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Research paper

Discorhabdin alkaloids from Antarctic *Latrunculia* spp. sponges as a new class of cholinesterase inhibitors

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ABSTRACT

The brominated pyrroloiminoquinone alkaloids discorhabdins B, L and G and 3-dihydro-7,8- dehydrodiscorhabdin C, isolated from methanol extracts of two specimens of *Latrunculia* sp. sponges collected near the Antarctic Peninsula, are here demonstrated for the first time to be reversible competitive inhibitors of cholinesterases. They showed K_i for electric eel acetylcholinesterase of 1.6–15.0 μM, for recombinant human acetylcholinesterase of 22.8–98.0 μM, and for horse serum butyrylcholinesterase of 5.0–76.0 μM. These values are promising when compared to the current cholinesterase inhibitors used for treatment of patients with Alzheimer's disease, to counteract the acetylcholine deficiency in the brain. Good correlation was obtained between IC_{50} data and results by molecular docking calculation on the binding interactions within the acetylcholinesterase active site, which also indicated the moieties in discorhabdin structures involved. To avoid unwanted peripheral side effects that can appear in patients using some acetylcholinesterase inhibitors, electrophysiological experiments were carried out on one of the most active of these compounds, discorhabdin G, which confirmed that it had no detectable undesirable effects on neuromuscular transmission and skeletal muscle function. These findings are promising for development of cholinesterase inhibitors based on the scaffold of discorhabdins, as potential new agents for treatment of patients with Alzheimer's disease.

1. Introduction

Cancer and cardiovascular dysfunction are currently the most diffuse diseases in the developed world, followed by Alzheimer's disease. It is a progressive neurodegenerative disease that leads to gradual memory decline and loss of intellectual abilities, and is associated with behavioural disorders and personality change [1]. Alzheimer's disease is the most common type of dementia and because of the aging population; it has become an ever-increasing social and economic problem. Currently, there are some 46.8 million people around the world who live with Alzheimer's disease or some other kind of dementia, and this number is expected to double over the next 20 years [2]. Unfortunately, at present there is no treatment that can stop, or preferably reverse, the progression of this neurological disorder. With the current selection of therapeutic agents, it is only possible to slow the progression of the disease, and to reduce the symptomatology.

Patients diagnosed with Alzheimer's disease show the characteristic neurochemical deficit of the neurotransmitter acetylcholine, especially in the basal forebrain. This is a consequence of enhanced activity of their acetylcholinesterase (AChE; E.C. 3.1.1.7), an enzyme that is mainly located in nerve synapses and

Abbreviations: AChE, acetylcholinesterase; BChE, butyrylcholinesterase; eeAChE, electric eel acetylcholinesterase; ESI-MS, electrospray ionisation-mass spectrometry; hAChE, recombinant human acetylcholinesterase; HMBC, heteronuclear multiple bond coherence; HPLC, high performance liquid chromatography; NMR, nuclear magnetic resonance; PDB, protein data bank; TFA, trifluoracetic acid.

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neuromuscular junctions, where it catalyses cleavage of acetylcholine. As well as decreasing the levels of acetylcholine, AChE is partially responsible for promotion of β-amyloid peptide aggregation, and the consequent formation of amyloid plaques [3,4]. Clinical studies have shown that inhibition of AChE to decrease the rate of acetylcholine hydrolysis is a promising strategy in the therapy of Alzheimer's disease, as it can reverse the reduced cognition and aberrant behavioural functions associated with Alzheimer's disease [5]. Indeed, most of the currently available anti-Alzheimer's drugs are AChE inhibitors, the standard use of which is supported by clinical evidence [4,6]. These inhibitors cannot reduce the rate of decline in the cognitive and functional capacities of patients with Alzheimer's disease, although over the long term, they can provide meaningful symptomatic benefits.

Alzheimer's disease is also associated with progressive increase in the activity of the related enzyme butyrylcholinesterase (BChE; E.C. 3.1.1.8). BChE is mainly located in the blood plasma [7], and it is assumed to serve as a 'back-up' for when AChE activity is compromised or absent, thus providing further support for and regulation of cholinergic transmission [8]. In this regard, AChE and BChE both represent interesting therapeutic targets for amelioration of the symptoms of Alzheimer's disease.

The US Food and Drug Administration has approved a number of drugs for the treatment of patients with Alzheimer's disease, which include the synthetic pharmaceuticals donepezil, rivastigmine, tacrine and galantamine. Galantamine was initially isolated from the common snowdrop, *Galanthus woronowii* [9], and it has contraindications for therapeutic use due to heart problems, epilepsy, and gastrointestinal symptoms. This requires the search for new drugs for the treatment of Alzheimer's disease that have reduced side effects.

Other important commercially available plant-derived natural AChE inhibitors include physostigmine from the Calabar bean *Physostigma venenosum* [10] and huperzine A from the Chinese club moss *Huperzia serrata* [11]. In recent years, several AChE inhibitors have also been isolated from marine organisms, such as soft corals, molluscs, ascidians, and in particular, sponges (reviewed in Orhan et al. [12]). Indeed, nearly half of the marine natural bioactive compounds now derive from sponges, which are considered to be the most bio-prospective [13] and pharmacodiverse marine taxa [14].

Sponges from the genus *Latrunculia* have been recognized as sources of the discorhabdin alkaloids [15–17]. These metabolites are pigments with a very particular structure that is characterised by azacarbocyclic spirocyclohexanone and pyrroloiminoquinone units. Over 40 members of the discorhabdin classes have been reported to date, with these defined according to: (i) the presence of sulfur cross-linkage (discorhabdins A, B, D, I, K, L, Q, R, X); (ii) the presence of a methyl sulfide substituent (discorhabdins S, T, U); (iii) the absence of a sulphur atom (discorhabdins C, D, G, E, F, O, P); and (iv) the presence of a rare dimeric structure (discorhabdin W). The discorhabdins and discorhabdin-related alkaloids show a plethora of biological effects, which include strong cytotoxic, antimicrobial, antiviral, antimalarial, immunomodulatory, caspase-inhibitory, and feeding-deterrent activities [16]. Due to these biological properties, the isolation, structural determination, reactivity and synthesis of these alkaloids have attracted considerable attention [18].

In 1975, Nèeman et al. [19] reported on a structurally unidentified toxin that they isolated from the Red Sea sponge *Latrunculia magnifica* that showed strong BChE inhibition. More recently, Turk et al. [20] evaluated AChE-inhibitory effects of ethanolic extracts obtained from two specimens of Antarctic *Latrunculia* spp. sponges, namely *Latrunculia* cf. *lendenfeldi* and *Latrunculia* cf. *bocagei*. These extracts induced 50% inhibition of electric eel AChE (eeAChE) at concentrations of 1.3 ng and 9 ng dried extract/mL, respectively. Thus, the identification of the metabolites responsible for these bioactivities and investigations into their mechanisms of action require further studies.

We initially report here on the isolation of four discorhabdins from methanol extracts of *Latrunculia* spp. sponges that were dredged from the deep Antarctic shelf in the Bransfield Strait near the Antarctic Peninsula in 2013. We further report on the evaluation of these metabolites as AChE inhibitors, with experimental data supported by docking calculations and related to their corresponding anti-BChE activities. One of the most active of these compounds, discorhabdin G, also underwent electrophysiological analysis to define any undesirable effects on neuromuscular transmission.

2. Results and discussion

2.1. Isolation and structural characterisation of discorhabdin alkaloids 1-4

(+)-Discorhabdin G (**1** in Fig. 1) and (-)3-dihydro-7,8-dehydro discorhabdin C (**2** in Fig. 1) were isolated in pure form starting from a crude methanol extract of *L. biformis*. The first purification step was on a cyano HPLC column eluting with water/methanol/TFA (60:40:1, v/v/v). The fraction collected at 7 min underwent further preparative workup on an RP-18 column with acetonitrile/water/TFA (30:70:1, v/v/v), obtaining the fraction eluted at 11 min which was further purified on a cyano HPLC column by elution with acetonitrile/water/TFA (30:70:1, v/v/v), to give the pure discorhabdin G triflate salt (**1**) as a brown solid. Electrospray ionisation mass spectrometry (ESI-MS) spectra, recorded in positive ion mode by direct infusion from a methanolic solution, showed a double signal at *m/z* 384/386 in a 1:1 ratio, corresponding to the M^+ ion, which indicated the presence of one bromine atom. It was confirmed by fragmentation of *m/z* 384 producing a signal at *m/z* 305, due to the loss of the bromine atom. NMR analysis, including ^{1}HNMR spectrum in deuterated methanol (CD_3OD) and the connectivity deduced by heteronuclear multiple bond coherence (HMBC) heterocorrelations, allowed the structural assignment, which was in agreement with the reported data for discorhabdin G isolated from *L. apicalis* [21].

The same three-step sequence was required for pure compound **2**, obtained evaporating the fraction eluted at 15.2 min in the last step of the work-up for the *L. biformis* extract by cyano HPLC preparative analysis. ESI-MS spectrum recorded in positive ion mode showed signals at: (i) *m/z* 462/464/466, corresponding to the M^+ ion and diagnostic for the presence of two bromine atoms, (ii) *m/z* 444/446/448, attributable to the $[M-H_2O]^+$ ion which supports the presence of a hydroxyl group in the structure and (iii) *m/z* 365/367, which are typical of the contribution of two bromine atoms and are due to the $[M\text{-}H_2O\text{-}Br]^+$ ion. NMR assignments, supported by long-range ^{1}H,^{13}C correlations by HMBC experiment, were in agreement with previous data reported for 3-dihydro-7,8-dehydro discorhabdin C [22].

The ^{1}H NMR spectrum of the crude methanol extract of *L. bocagei* showed a less complex mixture than that from *L. biformis*, with signals corresponding to two main compounds. These required only a two-step sequence of HPLC purification using a cyano stationary phase and elution with water/methanol/TFA 60:40:1 (v/v/v) to isolate discorhabdin B (**3**) and discorhabdin L (**4**) as trifluoroacetate, respectively (Fig. 1). They belong to the discorhabdin subclass that is structurally characterised by an additional sulfur bridge between the C-5 and C-8 positions.

(+)-Discorhabdin B was recovered by evaporation of the fraction eluted at the later retention time (15.8 min). The assignment that identified the structure of this monobrominated metabolite was