published online: 18 November 2021

HPLC	High-performance liquid chromatography
IMER	Immobilized enzyme reactor
MS	Mass spectrometry
PAS	Peripheral anionic site
PBS	Phosphate buffer solution
rhAChE	Recombinant human AChE
RA	Relative activity
RMSD	Root-mean-square deviation
SF	Scoring function
TcAChE	*Torpedo californica* AChE
TS	Transition state

Introduction

The Amaryllidaceae alkaloids are a distinctive chemotaxonomic feature of the long-known Amaryllidaceae family, whose status, based on molecular phylogenetic studies, has been reduced during the last ten years to a subfamily, the Amaryllidoideae (Chase et al. 2009, 2016). The Amaryllidoideae subfamily consists of 59 genera and over 800 perennial bulb species distributed throughout the tropics and warm temperate regions (Stevens 2001). The Amaryllidaceae alkaloids provoke interest due to their wide range and potent biological activities. Till the end of March 2021, the term "Amaryllidaceae alkaloids" has been mentioned in about 2296 papers in PubMed, more than 1800 of which have been published since 2001 when the acetylcholinesterase (AChE) inhibitor galanthamine was approved by the FDA for treatment of Alzheimer's disease (AD).

Alkaloids such as galanthamine, haemanthamine, lycorine and pancratistatin are lead molecules in AD and anticancer drug research. Some Amaryllidaceae alkaloids have recently shown inhibitory activity of glycogen synthase kinase-3β (GSK-3β), associated AD, bipolar disorder, strokes, more than 15 types of cancer, and diabetes (Hulcová et al. 2018). Excellent reviews have been published over the years on their biological (Bastida et al. 2006, 2011), antiprotozoal (Nair and van Staden 2019), proapoptotic, antitumor, cytotoxic (Kornienko and Evidente 2008; Lamoral-Theys et al. 2010; Nair and van Staden 2014; Nair et al. 2012), antifungal (Nair and Staden, 2020) and antibacterial (Nair et al. 2017) activities. The AChE inhibitory activity of the Amaryllidaceae alkaloids, however, is one of the most intensively studied activities, probably due to the accessibility and easiness for application of the Ellman method. In the last years, the AChE inhibitory activity has been reported in most of the articles dealing with extracts or isolated compounds from the Amaryllidoideae plants. With exception of a few partial reviews on the AChE inhibitory activity of Brazilian Amaryllidaceae plants (de Andrade et al. 2012) and compounds from the lycorine type (Nair and van Staden 2012), no systematic review on the whole family or alkaloids have been done over the last two decades. Partial data on the AChE inhibitory activity of extracts or isolated compounds from the plants of Amaryllidoideae appear in more general reviews of natural AChE inhibitors (dos Santos et al. 2018) or in reviews on determined periods of time (Jin 2011, 2013; Jin and Yao 2019). A number of articles have been recently published on in silico approaches to study the interactions between Amaryllidaceae alkaloids and the AChE enzyme, which also needs to be appropriately reviewed (Cortes et al. 2015, 2018; Tallini et al. 2017, 2018; Moreno et al. 2020; Acosta et al. 2021, etc.).

To the best of our knowledge, about 214 (*ca*. 33%) of all known Amaryllidaceae alkaloids and extracts from over 106 species (*ca*. 13%) have been tested for their ability to inhibit the AChE. In this article, we attempt to provide a comprehensive overview on the AChE inhibitory activity and the recent in silico advances in the research of alkaloids and extracts from the Amaryllidoideae subfamily.

Amaryllidaceae alkaloids—distribution and chemodiversity

More than 636 Amaryllidaceae alkaloids had been reported till the end of 2018 (Berkov et al. 2020) since the isolation of lycorine from *Narcissus pseudonarcissus* in 1877 by Gerrard (Collins et al. 2010). Isolation of typical Amaryllidaceae alkaloids from plants of other plant families has been also reported in a few cases. For example, eighteen Amaryllidaceae alkaloids have been found in *Hosta plantaginea* (Asparagaceae) (Wang et al. 2007). Crinamine from *Dioscorea dregeana* (Dioscoreaceae) (Mulholland et al. 2002), lycorine and acetylcaranine from *Urginea altissima* (Hyacinthaceae) (Miyakado et al. 1975) have been isolated. Vice versa, alkaloids typical for dicotyledonous plants of the genus *Sceletium*

(Aizoaceae) have been found in *Narcissus triandrus* (Pigni et al. 2013). Capnoidine, characteristic of the Fumariaceae, and bulbocapnine, found in the Papaveraceae have been isolated from *Galanthus nivalis* subsp. *cilicicus* (Kaya et al. 2004).

Most of the Amaryllidaceae alkaloids are tertiary monomer bases, but *N*-oxides, quaternary, dimer, glucosylated and conjugated with fatty acids alkaloids have also been found. According to their skeleton, the Amaryllidaceae alkaloids are grouped into 42 structural types, but the majority of them are distributed in seventeen structural types. The most structurally diverse types are that of lycorine (19%), followed by haemanthamine- (15%), homolycorine- (13%) and crinine-(12%) types (Berkov et al. 2020; Table 1).

Alkaloids with new skeleton types as well as atypical compounds are constantly being described for the Amaryllidoideae subfamily. About 350 (44%) of the Amaryllidoideae species, have been studied for their alkaloid pattern and they appear to be an untapped source of new bioactive molecules. The main Amaryllidaceae skeleton types and their representative alkaloids are shown in Fig. 1.

Acetylcholinesterase

The acetylcholinesterase (AChE, EC 3.1.1.7), located on the post-synaptic membrane, hydrolyzes the neurotransmitter acetylcholine (ACh) to choline (Ch) blocking the impulse transmission in the interneurons of the central nervous system, in the preganglionic sympathetic and parasympathetic neurons of the autonomic nervous system and in the neuromuscular junction between the motor nerve and skeletal muscle of the peripheral nervous system (Čolović et al. 2013). ACh is released from the presynaptic neurons into the synaptic cleft where it binds to ACh receptors (nicotinic and muscarinic) on the postsynaptic membrane relaying the impulse from cell to cell(s). Ch is taken up again by the presynaptic neuron cells and converted to ACh with the participation of cholineacetyltransferase and Acetyl-CoA (Fig. 2) (Čolović et al. 2013).

The hydrolysis of ACh by AChE follows the double displacement mechanism and it can be separated in two stages, acetylation and deacetylation stage each accompanied with a tetrahedral transition state (TS) (Fisher and Wonnacott 2012; Lockridge and Quinn 2010). The γ-O atom of the Ser203 side chain makes a nucleophilic attack on the carbonyl carbon of the substrate thus forming the first tetrahedral transition state (Supplementary file S1). The imidazole side chain of His447 functioning as a general base picks up the proton of the OH group of the attacking serine. Next, the ester bond is breaked down accompanied with a proton transfer from the hisitidine imidazolium ion to the choline oxygen atom. Thus, the tetrahedral transition state decomposes to the trigonal acetylserine intermediate and leaving choline. In the deacetylation stage the resultant acyl enzyme intermediate is attacked by a water molecule serving as a nucleophile to form the second tetrahedral transition state. Analogically as in the first stage of catalysis, the imidazole side chain of His447 acts as a general base in the formation of the second transition state accepting a proton from the water molecule and as a general acid in the decomposition of the TS to acetate and restoring the active site of serine.

The binding site (BS) of AChE is well studied. The first crystallographic structures of *Torpedo californica* AChE (TcAChE) in complex with tacrine and

Table 1 Chemodiversity of the main Amaryllidaceae alkaloid types

Structural type	Number of alkaloids	Structural type	Number of alkaloids
Norbelladine	13	Pretazettine	32
Lycorine	119	Plicamine	23
Homolycorine	80	Secoplicamine	8
Galasine	5	Graciline	6
Galanthindole	15	Montanine	14
Crinine	85	Galanthamine	47
Haemanthamine	76	Ismine	5
Cripowelline	8	*Sceletium*	15
Narciclasine	33	Miscellaneous	51

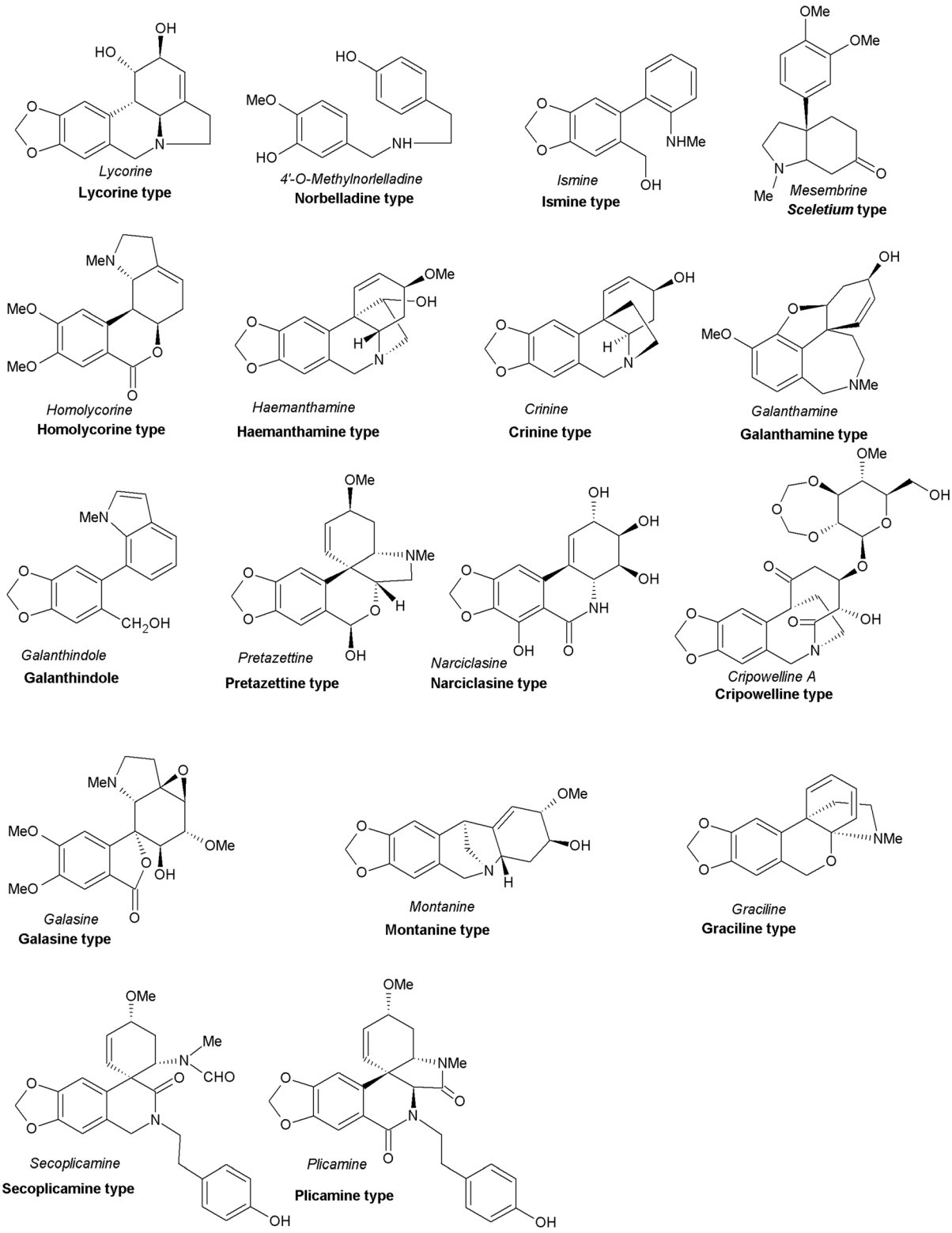


Fig. 1 Main skeleton types of the Amaryllidaceae alkaloids

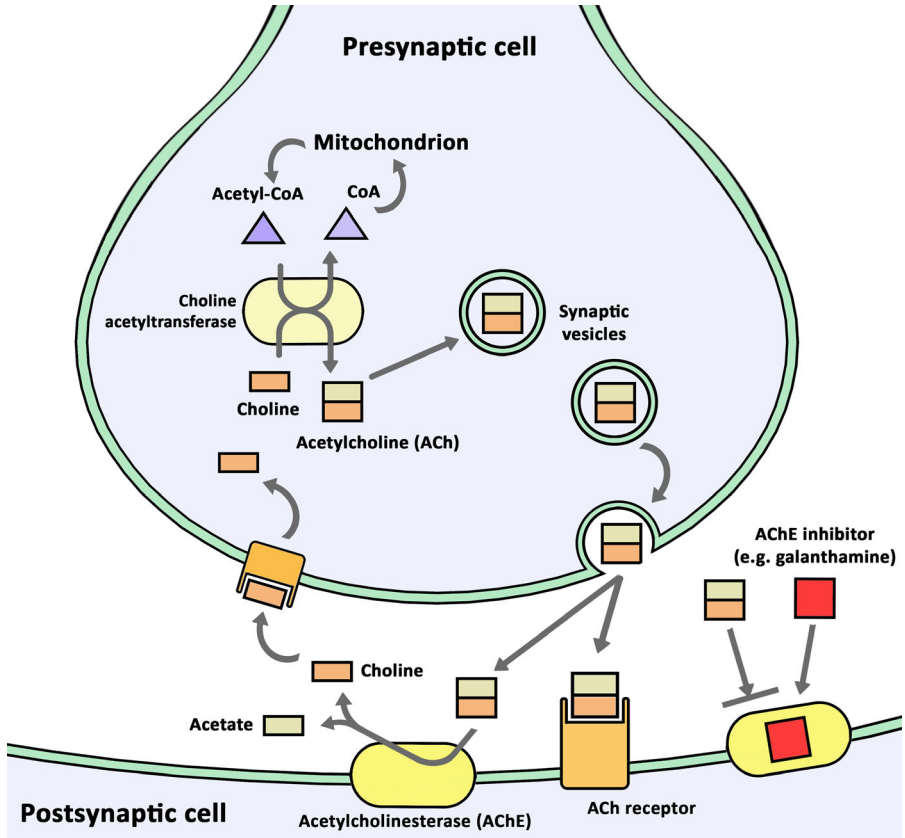


Fig. 2 Acetylcholine neurotransmission

edrophonium (with PDB codes, respectively: 1ACJ and 1ACL resolved at 2.8 Å) date since 1991 (Sussman et al. 1991). Since then at the Protein Data Bank (https://www.rcsb.org/) there are available above 250 crystallographic *apo*/*holo* structures of AChE with different origins. The BS of recombinant human AChE (rhAChE) presents a deep 20 Å and narrow gorge consisting of several sites—catalytic, anionic, acylic, oxyanionic and peripheral anionic (Fig. 3) (Harel et al. 1993; Ordentlich et al. 1993; 1998; Radic et al. 1993; Sussman et al. 1991). The catalytic subsite consists of the catalytic triad—Ser203, Glu334 and His 447 and is located almost at the bottom of the binding gorge (Fig. 3b). The anionic site binds the choline part of the ACh and is located next to catalytic one. This site is composed of only aromatic amino acids—Trp86, Tyr133, Tyr337, and Phe338. They play a key role in binding, as they bind the positively charged trimethylammonium part of ACh via cation-π interactions. The selective binding of ACh is determined by the acyl pocket. It consists of two bulky amino acids—Phe295 and Phe297 that restrict the access of bulkier choline esters. This site stabilizes and orients the acetyl portion of ACh. A structural water molecule is hosted within the oxyanion hole and stabilizes the substrate tetrahedral transition state via hydrogen-bond network between the enzyme and substrate. The domain consists of Gly121, Gly122, and Ala204. There is a large dipole moment oriented along the axis of the gorge due to the aromatic amino acids lined within the groove. Thus the positively charged ACh is attracted down to the active site, facilitated by the cation-π interactions. Aromatic residues are included in the peripheral anionic site (PAS)—Tyr72, Asp74, Tyr124, Trp286 and Tyr341. The PAS is located at the entrance of the binding gorge and is proposed to trap the substrate via weakly binding of ACh through π interactions and thus enhancing its entry to the BS (Silman and Sussman 2008). The PAS allosterically modulates the catalysis (Kitz et al. 1970) and it is involved in non-cholinergic functions as amyloid deposition (De Ferrari et al.

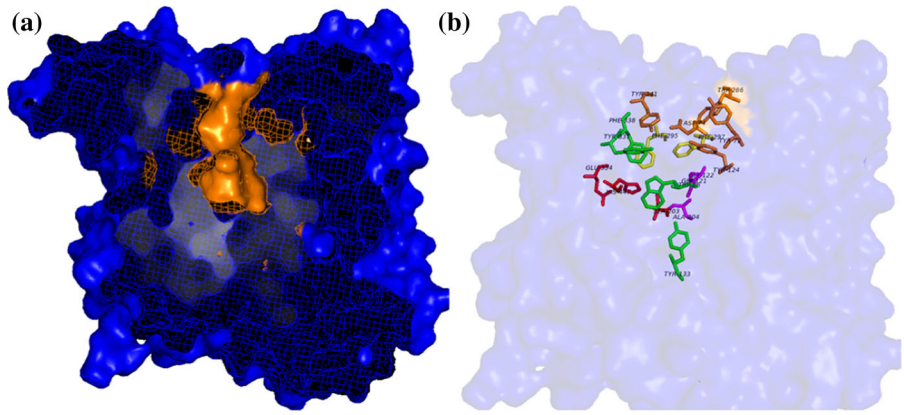


Fig. 3 The binding site (BS) of rhAChE is presented. **a** The surface of the binding gorge is shown in orange. **b** The amino acids of the subsites are shown in different color: the catalytic, consisted of catalytic triad (Ser203, Glu334 and His 447)—in red, the anionic (Trp86, Tyr133, Tyr337, and Phe338)—in green; the acyl pocket (Phe295 and Phe297)—in yellow, the oxyanion hole (Gly121, Gly122, and Ala204)—in magenta and the peripheral anionic site (Tyr72, Asp74, Tyr124, Trp286 and Tyr341) in orange. (Colour figure online)

2001), cell adhesion and neurite outgrowth (Johnson and Moore 2004, 2006). Thus the PAS is the second important drug target at the BS after the catalytic one.

AChE inhibitors

The inhibition of AChE leads to ACh accumulation into the synaptic cleft which results in hyperstimulation of nicotinic and muscarinic receptors increasing the level and duration of neurotransmission. Due to their biological effects, the AChE inhibitors are applied as drugs in medicine for treatment of various diseases, as insecticides in agriculture and as chemical weapons. AChE inhibitors are classified into irreversible and reversible according to their mode of binding with the active center of the enzyme.

Irreversible AChE inhibitors

The toxic effects/applications of the irreversible AChE inhibitors are associated mainly with the organophosphorus compounds (OPs; esters or thiols of phosphoric, phosphonic, phosphinic or phosphoramidic acids), which non-reversibly phosphorylate AChE. They are manifested as the muscarinic (lacrimation, salivation, miosis), nicotinic (neuromuscular blockade) or central (breath depression) symptoms and death (Bajgar 2004). OPs are commonly used as insecticides in agriculture, causing environmental pollution, health risks to humans and non-target animals and reducing the biodiversity in the treated areas. OPs are also used as therapeutic agents in ophthalmology or as nerve agents in chemical weapons (sarin, tabun, soman, cyclosarin and VX, Lane et al. 2020).

Reversible AChE inhibitors in the management of AD

The reversible AChE inhibitors are classified into synthetic, semi-synthetic and natural, considering their origin, and into carbamates (derived from carbamic acid), quaternary or tertiary compounds, according to their functional groups. They are widely applied in medicine for treatment of various diseases such as AD, myasthenia gravis, postoperative ileus, bladder distention, glaucoma, antidote to anticholinergic overdose, as well as in agriculture as insecticides (mainly carbamates).

The reversible AChE inhibitors, however, are mostly used in the treatment of AD, which is among the most costly diseases for society of Europe and the United States (Takizawa et al. 2015). AD is a chronic, progressive, and neurodegenerative disorder which is the most common form of dementia among people over 60 years. Over 45 million people are suffering from AD worldwide and this number is expected to double every 20 years (Fiest et al. 2016; Scheltens et al. 2016; Anonymous 2020). AD is characterized by

cognitive and memory decline, progressive impairment of daily activities, and a variety of neuropsychiatric symptoms and behavioral disturbances (Tarawneh and Holtzman 2012). Pathophysiology of AD involves neuronal and synaptical degeneration as a result from beta-amyloid (Aβ) protein aggregation and neurofibrillary tangles (aggregates of hyperphosphorylated tau protein), neuroinflammation, oxidative stress and excitotoxicity interfering neurotransmission (Madeo and Elsayad 2013; Godyn et al. 2016; Henstridge et al. 2016).

There is no cure for AD, though some drugs may temporarily improve symptoms. Most currently available drug therapies for AD are based on the cholinergic hypothesis, which proposes that AD is caused by reduction in the activity of the cholinergic neurons and the synthesis of acetylcholine, in particular (Bartus et al. 1982). The AChE inhibitors galanthamine (**1**, approved by FDA in 2001), donepezil (**2**, approved by FDA in 1996), and rivastigmine (**3**, approved by FDA in 1997, Fig. 4), and the glutamate antagonist memantine are the four currently marketed drugs to treat cognitive problems of AD and improve life quality. The first reversible AChE inhibitor approved by FDA in 1995 for treatment of AD was tacrine (**4**), but it was soon withdrawn due to its hepatotoxicity (Castillo-Ordoñez and Cajas-Salazar 2020).

Donepezil (**2**) is a reversible AChE inhibitor, delaying the deposition of amyloid plaque and improving cognitive function in patients with severe AD symptoms (Camps et al. 2008). Rivastigmine (**3**) is a powerful, slow, reversible carbamate inhibitor of both butyrylcholinesterase (BuChE) and AChE (Kandiah et al. 2017). Galanthamine (**1**) is a natural alkaloid isolated from several plants from the Amaryllidaceae family (Berkov et al. 2009) which is used for treatment of mild to moderate AD (Heinrich 2010). In contrast to donepezil (**2**) and rivastigmine (**3**), galanthamine (**1**) interacts with the nicotinic receptors enhancing their activity in the presence of ACh (Akk and Steinbach 2005). This effect appears to be beneficial for the AD treatment as the cognitive impairment correlates with loss of nicotinic receptors (Buckingham et al. 2009). The allosteric potentiating effects of galanthamine (**1**) on nicotinic receptors also affect monoamine-, glutamate-, and γ-aminobutyric acid (GABA) neurotransmitter systems, improving cognitive dysfunction and psychiatric illness in schizophrenia, major depression, bipolar disorder and alcohol abuse (Ago et al. 2011). A clinically relevant oral dose of galanthamine effectively prevents lethality and neuropathology induced by a supralethal dose of the nerve agent soman in *Cynomolgus* monkeys posttreated with conventional antidotes (Lane et al. 2020). There is no evidence that any of donepezil (**2**), rivastigmine (**3**) and galanthamine (**1**) is superior in terms of efficacy, however, donepezil is better tolerated. These drugs do not reduce the rate of decline in cognitive or functional

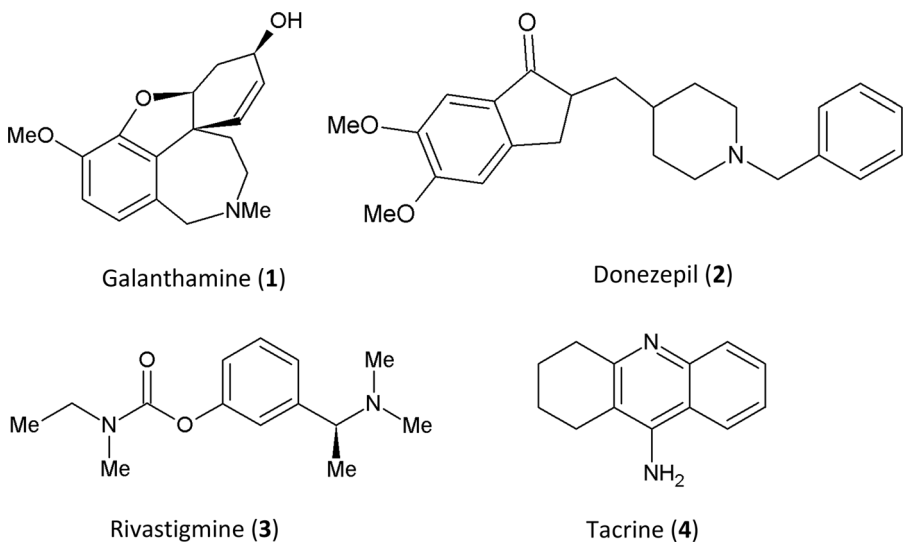


Fig. 4 Structures of AChE inhibitors approved for treatment of AD

capacities over the long term but provide meaningful symptomatic benefits (Čolović et al. 2013).

Over the last three decades, a number of reversible AChE inhibitors have been studied in clinical trials for their efficacy in AD management. Many of them failed to reach significant efficacy in phase III of development. Among them are the natural compound huperzine A, and eptastigmine, a carbamate derivative of physostigmine (isolated from *Physostigma venenosum*). Other compounds, such as the galanthamine prodrug Memogain® and ladostigil are still under evaluation (for a review, see Galimberti and Scarpini 2016).

AChE interacts with the Aβ peptide throughout the PAS forming highly neurotoxic AChE-Aβ complexes. These complexes promote the assembly of Aβ into amyloid fibrils that grow irreversibly to the characteristic for the AD amyloid plaques (Inestrosa et al. 2008). In the last years, considerable efforts have been focused on in silico design and synthesis of so called dual-site binding AChE inhibitors which are able to bind simultaneously both the CAS and the PAS of AChE. Many novel hybrid derivatives, such as donepezil-tacrine, oxoisoaporphine-tacrine, galanthamine-curcumine and other dual-site binding AChE inhibitors, exhibiting a high AChE inhibitory activity with IC_{50} values in the nanomolar range, have been studied from pharmacological and biochemical point of view or in mouse and rat models (Alonso et al. 2005; Camps et al. 2008; Sharma 2019; Stavrakov et al. 2020).

Methods for AChE inhibitory assay of plant extracts and natural compounds from Amaryllidoideae plants

Colorimetric, photometric, fluorimetric, chemo-luminescent, and electrochemical methods are known to assay the AChE inhibitory activity (Rhee et al. 2004). The Ellman's spectrophotometric method has been most frequently applied in plant extracts and compounds from the Amaryllidoideae in the past twenty years. It is based on measurement of 5-thio-2-nitrobenzoic acid, which is produced after reaction of thiocholine (liberated after enzymatic hydrolysis of acetylthioholine iodide, ATCI) with Ellman's reagent (dithiobisnitrobenzoic acid, DTNB, Ellman et al. 1961). The method has been modified and applied in spectrophotometric, microplate, TLC-bio-autographic and HPLC-on-line flow assays.

Spectrophotometric method

The method described by Ellman had been largely used to assay the AChE inhibitory activity of plant extracts and compounds before 2000 (for example in Ryan and Byrne 1988). It has undergone various modifications during the years with respect to enzyme source, concentrations of enzyme, DTNB, and ATCI. In a typical run, Ellman et al. (1961) use 50 µl of bovine erythrocyte cholinesterase at concentration of 5 U/ml, 3 ml of 0.1 M phosphate buffer at pH 8, 100 µl of 10 mM DTNB in phosphate buffer at pH 7 (DTNB is more stable at pH7), and 20 µl of 75 mM ATCI. Perry et al. (2000), assaying essential oils and terpenes from *Salvia lavandulifolia,* mix 50 µl of 0.39 U/ml of AChE from human erythrocytes in phosphate buffer (pH 8) and 20 µl of sample solution in 2.0 ml of phosphate buffer and incubate the mixture on ice at 4 °C for 30 min. Then the authors add 20 µl of DTNB (10 mM) in phosphate buffer (pH 7) and 20 µl of ATCI (0.03–0.05 mM in water) incubating the mixture at 25 °C for 6 min before spectrophotometric measurement at 412 nm. Moodley et al. (2021) assayed the AChE inhibitory activity of alkaloids from *Crinum stuhlmannii* applying a method described by Ren et al. (2004). The authors use 40 µL of 0.86 U/ml AChE solution (pH 8) and 20 µl of sample solutions in 2.0 ml phosphate buffer starting the reaction after 20 min with 20 µl of DTNB (0.05 mM) in phosphate buffer (pH 7) and 20 µl of ATCI (0.06 mM in buffer, pH7).

The different modifications of the Ellman method may result in different IC_{50} values of the tested compounds. The thiocholine production depends on the enzyme activity, which may be variable from different sources, and even in different batches from the same source (Ren et al. 2004) and the enzyme concentrations (Ellman et al. 1961). That is why, the use of positive control is obligatory. Most often, galanthamine has been used as positive control in the AChE assays of extracts and compounds from the Amaryllidoideae plants.

The Ellman's method, however, is known to give many false-positive effects with aldehydes and amines, such as heptanal, decanal, cinnamaldehyde, anisaldehyde, benzaldehyde, hexylamine and

tryptamine. These compounds inhibit the chemical reaction between thiocholine (product of enzymatic reaction) and DTNB reducing the formation of yellow-colored 5-thio-2-nitrobenzoic acid (Rhee et al. 2003). Therefore, the plant extracts, fractions and isolated compounds assessed by the Ellman's method have to be verified for possible false-positive effects. Rhee et al. (2003) have developed a TLC method to distinguish the false-positive effects from true enzyme inhibition in plant extracts, which will be discussed below.

Microplate assay

The microplate AChE assay has been routinely used in bioactivity studies of extracts and compounds from the Amaryllidoideae plants. Ingkaninan et al. (2000) are one of the first who apply microplate assay to measure the AChE activity of samples including galanthamine. The authors' aim was the optimization of solvent composition for high-performance liquid chromatography-ultraviolet/mass spectral (HPLC–UV/MS) biochemical detection and identification of AChE inhibitors and found that solvents such as methanol, acetonitrile and acetic acid affect the AChE activity. Methanol at concentration of 2.5% in the sample inhibits the enzyme activity about 20%. The Ellman method is modified mixing in the wells 125 µl of 3 mM DTNB, 25 µl of 15 mM ATCI and 50 µl of buffer (50 mM Tris–HCl, pH 8) with 25 µL of samples diluted in buffer. Than 25 µL of 0.226 U/ml AChE (from electric eels, type VI-S lyophilized powder) solution in buffer with 0.1% bovine serum albumin (BSA) is added to the reaction mixture. The absorbance read at 405 nm was found to increase for more than 2 min, meaning that the microplates should be read after 2–3 min.

López et al. (2002), in their pioneering study on the AChE inhibitory activity of extracts and compounds from Amaryllidaceae plants, use 96 well plates mixing 50 µL of the samples (diluted from 10^{-2} to 10^{-8} M) in phosphate buffer solution (PBS: 8 mM K_2HPO_4, 2.3 mM NaH_2PO_4, 0.15 M NaCl, 0.05% Tween 20, pH 7.6) with 50 µL 0.25 U/ml of AChE (from electric eels, type VI-S lyophilized powder) solution in PBS. After incubation of 30 min at room temperature, 100 µL of substrate solution (0.1 M Na_2HPO_4, 0.5 M DTNB, 0.6 mM ATCI in water, pH 7.5) is added and the absorbances are read in a microplate reader at 405 nm three minutes later. Enzyme activity is calculated as a percentage compared to an assay using a buffer mixture without any inhibitor.

Later, different research groups have described some variations in the concentration of ATCI, DTNB or AChE. For example, Tallini et al. (2018) and Rojas-Vera et al. (2021) apply an AChE working solution with concentration of 6.24 U/ml. Bozkurt et al. (2021) use solutions with concentrations of 0.2 mM for DTNB and 0.24 mM for ATCI while Zhan et al. (2017) use 3 mM and 15 mM for DTNB and ATCI, respectively.

Thin-layer chromatography (TLC) bio-autographic assay

The TLC bio-autographic assay of AChE inhibitory activity of plant extracts and compounds is a rapid and simple screening approach. It is not applicable to calculate IC_{50} values. Two methods have been used to detect active compounds in Amaryllidoideae plants. Rhee et al. (2001) stain developed TLC plates with Ellman's reagent (DNTB) to detect enzyme activity resulting in white spots for active compounds on yellow background. The enzyme at final concentrations of 3 U/ml is dissolved in a buffer with 50 mM Tris–HCl (pH 8) containing 0.1% BSA as a stabilizer. The detection limits for galanthamine is lower (0.014 µg/spot) as compared to UV (0.6 µg/spot at 254 nm), Dragendorff's reagent (0.1 µg/spot), and microplate (0.092 µg/well) detection. The method has been used for screening of a number of plants and detection of AChE inhibitors on TLC plates, which later were identified by mass spectrometry (Berkov et al. 2011).

In order to detect false-positive effects, Rhee et al. (2003) run in parallel another TLC plate under the same chromatographic conditions. The plate first is sprayed with a solution of DTNB and than with a solution of thiocholine obtained by premixing AChE with ATCI. Thus, compounds which inhibit the chemical reaction between thiocholine and DNTB give false-positives (white spots on yellow background) and are easily distinguished by comparison between both TLC plates. Using this approach, the authors detected a number of false-positive spots (compounds) in *Nerine bowdenii* facilitating the choice of fractions with true AChE inhibitors for further investigation.

In the method described by Marston et al. (2002), the enzyme activity is detected by the conversion of naphthyl acetate into naphthol and the formation of the corresponding purple-colored diazonium dye with Fast Blue B salt. AChE inhibitors produce white spots on the dye-colored background of the TLC plates. The enzyme solution is with concentration of 6.7 U/ml. The detection limits for galanthamine is comparable with the method of Rhee et al. (2001). Mroczek et al. (2020) applied this method for bio-guided isolation of AChE inhibitors from *Narcissus* cv. Hawera extracts. Sibanyoni et al. (2020) also preferred the method for screening and isolation of AChE inhibitors from 28 South African Amaryllidaceae species.

HPLC on-line flow assay

The main advantage of HPLC on-line flow assay is the simultaneous separation of complex mixtures of natural compounds and selective detection and identification of AChE inhibitors. Although HPLC on-line flow assay is very effective in terms of detection and isolation of active compounds, it has found limited application in the search of Amaryllidoideae plants for AChE inhibitors. Probably, this is due to the lack of appropriate devices on the market for on-line AChE inhibitory measurements and their sophisticated structure. Ingkaninan et al. (2000) describe the development of a method for detection of AChE inhibitors with HPLC coupled on-line with UV–MS-flow biochemical detection system. The biochemical detection is based on colorimetric method using Ellman's reagent. The system consists of a HPLC with UV and MS detectors for separation and identification of compounds, and a biochemical detection system with three pumps first mixing DTNB and AChE and then ATCI solutions. Than the flow is pumped into a reaction coil and the AChE inhibitors are monitored by a UV detector set at 405 nm. The sensitivity for galanthamine was found to be 0.3 nM. Rhee et al. (2004) describe the isolation of a potent AChE inhibitor of lycorine type, ungeremine, from *Nerine bowdenii* by preparative HPLC coupled on-Line to a flow assay system. After separation of the compounds in the preparative HPLC column, the flow is split into three parts, to an assay system, to a MS detector, and to a photodiode array detector to get the UV chromatogram and to isolate compounds in a fraction collector. 7-Acetoxy-1-methylquinolinium iodide is used as a substrate and the AChE inhibitors are detected by a fluorescence monitor.

Yuan et al. (2020) describe a HPLC system hyphenated on-line with an immobilized enzyme reactor (IMER) which is used for on-line monitoring of AChE inhibitory activity of compounds in extract from *Lycoris radiata*. It consists of a HPLC separation part and an enzyme reaction part. The enzyme reaction part consists of a substrate pump, IMER kept at a stable room temperature (25 °C) and an electrospray ionization-mass spectrometry (ESI–MS) detector. The HPLC separation and the substrate flows are combined by a tee before entering to the IMER. The substrate solution (600 μM ACh in 15 mM NH_4HCO_3) is pumped into the IMER. ESI–MS operates in positive full scan MS mode in the range of *m/z* 50–600. The passing of ACh (substrate) through the IMER with immobilized AChE results in constant MS signals of formed Ch (*m/z* 104) and of remaining ACh (*m/z* 146). When an eluted AChE inhibitor passes through the IMER, the quantity of the formed Ch decreases and that of remaining ACh increases resulting in a negative peak for the ion at *m/z* 104 (Ch) and a positive peak for the ion at *m/z* 146 (ACh).

Fluorimetric AChE assay

Recent technological advances in high-throughput screening platforms result in miniaturization of assays toward microarrays for detection of enzyme activity, saving significant efforts, time and costs. In this respect, the work of Monton et al. (2010) is notable, reporting a reliable, robust, reproducible and high-throughput fluorimetric screening method for AChE inhibitors. The method is based on a disulfide-thiol interchange reaction between the fluorigenic dimeric dye 4,4-difluoro-1,3,5,7-tetramethyl-4-bora-3a,4a-diaza-s-indacene (BODIPY®FL) L-cystine and thiocholine generated by the AChE hydrolysis of ATCh. The result is a brightly fluorescent (green) monomeric product with exceptional spectral and photophysical stability which is not sensitive to pH. In fact, the Ellman's reagent DNTB is replaced with BODIPY. Fabrication of AChE microarrays of 1200 spots (400 μm in diameter and 16 μm in thickness) is achieved printing onto slides nanoliter volumes of solutions containing the dye, ATCh, and potential inhibitors.

The new fluorimetric assay was found to be comparable with the Ellman's assay with respect to the K_i and IC_{50} values of known AChE inhibitors such as the Amaryllidaceae alkaloids galanthamine (**1**) and sanguinine. It was used to screen two libraries of small molecules, including synthetic derivatives of Amaryllidaceae alkaloids. Mixtures of 20 compounds at a concentration of 2 μM per a compound were screened to increase throughput. The compounds of mixtures showing activity were screened individually at a concentration of 5 μM each, in order to identify the active one(s). A total of 1040 compounds were assayed rapidly using 52 mixtures. The lycorine derivatives 2-*tert*-butyldimethylsilyllycorine and 1-benzoyl-2-acetyllycorine were found to be the most active among the screened compounds with IC_{50} values of 16 μM and 21 μM, respectively. The calculated K_i values of these compounds (2.6 μM and 3.9 μM, respectively) were in agreement with the values obtained using Ellman assay (0.9 μM and 1.0 μM, respectively, Monton et al. (2010).

The Amaryllidaceae family as a source of AChE inhibitors

Extracts from Amaryllidaceae plants with AChE inhibitory activity

The first step towards the discovery of natural AChE inhibitors is the assay of AChE inhibitory activity of plant extracts. AChE microplate and TLC bio-authographic (Calderón et al. 2010) assays allow high throughput screening of crude extracts and alkaloid fractions from a large number of plant samples. Determination of IC_{50} values by microplate assays is the most applied and simple approach encompassing bioactive extracts and fractions for further bio-guided isolation of active compounds. To the best of our knowledge, alkaloid extracts and fractions from 106 wild species from 20 genera and over 100 cultivars from the Amaryllidoideae subfamily have been studied for their AChE inhibitory activity. When applicable, in Fig. 5 are presented the IC_{50} values of plant extracts and compounds, expressed as relative (to galanthamine in the same study) IC_{50} for better comparison of the results between different studies. The original IC_{50} values are presented in Supplementary file S2. Both, crude alcoholic, alcohol/water or ethyl acetate extracts (requiring simple and fast sample preparation) and alkaloid fractions have been used in screening studies. As examples, Sibanyoni et al. (2020), after measuring the IC_{50} values of methanol extracts from 28 South African Amaryllidoideae plants, selected the extract with highest activity (from *Amaryllis belladonna*, IC_{50} of 14,3 μg/ml) and isolated a strong AChE inhibitor, acetylcaranine (IC_{50} of 11.7 μM). Naidoo et al. (2018) isolated three AChE inhibitors after bio-guided fractionation of a crude ethanol extract from *Scadoxus puniceus* (IC_{50} of 70 μg/ml) by column chromatography. Two of them, haemanthamine and haemanthidine are typical Amaryllidaceae alkaloids, while metolachlor is known as herbicide (Pothuluri et al. 1997). Among the crude extracts inhibiting most effectively AChE are those of *Scadoxus puniceus* (IC_{50} value of 0.3 μg/ml) and *Crinum bulbispermum* (IC_{50} of 2.1 μg/ml, Adewusi and Steenkamp 2011), *Z. candida* (IC_{50} of 4.23 μg/ml) and *Habranthus robustus* (IC_{50} of 7.92 μg/ml, Shawky et al. 2019).

López et al. (2002) use solid-phase extraction with C18 SPE cartridges to prepare alkaloid fractions from methanol extracts of 26 wild *Narcissus* species for microplate assay. Most often, however, the alkaloid fractions for AChE inhibitory activity assays are obtained by the classical liquid–liquid acid–base extraction. In the final step of purification procedure, the alkaloids are fractionated at $pH > 8$ by ethyl acetate, chloroform, dichloromethane, petroleum ether or butanol. The organic solvent used for fractionation of alkaloids affects both the content of non-alkaloid compounds and alkaloid patterns, and consequently the IC_{50} values (Elisha et al. 2013; Gasca et al. 2020). The alkaloid fractions from *Ammocharis coranica* (IC_{50} value of 0.05 μg/ml, Elisha et al. 2013), *Crinum amabile* (IC_{50} value of 0.05 μg/ml, Rojas-Vera et al. 2021), *Galanthus cilicicus* (IC_{50} value of 0.154 for leaf alkaloid fraction and IC_{50} value of 0.407 for bulb alkaloid fraction, Kaya et al. 2017), *Galanthus woronowii* (IC_{50} value of 0.027 μg/ml for leaf alkaloid fraction and IC_{50} value of 0.084 μg/ml for bulb alkaloid fraction, Bozkurt et al. 2017), *Habranthus jamesonii* (IC_{50} value of 0.7 μg/ml, Cavallaro et al. 2014), *Phaedranassa cuencana* (IC_{50} value of 0.88 μg/ml, Moreno et al. 2020) and *Narcissus confusus* (IC_{50} value of 0.053 μg/ml, López et al. 2002) have shown potent AChE inhibitory activity with IC_{50} values below 1 μg/ml. From the above

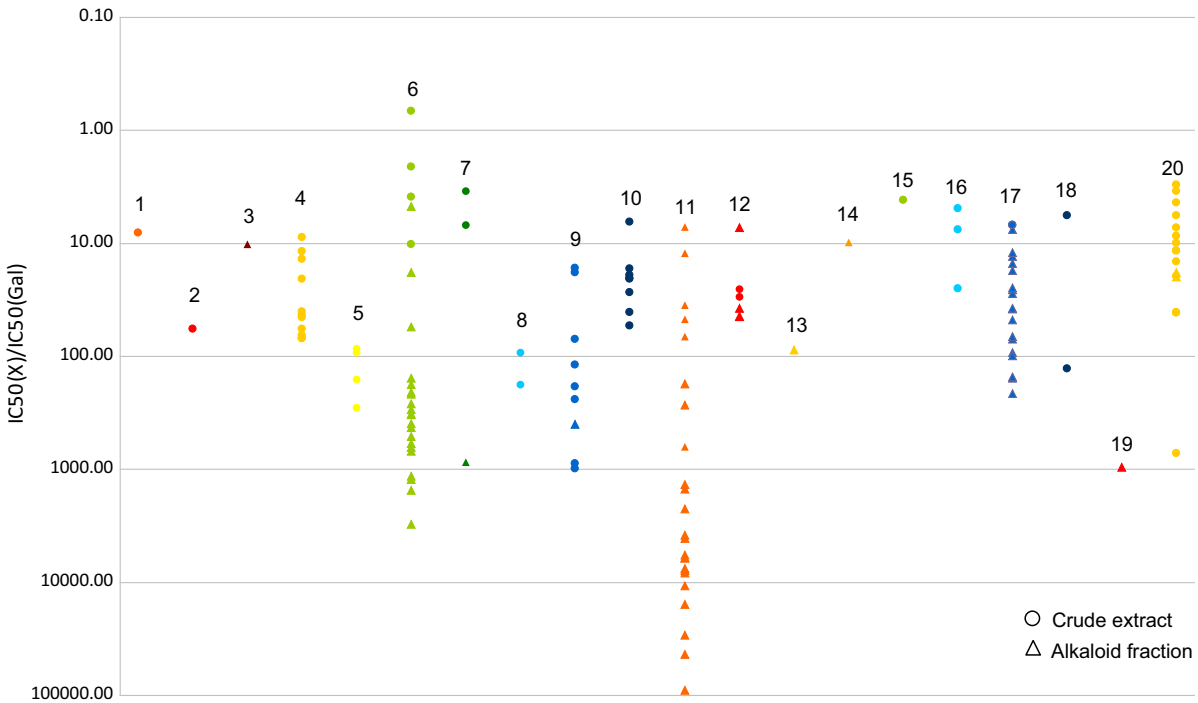


Fig. 5 AChE inhibitory activity of extracts from wild Amaryllidoideae plants. The values are expressed as IC_{50} (μg/ml) of crude extract or alkaloid fraction/IC_{50} of galanthamine (μg/ml). 1-*Amaryllis*; 2-*Brunsvigia*; 3-*Chlidanthus*; 4-*Crinum*; 5-*Cryptostephanus*, 6-*Galanthus*; 7-*Habranthus*; 8-*Haemanthus*; 9-*Hippeastrum*; 10-*Hieronymiella*; 11-*Narcissus*; 12-*Nerine*; 13-*Pancratium*; 14-*Phaedranassa*; 15-*Phycella*; 16-*Rhodolirium*; 17-*Rhodophiala*; 18-*Scadoxus*; 19-*Sternbergia*; 20-*Zephyranthes*

mentioned alkaloid fractions containing galanthamine, only that of *Galanthus woronowii* leaves has an IC_{50} value below that of galanthamine (IC_{50}—0.04 μg/ml, Bozkurt et al. 2017) indicating that not only galanthamine contribute to the bioactivity (Fig. 5 and Supplementary file S2).

The high AChE inhibitory activity of the alkaloid extracts and fractions is attributed to the content of galanthamine and other known and potent AChE inhibitors such as sanguinine, 11-hydroxygalanthamine and epinorgalanthamine (López et al. 2002; Torras-Claveria et al. 2013; Moreno et al. 2020). The lack or low percentage contribution of known potent AChE inhibitors in the alkaloid fractions with high AChE inhibitory activity is an indication for the presence of other strong AChE inhibitors (Cahlíková et al. 2011). Torras-Claveria et al. (2013) have studied the AChE inhibitory activity of extracts from leaves and bulbs of 105 *Narcissus* cultivars and correlated their activity with the galanthamine content. The authors found extracts with activity higher than the predicted and concluded that sanguinine (detected in the leaves of 22 out of 105 cultivars) contribute significantly to the AChE inhibitory activity. GC–MS is the most applied approach to identify Amaryllidaceae alkaloids and to determine their relative percentage in the alkaloid mixtures (Cortes et al. 2015, Bozkurt et al. 2017, etc.). In the recent years, in silico approaches have been frequently used for both, searching for AChE inhibitors within the studied extracts and to better explain their activity (Moraga-Nicolás et al. 2018; Moreno et al. 2020; Ortiz et al. 2018; Rojas-Vera et al. 2021; Shawky et al. 2019; Sibanyoni et al. 2020, etc.).

Amaryllidaceae alkaloids with AChE inhibitory activity

To the best of our knowledge, about 224 (*ca.* 35%, Supplementary file S3) of all known Amaryllidaceae alkaloids have been tested in vitro for their ability to inhibit AChE. The direct comparison of the activity

using IC_{50} values from different studies is problematic due to variations in the IC_{50} values reported for the positive control galanthamine (**1**). The IC_{50} value of galathamine has been found to range from 0.04 μM (Endo et al. 2019) to 27.90 μM (Lee et al. 2014) which is a difference between the highest and lowest value of about 700-times. The possible reasons for the inter-laboratory variations of IC_{50} value are discussed above. As galanthamine has been applied as a positive control in all the reviewed articles, we decided to use the ratio between the IC_{50} of a compound and that of galanthamine reported in the same article as a value for relative activity (RA) in order to compare more accurately the activity of the alkaloids. Although the RA may depend on variations in Ellman's assay in the different laboratories, it better compares the AChE inhibitory activities among compounds assayed in different laboratories. As an example, López et al. (2002) reported IC_{50} of 1.07 μM and 0.1 μM for galanthamine (**1**) and sanguinine (**5**, Fig. 6), respectively. On the other hand, Sarikaya et al. (2013) found IC_{50} of 0.15 μM and 0.007 μM for galanthamine (**1**) and sanguinine (**5**), respectively. The RA values of sanguinine (**5**) are 0.093 and 0.046, respectively. The difference between the IC_{50} values of sanguinine (**5**) is 14.3 times, while it is 2-times when the value of RA is used.

The IC_{50} and RA values of the assayed Amaryllidaceae alkaloids are presented in Supplementary file S3. In Fig. 7, the compounds are subjectively grouped into potent (RA values between 0.00 and 1.00), strong (RA values between 1.01 and 10.00), moderate (RA values between 10.01 and 100), and weak (RA values over 100) AChE inhibitors. The RA value of the positive control galanthamine is 1. Compounds that are stronger AChE inhibitors than galanthamine have RA values less than 1, and vice versa, the weaker AChE inhibitors have RA values higher than 1.

Sixty-eight (*ca.* 30%) out of 224 tested alkaloids have shown AChE inhibitory activity with RA values below 100. The AChE inhibitory activity of the Amaryllidaceae alkaloids is associated with galanthamine and the galanthamine-type skeleton. However, among the most potent AChE inhibitors, with RA values below 1.0, two compounds out of six (including galanthamine) possess lycorine-type skeleton (Fig. 6). Sanguinine (**5**) is the most potent known natural Amaryllidaceae alkaloid with RA of 0.09 (IC_{50} of 0.09 μM, López et al. 2002). *N*-allylnorgalanthamine (**6**) and *N*-(14-methylallyl)norgalanthamine (**7**), minor compounds found in *Leucojum aestivum* (Berkov et al. 2009), show RA values comparable with that of sanguinine, 0.10 and 0.09, respectively. Galanthamine has been used as a scaffold for synthesis of a number of potent AChE inhibitors, which will be discussed below. Ungeremine (**8**) is a lycorine-type compound showing a potent AChE inhibitory activity (IC_{50} of 0.33 μM) with RA value of 0.15 (Rhee et al. 2004). 1-*O*-Acetyllycorine (**9**), another lycorine-type compound from *Crinum moorei*, has shown IC_{50} of

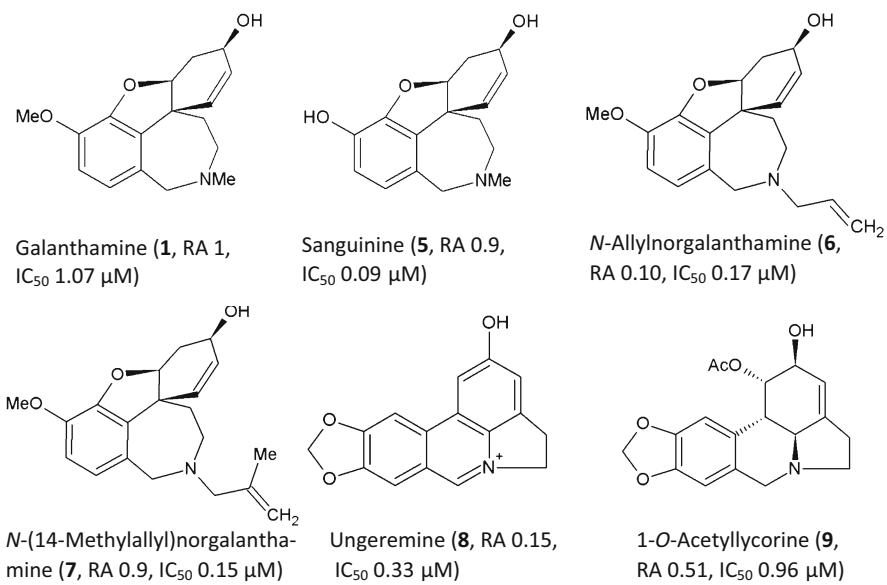


Fig. 6 Amaryllidaceae alkaloids with higher AChE inhibitory activity than galanthamine

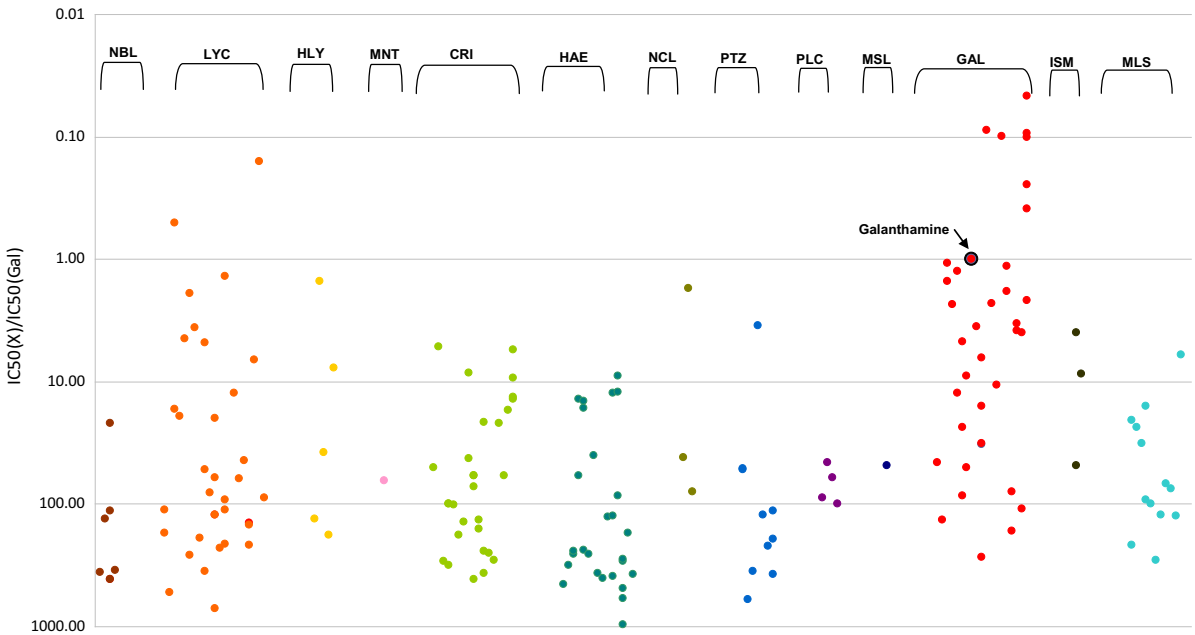


Fig. 7 Distribution of the alkaloids according their structural type and AChE inhibitory activity. The values are expressed as IC_{50} (μM) of alkaloid /IC_{50} of galanthamine (μM). NBL—Norbelladine type; LYC—Lycorine type; HLY—Homolycorine type; MNT—Montanine type; CRI—Crinine type; HAE—Haemanthamine type; NCL—Narciclasine type; PTZ—Pretazettine type; PLC—Plicamine type; GLD—Galanthindole type; GAL—Galanthamine type; ISM—Ismine type; MSL—Miscellaneous type

0.96 μM with RA value of 0.51 (Elgorashi et al. 2004). Lycorine has also been used as a scaffold for a synthesis of a number of lycorine analogs with AChE inhibitory activity (McNulty et al. 2010). 2-*O*-Acetyllycorine, lycorine and 1,2-*O*-diacetyllycorine, have significantly diminished activity while 1-acetoxy-2-TBS-lycorine (*Ki* = 0.34 μM) and 1-benzoate-2-TBS-lycorine (*Ki* = 0.39 μM) have shown activity comparable with that of galanthamine (*Ki* = 0.30 μM; McNulty et al. 2010; Nair and Van Staden 2012). Synthetic seco-lycorine derivatives, 5-butyl-8,9-methylenedioxy-4-vinyl-5,6-dihydrophenanthridine and 5-methyl-8,9-methylenedioxy-4-vinyl-5,6-dihydrophenanthridine with RA values of 0.83 and 0.91, respectively, were found to be more active AChE inhibitors than galanthamine (Lee et al. 2007).

Thirty-two of the assayed alkaloids (14%) have shown strong AChE inhibitory activity with RA values between 1.0 and 10 (Fig. 7 and Supplementary file S3). Among them, compounds with galanthamine- and lycorine-type skeletons are dominant. A few typical Amaryllidaceae alkaloids of crinine- (8-hydroxy-9-methoxycrinine, buphanidrine, and undulatine), haemanthamine- (haemanthidine), pretazettine-(deoxytazettine), and narciclasine-type (*N*-methylcrinasiadine), homolycorine (deoxylycorenine and *O*-demethylhomolycorine), ismine-(ismine and zephycandidine III) are in this group. In fact, the structure of *O*-demethylhomolycorine in the article reporting its activity is presented as *O*-methyllycorenine (Yang et al. 2010). The AChE inhibitory activity of the haemanthamine-type compounds provoked research on their semisynthetic derivatives. Haemanthamine itself has a weak AChE inhibitory activity ($IC_{50} > 100$ μM) but it is abundant in some Amarylidoideae plants and like galanthamine and lycorine can be easily isolated and used for semisynthesis. Aromatic esters of haemanthamine have shown RA values of 8.6 ($IC_{50} > 14.7$ μM, Kohelová et al. 2019) and RA 2.35 ($IC_{50} > 4.0$ μM, Peřinová et al., (2020). It should be noted that the RA values of some compounds in this group have shown a wide range of variability. As examples, the RA values of lycorine vary from 1.38 (Yang et al. 2010) to 211 (Tallini et al. 2018) while those of galanthine vary from 4.80 (Zhan et al. 2017) to 354 (Kulhánková et al. 2013,

Supplementary file S3). Such varying results have to be interpreted cautiously considering the IC_{50} values of the positive control and results from other research groups. On the other hand, four independent measurements of the AChE inhibitory activity of undulatine have shown RA values between 5.48 and 13.82 indicating that the compound is a strong AChE inhibitor. *N*-Chloromethylgalanthamine, reported as natural compound (Zhu et al. 2015), has RA value of 2.28, but it may be considered as an artifact of isolation procedure (Matusch et al. 1994). Compounds from all of the tested skeleton types have representatives in the group of moderate AChE inhibitors (Fig. 7).

Interactions of galanthamine within the active site of rhAChE

The first crystallographic structure of TcAChE was revealed in 1991 by Sussman and co-authors (Sussman et al. 1991). The first two independent crystallographic structures of TcAChE in complex with galanthamine are available at PDB database since the end of 1999 (Bartolucci et al. 2001; Greenblatt et al. 1999). In 2001 the first ad hoc docking methodology of galanthamine in the BS of TcAChE was performed with a remarkable success (Pilger et al. 2001). Pilger and co-authors used the seven available X-ray structures of TcAChE in complexes with different ligands excluding galanthamine in order to predict its pose, *i.e.* orientation and conformation, of a small molecule within the binding site. The authors combined a rigid docking procedure of AUTODOCK with flexible optimization of the resulting complexes with Tripos force field. Fourty-two residuals of the BS were allowed to relax during the optimization procedure. The authors accurately predict the pose of galanthamine in terms of RMSD (root-mean-square deviation) value $\sim$ 0.5 Å when compared to the later released crystalographic structure of the complex between the galanthamine and TcAChE.

The first crystallographic structure of rhAChE in a complex with galanthamine dates back to 2012 (Cheung et al. 2012). The structure of galanthamine fits well within the binding site of rhAChE (Fig. 3). The alkaloid forms four hydrogen bonds within the BS of the enzyme. The hydrogen and oxygen atoms of the hydroxy group of galanthamine form two hydrogen bonds—one with the carboxyl group of Glu202 and second, with the structural water molecule (Fig. 8). The structural water molecule serves as a bridge between the AChE and galanthamine as it stabilizes the complex via hydrogen bond network. One hydrogen bond is formed between the oxygen atom of the ligand's methoxy group and the hydroxy group of Ser203. The protonated ammonium group is involved in a fourth hydrogen bond with the oxygen atom of the hydroxy group of Tyr337. A π-π contact is formed between the phenyl ring of galanthamine and Phe338. Additionally, the quaternary ammonium group is involved in a cation-π interaction with Tyr337. Two hydrophobic contacts between the galanthamine cycloheptane moiety and Trp86 and Gly448 stabilize the complex.

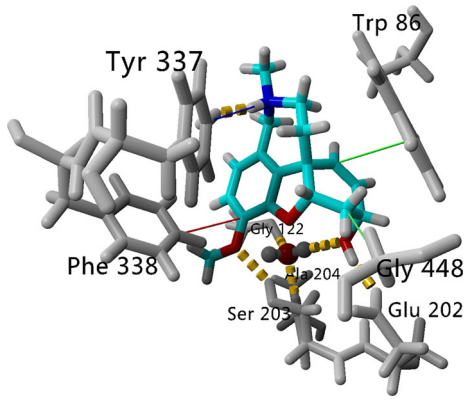


Fig. 8 The intermolecular interactions of galanthamine within the binding site of *rh*AChE are presented. Hydrogen bonds are shown in orange dashes, cation-π interactions are presented in blue dashes, π–π contacts are given in red lines, and hydrophobic contacts are shown in green lines. The structural water molecule is presented in ball-and-stick style. (Colour figure online)

Design of AChE inhibitors based on galanthamine

The well situated galanthamine and the great diversity and number of interactions within the binding site of AChE designates its moiety as a good anchor for AChE binding and as a lead structure for drug design. Molecular docking is a structure-based method playing a key role in rational drug design. It is used for binding predictions of newly designed ligands as well as for virtual screening of huge databases of small molecules. The predicted poses of binding, i.e. the

orientation and conformation of both partners, are estimated via scoring functions. Usually scoring functions rarely correlate with the experimental binding activity, though the opposite is observed in some occasions. Molecular docking is used solely or in combination with 2D- and 3D-QSAR, pharmacophore models or machine learning methods (Jansen and Martin 2004; Jain 2004; Kishan 2007; Villoutreix et al. 2007; Fukunishi 2009; Sobhia et al. 2010; Kirchmair et al. 2011; Scotti et al. 2012; Debnath 2013; Safavi et al. 2013; Kumar et al. 2014; Tomioka 2014; Siddiqi and Siddiqi 2014). In this section we will focus on galanthamine-based AChE inhibitors designed via molecular docking.

The first to our knowledge, application of molecular docking in the design of galanthamine-based derivatives as AChE inhibitors was performed by Simoni et al. in 2012 (Simoni et al. 2012). The authors investigated the length of the linker connecting galanthamine and memantine, via molecular docking. Docking simulations were performed on a human AChE modeled by superimposition and tethering of the crystal structure of human AChE (hAChE, PDB code 1BY1) to the crystal structure of TcAChE in complex with bis-acting galanthamine derivative (PDB code 1W4L) using ICM 3.7. The Biased Probabililty Monte Carlo stochastic optimizer implemented in ICM 3.7 was used for docking simulations and the resulted poses were evaluated with the standard empirical ICM scoring function. The docking score of compounds with five, six and seven methylene groups as a linker between galanthamine and memantine defines them as the most promising AChE inhibitors. These predictions were confirmed by AChE inhibitory test where compounds containing six and seven methylene groups showed prominent inhibitory activities to AChE with IC_{50} values of 1.16 and 1.73 nM, respectively. All newly designed compounds showed higher AChE inhibition with IC_{50} values in nanomolar range comparing to galanthamine IC_{50} value of 2.55 μM. Furthermore, two NMDAR assyas and SHSY-5Y viability assay were conducted with the new molecules indicating that the compound with six methylene groups, named memagal, is the most promising multitarget agent.

A good example of application of molecular docking in drug design is work of Atanasova et al. who derived a docking-based model for prediction of newly designed galanthamine derivatives (Atanasova, et al. 2015a, b). The model is able to predict the IC_{50} values of newly designed galanthamine- containing AChE inhibitors from the derived docking scores. For this purpose, the docking protocol was optimized in a stepwise manner in terms of scoring function (SF), flexibility/rigidity of the BS, presence/absence of a water molecule, radius of the BS, by GOLD software (CCDC Ltd., Cambridge, UK). The flexibility of the ligand as well as the flexibility of the residues within the BS are taken into account during the search. On each step, the correlation between the SF and pIC_{50} (-log IC_{50}) values of galanthamine derivatives was evaluated. The settings of the optimized protocol are as follows: GoldScore scoring function, flexible ligand, flexible binding site (Tyr72, Asp74, Trp86, Tyr124, Ser125, Trp286, Phe297, Tyr337, Phe338 and Tyr341), presence of a structural water molecule and radius of the BS 10 Å. Firstly, the protocol was applied on a training set of 22 synthetic galanthamine derivatives and external test set of 11 natural alkaloids as IC_{50} values were taken from the literature. The correlation between GoldScore and pIC_{50} values for compounds of the training set was moderate (0.536) while for the external test set it was 0.800 (Atanasova et al. 2015a). Next, the same protocol was applied on another training set of 41 synthetic galanthamine derivatives and a correlation coefficient of 0.826 was achieved (Atanasova et al. 2015b). In that study, the authors designed and docked as an external test set 10 N-substituted indole galanthamine derivatives. Four of them having the highest docking score were synthesized and tested for AChE inhibitory activity. All four compounds were more active than galanthamine as two of them, denoted as **10** (IC_{50} = 0.011 μM) and **11** (IC_{50} = 0.012 μM) are 95- and 93-times, respectively, more active than galanthamine (IC_{50} = 1.070 μM) (Table 2). The newly designed derivatives binds PAS which makes them dual-site binders in the groove of AChE. Later, a similar protocol was applied to 20 newly designed derivatives resulted from combination of five different terminal phenyl residues and four alkylamide linkers at the *N* atom with different length (butyl-, pentyl-, hexyl-, and heptylamide) that connect the galanthamine moiety and terminal parts of the compounds (Stavrakov et al. 2016). Among the designed and docked compounds, 11 with highest score were synthesized and tested for AChE inhibitory activity. Two of them, **12** (IC_{50} = 0.0008 μM) and **13** (IC_{50} = 0.0011 μM,

Table 2 Designed galantamine derivatives as dual-site binders

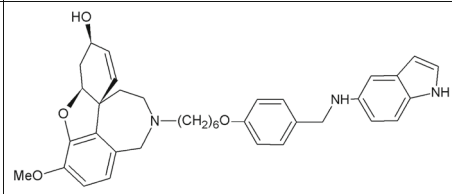
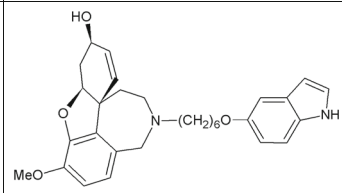
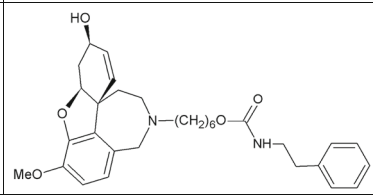
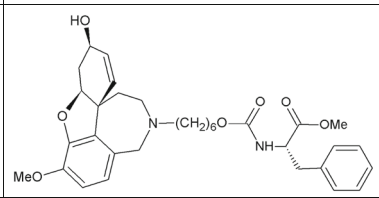
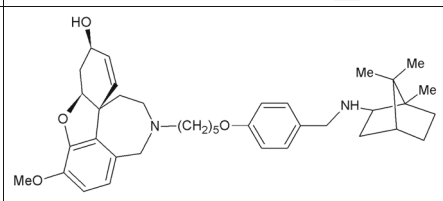
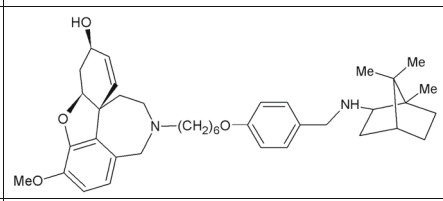
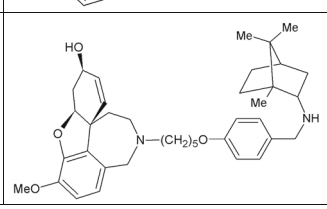
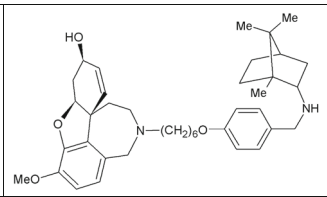
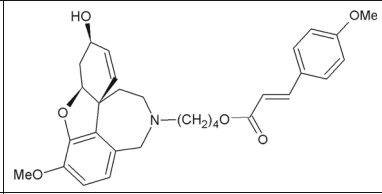
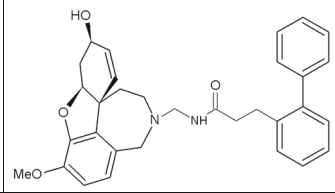
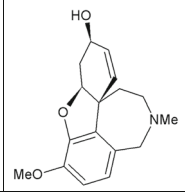
Compound ID	Structure	Docking score	IC_{50}, μM	Reference
10		119.15	0.011	Atanasova, Stavrakov, et al. 2015
11		112.32	0.012	Atanasova, Stavrakov, et al. 2015
12		109.94	0.0008	Stavrakov et al. 2016
13		116.95	0.0011	Stavrakov et al. 2016
14		111.683	0.0047	Stavrakov et al. 2017
15		112.340	0.0099	Stavrakov et al. 2017
16		111.013	0.0056	Stavrakov et al. 2017

Table 2 continued

17		114.433	0.0029	Stavrakov et al. 2017
18		94.31	0.020	Stavrakov et al. 2020
19		105.56	0.028	Stavrakov et al. 2021
Galantamine **1**		74.56	1.070 3.520* 1.92**	

*Value observed in Stavrakov et al. (2020)

**Value observed in Stavrakov et al. (2021)

showed, 1338- and 1008-fold higher activity, respectively, compared to galanthamine (IC_{50} = 1.070 μM) (Table 2). A common feature of both molecules is the longest heptylamide spacer responsible for a good filling of the binding gorge. The terminal phenyl ring of the most active compound **12** takes part in a sandwich-type π−π intercation with Tyr72 and His287. The docking-based model has proved its ability to predict the AChE inhibitory activity as good correlation (r = 0.828) between the ChemPLP scores and the pIC_{50} values of the novel designed and tested derivatives was found.

Recently, eight novel galanthamine-camphane hybrids were modeled, docked, synthesized and tested for inhibition on AChE applying the same protocol (Stavrakov et al. 2017). Two terminal bulky fragments, isobornylamine and bornylamine, in combination with four spacers, hexylamide, heptylamide, 4-(pentyloxy)benzylamine and 4-(hexyloxy)benzylamine, were designed. All of them have highter ChemPLP score compared to that of galanthamine and as expected, showed greater AChE inhibitory activity than the parent alkaloid. Four of them containing benzylamine linker, denoted as **14**, **15**, **16**, and **17**, have the highest inhibition affinity with IC_{50} values as follows 0.0047, 0.0099, 0.0056 and 0.0029 μM, respectively (Table 2). Thus, they are between 108- and 369-times more active than galanthamine. Obviously, the phenyl ring of the linker plays an important role in binding to AChE by taking part in π-π interactions with Tyr72, Trp286 and Tyr341 as in the case of **15** and **17** it forms a "sandwich" with Trp286 and Tyr341. The π-π interaction with Trp286 is responsible for the preferred "closed" conformation of Tyr286, thus reducing the cavity of the PAS as observed previously (Stavrakov et al. 2016).

Additionally, the camphane fragment makes hydrophobic interactions with Tyr72 or Leu76 and blocks PAS. Among the terminal substituents, the bornylamine showed higher docking scores. The value of Pearson correlation coefficient between the ChemPLP scores and experimental pIC_{50} values, $R^2 = 0.893$, confirms the good predicted ability of the docking-based model.

Recently, a combinatorial library of 72 galanthamine-curcumine hybrids as dual-side AChE inhibitors were designed (Stavrakov et al. 2020). The structures of the novel hybrids consist of galanthamine core, linkers and terminal aromatic fragments. Nine types of linkers that resemble the curcumine α–β unsaturated fragment and eight terminal aromatic substituents (a single phenyl ring, phenyl ring with methoxy or methyl groups at m- or p-position, or both, and a 1,3-dioxole ring that replaces the two methoxy groups) were combined. The compounds were screened for absorption, distribution, metabolism, and excretion (ADME) properties and blood–brain barrier (BBB) permeability as 44 of them passed these criteria. Then molecules were docked on rhAChE applying a previously derived protocol (Atanasova et al. 2015a, b). All compounds have higher scores compared to galanthamine and 14 hybrids with different score range—highest, moderate and low, were selected for synthesis. Compounds were tested for toxicity on NEURO-2A cells and five hybrids that are less toxic than curcumine were selected for tests to AChE. All tested molecules showed superior affinity to the enzyme with IC_{50} values between 0.086 and 0.020 μM, which means from 41- to 186-fold higher activity compared to that of galanthamine (IC_{50} = 3.520 μM). A molecular dynamics simulation of the complex between the most active compound **18** (IC_{50} = 0.020 μM) (Table 2) and AChE revealed that the oxygen atom at the methoxy group of the terminal phenyl ring is able to form a hydrogen bond with Tyr72 that is most probably responsible for the better binding and activity.

A similar in silico protocol, including BBB permeability prediction, and docking was applied on 1220 fragment-based designed galanthamine derivatives (Stavrakov et al. 2021). The lead structure among the top 10 best-scored derivatives was synthesized and tested for AChE inhibitory activity and neurotoxicity. The molecule (**19**) is non-toxic and showed IC_{50} of 27.79 nM which makes it 68 times more active than galanthamine (IC_{50} = 1.92 μM) (Table 2).

Docking studies on other Amaryllidaceae alkaloids

The docking programs are able to dock almost every ligand into a defined binding site at any conditions of the protocol. There is no universal SF, docking protocol and conditions that can be applied in all case studies and therefore, careful optimization of the docking protocol, its validation, choosing a SF (if more than one are available) and etc. should be done including: (1) A well selected crystallographic structure with holo protein (with a bound ligand) in a complex with a structurally similar to the studied molecules ligand when it is available. (2) Consideration of the protonation state of the studied molecules at physiological pH. The knowledge of the BS of AChE indicates that positively charged ligands are attracted by PAS and moved to the bottom of the gorge where CAS is situated. Therefore, one of the key points of the ligand preparation is the calculation of pKa value and using the corresponding ionization state at physiological pH for docking calculations. (3) Validation of the docking protocol according to the RMSD (root-mean-square deviation) value of the re-docked ligand from the crystal structure. For this reason, the protocol should be optimized in terms of SF (when more than one is available), flexible residues within the binding site, structural water molecules and other parameters implemented in the docking software, until the best predicted pose of the re-docked ligand is in accordance with the experimental ones.

In the recent years, molecular docking on AChE has been applied in many phytochemical studies on Amaryllidoideae plants in order to reveal the possible binding modes of detected or isolated alkaloids and to give an approximation of their binding affinity. One of the first attempts in this direction is the work of Cortes et al. (2015). The authors analyze the alkaloid pattern and AChE inhibitory activity of alkaloid extracts from five Amaryllidoideae plants and choose for further in silico studies the most active one, from *Zephyranthes carinata*. Lycoramine, 3-*epi*-macronine, 3-oxocrinine, tazettine, maritidine, galanthine, vittatine, lycorine, 3-*O*-demethylmaritidine, 11,12-dihydrolicorene, trisphaeridine, and 11,12-didehydroanhydrolycorine were docked on TcAChE using Autodock 4.2

software. The ligand structures were optimized using the semiempirical AM1 method implemented in MOPAC 07. The protocol was validated as galanthamine was docked in two TcAChE X-ray structures—one in complex with galanthamine itself (PDB code 1DX6) and the second, in complex with donepezil (PDB code 1EVE). The docking scores and inhibitory constants K_i were compared with each other and were found to be similar. A similar docking protocol was applied on anhydrolycorine, dihydrolycorine, galanthamine-*N*-oxide, 8-*O*-demethylmaritidine, sanguinine, galanthamine, narwedine, lycorine, methylpseudolycorine, anhydrolycorine, vittatine, deacetylbowdensine, powelline, oxopowelline, undulatine-diol, buphanidrine and undulatine derived from *Zephyranthes carinata*, *Phaedranassa lehmannii*, *Eucharis bonplandii*, *Eucharis caucana*, *Crinum jagus*, *Hippeastrum elegans*, and *Clivia miniata* (Cortes et al. 2018). The predicted energies of binding via rigid protein docking with Autodock 4.2 software was conducted on the binding site of TcAChE (PBD code: 1DX6, galanthamine-AChE complex) and rhAChE (PBD code: 4EY7, donepezil-AChE complex). Several subsequent docking studies of Amaryllidaceae alkaloids using the same docking protocol were published examining the mode of binding of 6β-hydroxymaritidine, 6α-hydroxymaritidine, reticulinine and isoreticuline from *Hippeastrum reticulatum* (Tallini et al. 2017), and 11-hydroxyvittatine, hamayne, deacetylcantabricine, haemanthamine, *O*-methyltazettine, 11,12-dehydroanhydrolycorine, galanthindole, trisphaeridine, dihydrobicolorine, and ismine from *Rhodophiala splendens* (Tallini et al. 2018). Similarly, 1-*O*-acetylcaranine, galanthine, 1-*O*-acetyllycorine, sternbergine, 2-methoxypratosine, cantabricine, galanthamine, *N*-demethylgalanthamine, *N*-formylnorgalanthamine, 6-epimesembranol, mesembrine, and pancratinine C identified in *Phaedranassa species* (Moreno et al. 2021) and vittatine, crinine and crinamine, originating from *Phaedranassa brevifolia* Meerow were subjected to molecular docking (Acosta et al. 2021). In another docking study of buphanisine, lycorine and sanguinine, detected in the leaf extract of *Crinum amabile*, the X-ray complexes between hAChE and donepezil and paraoxon (PDB codes: 4EY7 and 5HF5) were used in AutoDock software (Rojas-Vera et al. 2021). In general, the corresponding estimated binding free energies in the above mentioned reports were lower than those for galanthamine. The poses of different alkaloids with high affinities within the AChE binding side were presented and discussed.

Buphanidrine, isolated from an extract of *Amaryllis belladonna* and showing in vitro high AChE inhibitory activity, was subjected to molecular docking using the apo (no ligand bound) form of *Electrophorus electrus* AChE (eeAChE) (PDB code: 1C2B) (Mella et al. 2021). The predicted pose of binding reveals that buphanidrine is located at the PAS region, with higher score (8.3 kcal/mol) than that of galanthamine (7.9 kcal/mol) which is not in agreement with their in vitro AChE inhibitory activities (IC_{50} value of 17.56 μg/ml, and 0.39 μg/ml, respectively). A similar docking study of galanthamine, lycoramine, galanthaminon (narwedine), 6α-deoxytazettine, norpluvine diacetate, 3-*O*-acetyl-1,2-dihidro-galanthamine, haemanthamine, undulatiane-diol, tazettine, acetylnatalensine, undulatine, 3-*epi*-macronine, and crinan-3-one detected by GC–MS in *Rhodolirium andicola* (Poepp.) Traub, was conducted using the apo X-ray structures of eeAChE (PDB code: 1C2B) and hAChE (PDB code: 4PQE, Moraga-Nicolás et al. 2018). Lycoramine and norpluvine diacetate show higher score (8.83 and 8.92 kcal/mol, respectively) than that of galanthamine (8.58 kcal/mol) in this study.

Three Amaryllidaceae alkaloids, named zephycandidines I-III, isolated from *Zephyranthes candida*, were subjected to molecular docking calculations (Zhan et al. 2017). The authors used Autodock 4.2. software for pose generation and MM/GBSA tool implemented in AMBER 11 for pose evaluation and ranking. The docking protocol was validated as ligands from five crystallographic complexes with hAChE (PBD codes: 4EY5 (huperizine-A-rhAChE), 4EY6 (galanthamine-rhAChE), 4EY7 (donepezil-rhAChE), 4M0E (dihydrotanshinone I- rhAChE), and 4M0F (territrem B-rhAChE). The authors separated the enzyme's binding gorge into two parts, one corresponding to the CAS area, and second, located at the PAS region. Molecular docking calculations were performed into both defined sites in order to confirm the ligand's primary binding at the PAS. Galanthamine was used as a structure for comparison. The results revealed that an initial contact with Trp286 from the PAS as well as an interaction with Tyr337 play a key role in AChE inhibition. These findings confirm the activity of zephycandidine III (IC_{50} = 8.82 μM) and galanthamine (IC_{50} = 1.02 μM), as

well as the lack of activity of zephycandidine I and II. Zephycandidine A, an imidazo[1,2-f]phenanthridine alkaloid, isolated from *Zephyranthes candida* was docked under the same methodology (Zhan et al. 2016). In this case, the docking results show that the Zephycandidine A binds to the PAS. This result is in a good accordance with the much weaker AChE inhibitory activity of the Zephycandidine A (IC_{50} = 127.99 μM) compared to galanthamine activity (IC_{50} = 1.02 μM).

A molecular docking study was performed on 6-hydrohymaritidine by Adessi et al. (Adessi et al. 2019). The β:α epimeric mixture of 6-hydroxymaritidine, isolated from *Hippeastrum reticulatum* that showed the highest activity within the studied seven haemanthamine skeleton type alkaloids, was subject to deep pre- and post-docking analysis, including a detailed consideration of the ligand protonation state at physiological pH, and ab-initio DFT calculations for estimation the conformers ratio in water. Four forms were subject to molecular docking—both isomers in neutral and protonated form. The TcAChE X-ray structure in complex with galanthamine (PBD code: 1W6R) was used for docking simulations as galanthamine was re-docked for protocol validation. The most theoretically stable complex having the lowest energy, -21.51 kcal/mol, corresponds to the protonated β-epimer of 6-hydroxymaritidine. The calculated energy for galanthamine is -6.62 kcal/mol, while its inhibitory activity was IC_{50} = 0.76 μM. The measured AChE activity for 6-hydroxymaritidine was IC_{50} = 10.53 μM. A detailed discussion of the docking results and docking-based structure–activity relationships between the seven haemanthamine skeleton type alkaloids and galanthamine were performed.

An extensive in silico study of 313 Amaryllidaceae alkaloids was performed by Shawky in 2017 in order to profile the various biological activities of the alkaloids (Shawky 2017). For this reason, seven proteins were used as targets—rhAChE, HIV-1 PR, HIV-1 RT, influenza NA, Aurora kinase A, HDAC2 and VEGF-R2. The studied alkaloids consists of nine chemical classes as 12 are of belladine-type, 80 of the lycorine-type, 82 of the crinine-type, 52 of the lycorenine(homolycroine)-type, 26 of the pretazettine-type, 15 of the narciclasine-type, 35 of the galanthamine-type and 8 of the montanine-type. The applied methods for in silico screening study were molecular docking, pharmacophore and 3D-QSAR models against the seven targets. The molecular docking was performed with XP-Glide. The protocol was validated in terms of pose and activity accuracy using different sets of target protein structures, cognate ligands obtained from DEKOIS (Demanding Evaluation Kits for Objective in silico Screening) and the 1000 decoys provided by Schrödinger in Glide enrichment studies. The results indicated that 7 galanthamine-type, 5 lycorine-type, 5 lycorenine-type and 5 pretazettine-type alkaloids would interact with hAChE. Within the top 30 alkaloids 10 are galanthamine-type including the highest scored apogalanthamine (GlideScore denoted as G-score = − 13.05 kcal/mol), nivalidine hydrochloride (apochlorine) with G-score = − 12 kcal/mol, *N*-allylnorgalanthamine (− 11.37 kcal/mol), sanguinine (− 11.04 kcal/mol) and galanthamine with G-score of − 10.08 kcal/mol. Among the top 10 scored alkaloids four lycorenine-type are included as clivacetine (− 12.59 kcal/mol), clivatine (− 12.32 kcal/mol), hippeastrine N-oxide (− 12.1 kcal/mol) and clivasine (− 11.3 kcal/mol). The predicted poses of apogalanthamine and clivacetine within the hAChE binding groove are compared with those of galanthamine and discussed.

Eight Amaryllidaceae alkaloids (−)-lycorine, (−)-pseudolycorine, (−)9-*O*-methylpseudolycorine, (−)-narcissidine, (+)-11-hydroxygalanthine, (−)-galanthamine, (+)-9-*O*-demethyl-2-α-hydroxyhomolycorine and (−)-pancratinine C, isolated form Turkish *Narcissus tazetta* were subject to a comprehensive molecular docking (Karakoyun et al. 2020). The X-ray structure of eeAChE in complex with donepezil (PBD code: 1EVE) was used as a target for the studied alkaloids. Water molecules within the biding site were kept for docking calculations. Energy minimization was conducted to protein structure and the protonated ligands using MOE.2016.08. The docking was performed with GOLD v.5.6.2 software. The generated poses were evaluated with GoldScore function. The protocol is described in detail and validated via re-docking of galanthamine and donepezil in the corresponding crystallographic complexes (PDB codes 1DX6 and 1EVE, respectively). The resulted Goldscore values of the studied compounds are very similar between 64.6201 and 58.7662, as for galanthamine is the highest, followed by 9-O-demethyl-2-α-hydroxyhomolycorine and (+) 11-hydroxygalanthine, with scores of 62.7722 and 62.7646, respectively. The last

alkaloid, (+) 11-hydroxygalanthine showed the highest inhibitory activity among the newly tested alkaloids with IC_{50} = 0.67 μM, while for galanthamine it was 0.15 μM. The most active alkaloids, (+) 11-hydroxygalanthine and (−)-narcissidine (IC_{50} = 1.85 μM) were located into the PAS, as the complexes are stabilized via hydrogen bonds with residues from the BS and water molecules as well, π-π stacking, H-π contacts, and salt bridges.

The four most active alkaloids against AChE isolated from *Amaryllis belladonna*, acetylcaranine, undulatine, buphanidrine and belladine, were subject to molecular docking study (Sibanyoni et al. 2020). The authors used AutoDock Vina implemented in LigandScout 4.09. The X-ray structure of TcAChE in complex with galanthamine (PDB entry 1QTI) was used. The protocol was optimized and validated according to the predicted pose of the re-docked galanthamine. Among the studied alkaloids, galanthamine was the most active one with IC_{50} = 6.19 μM having the highest calculated binding affinity (CBA) of -10.60 kcal/mol. The most active compound of newly alkaloids, acetylcaranine, showed IC_{50} = 11.7 μM and CBA = − 10.40 kcal/mol. For the most potent and the weakest AChE inhibitors the calculated binding affinities are in accordance with the experimental activity. The authors made detailed discussion and good representations of intermolecular intercations of the ligands within the binding site.

A ligand-based drug design of Amaryllidaceae alkaloids with reported biological inhibitory activity against AChE in ChEMBL and NPASS databases led to identification of 11 alkaloids (lycoramine, 1,2-dihydro-13-norgalanthamine, galanthamine *N*-oxide, chlidanthine, *N*-demethyllycoramine, masonine, acetylleucovernine, memogain (a semi-synthetic galanthamine derivative), nivalin, apochlorine, and acetylsanguinine), that were subject to further molecular docking investigation using Molegro Virtual Docker v. 6.0.1 (MVD) software (López et al. 2021). The crystallographic structure of rhAChE in complex with galanthamine (PBD code: 4EY6) was used for calculations. The protocol was validated via re-docking of galanthamine and comparing the resulted pose to the X-ray structure (RMSD = 0.27 Å). The authors provided detail information of contacts between the alkaloids and the enzyme and exhaustive discussion was done linking the docking results to the QSAR descriptors.

Recently, two Amaryllidaceae alkaloids, 8-hydroxy-9-methoxycrinine and *N*-norgalanthamine, were docked into the active site of rhAChE (PDB code: 4EY6) (Orhan et al. 2021). Docking was performed on Glide implemented in Schrödinger Small-Molecule Drug Discovery Suite. Both alkaloids showed AChE inhibitory activity as follow: 8-hydroxy-9-methoxycrinine (IC_{50} = 6.92 μg/ml) and *N*-norgalanthamine (IC_{50} = 2.42 μg/ml), galanthamine (IC50 = 1.33 μg/ml). The corresponding predicted binding energy values were − 9.260 kcal/mol and − 9.427 kcal/mol. It was found that both alkaloids occupy the middle area of the active site between the CAS and PAS while contacts with residues of the PAS and catalytic triad were observed.

No information about the docking protocols was found in two docking studies of Amaryllidaceae alkaloids to the AChE target (Castillo-Ordóñez et al. 2017; Shawky et al. 2019). In the fist one nine alkaloids, norbelladine, crinine, lycorine, lycorenine, hemanthamine, montanine, narciclasine, pretazettine, and galanthamine, selected from public repository PubChem, were subjected to molecular docking using GOLD (Castillo-Ordóñez et al. 2017). In the second one, the XP-Glide was used for molecular docking of 30 alkaloids detected from aquatic and terrestrial Amaryllidaceae species like *Crinum naans* Baker, *C. calamistratum* Bogner & Heine, *Zephyranthes candida* (Lindl.) Herb., *Z. rosea* Lindl., *Habranthus robustus* Herb (Shawky et al. 2019).

Conclusions

Small fractions of the known alkaloids and species from the Amaryllidoideae subfamily have been assayed for their AChE inhibitory activity. The results, however, indicate that this subfamily is an outstanding source of AChE inhibitors and further studies can result in revealing of other potent AChE inhibitors. Compounds form different skeleton types have shown strong AChE inhibitory activity, revealing possibilities for structure optimization and semi-synthesis of potent AChE inhibitors. Alkaloids from galantnamine-, lycorine-, and haemanthamine-types have shown the most potent AChE inhibitory activity. Galanthamine, lycorine and haemanthamine, which are one of the most abundant Amaryllidaceae compounds, have been used as scaffolds for semi-synthesis

of AChE inhibitors. The molecular docking of Amaryllidaceae alkaloids on AChE provokes the interest of researchers as an alternative tool to reveal new AChE inhibitors and their possible binding mode. The utilization of powerful in silico tools, however, should be used with caution. The application of molecular docking combined with ADME optimization and BBB permeability prediction of newly designed galanthamine-based AChE inhibitors showed good success.

Funding This work was supported by the Bulgarian National Science Fund (Grant DN03/9/2016) and Grant No BG05M2OP001-1.001-0003, financed by the Science and Education for Smart Growth Operational Program (2014–2020) and co-financed by the European Union through the European Structural and Investment funds.

Declarations

Conflict of interest The authors declare that there is no conflict of interest.

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