JPP
Journal of
Pharmacy and Pharmacology

JPP 2009, 61: 103–107

Received April 23, 2008
Accepted September 22, 2008
DOI 10.1211/jpp/61.01.0014
ISSN 0022-3573

Research Paper

Triclisia sacleuxii (Pierre) Diels (Menispermaceae), a potential source of acetylcholinesterase inhibitors

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Abstract

Objectives To search for compounds possibly useful for the treatment of Alzheimer's disease.

Methods Alkaloid fractions from the roots, stems and leaves of *Triclisia sacleuxii* (Menispermaceae) and pure bisbenzylisoquinoline alkaloids isolated from the roots (phaeanthine, *N*-methylapateline, 1,2-dehydroapateline and gasabiimine) were assessed for acetylcholinesterase inhibitory activity.

Key findings All extracts and compounds tested inhibited acetylcholinesterase to varying degrees; the leaf tertiary alkaloid fractions and the root quaternary alkaloid fractions exhibited the strongest inhibitory potential (90% at 0.1 mg/ml). The leaf tertiary alkaloid fraction was selected for further analysis (the quaternary alkaloids, which are too polar for absorption and brain distribution, were presumed to be clinically uninteresting). TLC bioautography using Ellman's reagent allowed the detection of acetylcholinesterase inhibitors and the isolation of the major active constituent, which was identified as lindoldhamine, a one-bridged bisbenzylisoquinoline alkaloid. Lindoldhamine displayed high acetylcholinesterase inhibitory activity with a 50% inhibition concentration in the micromolar range.

Conclusions All *T. sacleuxii* alkaloid fractions tested exhibited anti-acetylcholinesterase activity; isolated bisbenzylisoquinoline alkaloids showed weak-to-high inhibition depending on their structural features. Structure modification could provide interesting derivatives with enhanced anti-acetylcholinesterase activity.

Keywords acetylcholinesterase; bisbenzylisoquinoline; Ellman's test; Menispermaceae; TLC bioautography

Introduction

Alzheimer's disease is a progressive neurodegenerative disorder that is extremely disabling and is closely linked to ageing and neurotransmission deficiency. Its aetiology is not yet fully understood but more than one factor appears to be involved. The most common symptoms are a decline in activities of daily living, behaviour disturbances and reductions in learning new things, retrieving information from the memory, planning and making decisions.[1,2] Among the neurotransmitter systems affected, degeneration occurs earlier and more consistently in the cholinergic system.[3] These cholinergic alterations are thought to be key factors in the cognitive and functional deficits associated with Alzheimer's disease.[4,5]

As there is no curative treatment for Alzheimer's disease, restoring and maintaining the choline transmitter stock at a near-physiological level through neutralisation of acetylcholinesterase (AChE) represents a major therapeutic option, although it only slows down disease progression and/or lessens its effects.

Since the end of the 20th century, a number of plant-derived compounds have shown efficacy in inhibiting AChE; these include galanthamine,[6–9] (–)-huperzine,[10–12] sanguinine[8] and bisbenzylisoquinoline alkaloids (BBIQ) and analogues.[13,14] Interestingly, bisindole alkaloids recently isolated from *Tabernaemontana divaricata* showed higher potency than galanthamine.[15] This underlines the fact that plants are a valuable source for potential protectors of the cholinergic system.

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As *Triclisia sacleuxii* (Pierre) Diels contains BBIQ,[16] it was considered worth investigating for AChE inhibitors.

Materials and Methods

Chemicals

Acetylthiocholine iodide (ATCI), AChE, bovine serum albumin (BSA), 5,5′ dithiobis [2-nitrobenzoic acid] (DTNB) and galanthamine were obtained from Sigma (St Louis, MO, USA). All other reagents and organic solvents (analytical grade) were purchased from Merck (Darmstadt, Germany). 50 mM Tris-HCl pH 8.0 was used as a buffer throughout the experiment unless otherwise stated.

The AChE used in the assay was from electric eel (type VI S lyophilised powder, 480 U/mg solid, 530 U/mg protein). The lyophilised enzyme was prepared in the buffer to obtain a 1130 U/ml stock solution, which was stored at −80°C. Further enzyme dilutions were prepared in 0.1% BSA in buffer. DTNB was dissolved in the buffer containing 0.1 M NaCl and 0.02 M $MgCl_2$. ATCI was dissolved in deionised water.

For thin-layer chromatography (TLC) assays, 3 U/ml enzyme, 1 mM ACTI (the substrate) and 1 mM DNTB (Ellman's reagent) were prepared in buffer.

Plant material

T. sacleuxii (Pierre) Diels was collected in the Pugu Forest, Tanzania, in July 2004 with the help of Dr F. Mbago of the University of Dar es Salaam (Tanzania), who authenticated the samples. Voucher specimens were deposited in the Dar es Salaam Botany Department Herbarium (Nos JBH Harris 4700; JBH Harris 6163) and in the herbarium of the Botany Department, Free University of Brussels, Belgium (No. 3319).

Extraction

Alkaloid extraction was performed as described previously by Kanyinda *et al.* (1992).[17] The dried and powdered leaf material (800 g) was macerated in ethanol/acetic acid (9 : 1) for 24 h and exhaustively extracted with the same mixture. The extract was evaporated to dryness under reduced pressure, and the residue dissolved in 2% acetic acid. The solution was kept at 4°C for 12 h then successively filtered, extracted with petroleum ether, alkalinised to pH 8 with ammonia and extracted with chloroform. The resulting organic phase was washed, dehydrated over anhydrous Na_2SO_4 and evaporated to dryness, yielding 2.99 g (0.37%) raw tertiary alkaloid fraction. The aqueous phase was filtered and acidified to pH 3 with 2 M HCl then precipitated using Mayer's reagent. The precipitate was dissolved in Me_2CO–MeOH–H_2O (6 : 2 : 1) and passed through an anionic exchange (Amberlite IRA400 Cl^-; Sigma-Aldrich-Fluka, St Louis, MO, USA) column to yield 2.82 g (0.32%) of quaternary alkaloid fraction.

This procedure was also applied to root (600 g) and stem (790 g) materials, yielding 30.67 g (5.1%) and 5.03 g (0.63%) of tertiary alkaloid residues, respectively, and 3.08 g (0.51%) and 2.25 g (0.28%) of quaternary alkaloid residues, which were candidates for assessment of AChE inhibition.

Bioassay-guided fractionation and isolation

Microplate assay for acetylcholinesterase activity

The assay for measuring AChE activity was modified from the assay described by Ellman *et al.* (1961)[18] and Ingkaninan *et al.* (2003).[9] Briefly, 125 μl 3 mM DTNB, 25 μl 1.5 mM ATCI, 50 μl buffer and 25 μl of sample dissolved in buffer containing not more than 4% methanol were added to the microplate wells followed by 25 μl 0.28 U/ml AChE. The microplate was then read at 405 nm every 5 s for 2 min using a CERES UV 900C microplate reader (Bio-Tek Instruments, Winooski, VT, USA). The velocities of the reactions were measured. Enzyme activity was calculated as a percentage of the velocity compared with that of the assay using buffer without any inhibitor. Inhibitory activity was calculated as 100 – % enzyme activity. All experiments were done in triplicate. AChE inhibitory data were analysed using the Prism software (Graph Pad Inc., San Diego, CA, USA).

Root, stem and leaf tertiary alkaloid fractions and root and stem quaternary alkaloid fractions were assessed for AChE inhibitory activity. BBIQ alkaloids previously isolated from roots (phaeanthine, *N*-methylapateline, 1,2-dehydroapateline and gasabiimine) were also tested. The structures of these compounds are shown in Figure 1.

Bioautography

This experiment was performed as described previously by Rhee *et al.* (2001).[7] A sample of the fraction or compound to be analysed was prepared by dissolving 10 mg in 1 ml chloroform or methanol; 2.5 μl was then spotted onto a silica gel plate; 2.5 μl 0.1 mM galanthamine in methanol was used as reference.

The leaf tertiary alkaloid residue, selected on the basis of the high potency displayed in the microplate assay and its low polarity, was spotted onto a silica gel PF_{254}. The plate was developed with two successive mobile phases (chloroform/methanol/ammonia: 30 : 4 : 0.3 v/v; chloroform/methanol/ammonia: 30 : 2 : 0.05 v/v) for a better separation of polar compounds first and then of non-polar compounds, dried and successively sprayed with the substrate, dye and enzyme for detection of inhibitory activity. White spots that correspond to inhibiting compounds appear on a yellow background. The comparison with a second plate developed under the same conditions and sprayed with the Dragendorff reagent allowed us to ascertain that the observed AChE inhibitors are alkaloids; this experiment thus gave a qualitative view of AChE-inhibiting alkaloids.

Fractionation and isolation

The leaf fraction was submitted to fractionation by column chromatography on silica gel (Si 0.063–0.200 mm; Merck), successively eluted with chloroform, chloroform/methanol (9 : 1, 8 : 2, 7 : 3, 5 : 5, 3 : 7) and finally with methanol. The fractions (89) were concentrated and analysed by bioautography for localisation of AChE inhibitors. The major active alkaloid present in fractions 62–65 was isolated by repetitive preparative TLC using chloroform/methanol/ammonia (30 : 2 : 0 : 25 v/v) as the mobile phase and identified by NMR and mass spectroscopy and compared with literature data.[19]

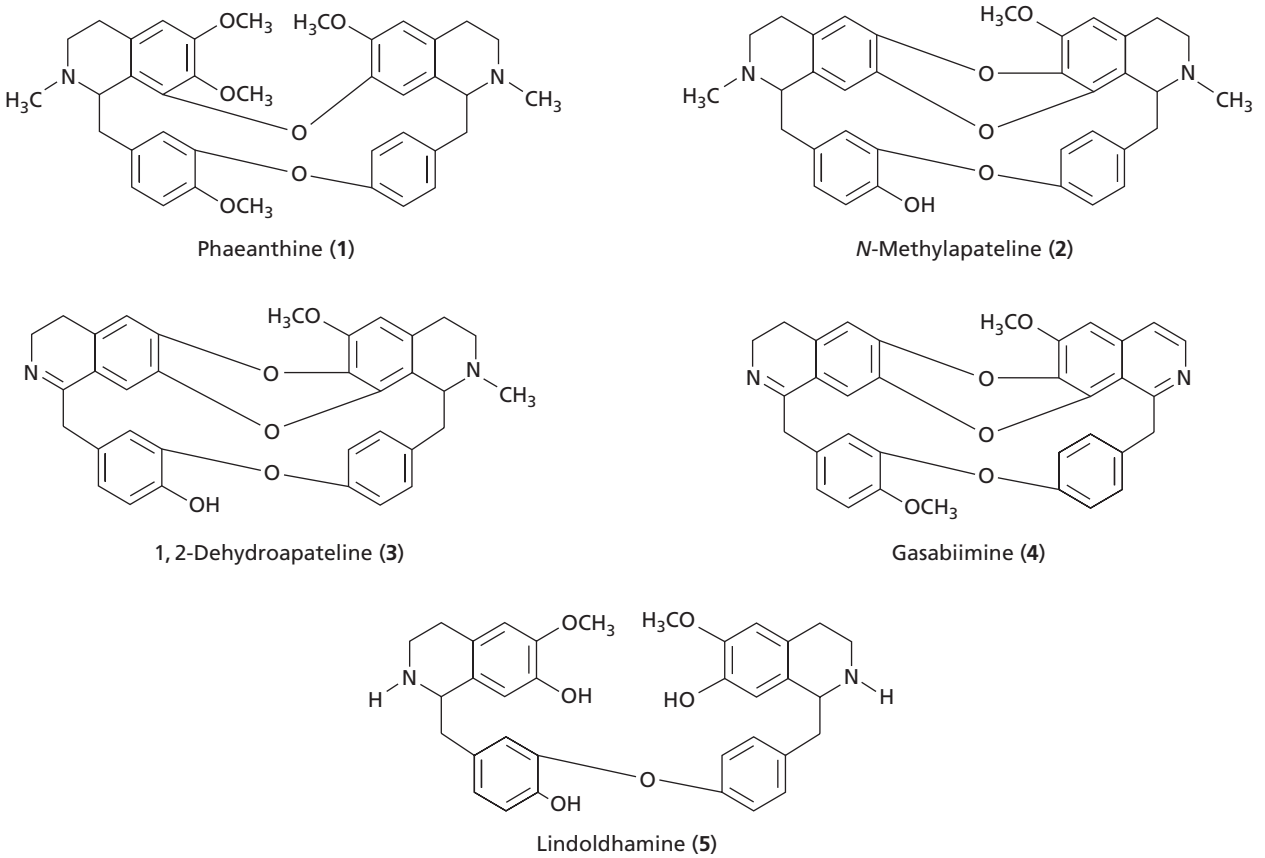

Figure 1 Structures of the compounds tested for acetylcholinesterase inhibitory activity.

Lindoldhamine

ESI-MS and MS/MS: m/z 583 M^+, 569, 552, 192, 178, 175. ^{1}H-NMR: 3.81 (3H, s, 6-OMe); 3.82 (3H, s, 6′-OMe); 2.70–3.22 (12 Al–H), 4.05 (1H, dd, J = 8.4/5.4, H-1); 4.10 (1H, dd, J = 9.0/4.2, H-1′); Ar-H: 6.55 (1H, s, H-5); 6.62 (1H, s, H-5′); 6.66 (1H, s, H-8); 6.67 (1H, s, H-8′); 6.87 (1H, d, J = 2.4, H-11′); 6.88 (1H, d, J = 2.4, H-13′); 6.92 (2H, m, H-13, H-14); 7.19 (1H, d, J = 1.8, H-10′); 7.19 (1H, d, J = 1.8, H-14′). The structure of lindoldhamine is shown in Figure 1.

Other alkaloids tested in this study, namely phaeanthine, *N*-methylapateline, 1,2-dehydroapateline and gasabiimine (Figure 1), were previously isolated from the root alkaloid fraction and identified as described in our recent publication.[20]

Statistics

Groups were compared by the Kruskal–Wallis test with post-hoc comparisons (Bonferroni correction) using Analyse-It software (Leeds, UK); the significance level was set at $P = 0.05$.

Results

Table 1 shows that all extracts tested inhibited AChE to differing degrees. The leaf tertiary alkaloid fraction and the root quaternary alkaloid fraction exhibited the strongest inhibitory potential (90% at 0.1 mg/ml). The more active leaf tertiary alkaloid fraction (IC50 6.7 μg/ml) was selected for further analysis. Quaternary alkaloids are probably too polar for absorption and distribution into the brain and were therefore presumed to be clinically uninteresting.

Table 1 Inhibition of acetylcholinesterase by alkaloid residues and the reference compound galanthamine

	% Inhibition at 100 μg/ml[a]	IC50 (μg/ml)
Root tertiary	61.7 ± 4.8	16.6 ± 3.8**
Root quaternary	90.3 ± 1.5	23.4 ± 3.6***
Stem tertiary	83.8 ± 2.9	14.2 ± 6.2*
Stem quaternary	71.2 ± 5.9	20.8 ± 7.7**
Leaf tertiary	90.4 ± 0.2	6.7 ± 1.0
Galanthamine	~ 100	0.15 ± 0.03

[a]Data presented for comparison purposes with published data.
Values are means ± SD from three experiments performed in triplicate. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ vs galanthamine (Kruskal–Wallis test followed by post-hoc comparison).

Table 2 details the inhibition of AChE measured for pure compounds obtained from *T. sacleuxii*; TLC bioguided isolation from the leaves yielded the most active compound, lindolhamine. All molecules tested belong to the three main groups of BBIQ; they bear one, two or three ether bridges.

Table 2 Inhibition of acetylcholinesterase by major alkaloids extracted from the leaves of *Triclisia sacleuxii* and reference compound galanthamine

	% Inhibition at 100 μg/ml	IC50 (μM)
Lindoldhamine	88.9 ± 1.0	3.52 ± 0.82
Phaeanthine	72.5 ± 4.2**	not determined
N-methylapateline	30.7 ± 4.5***	not determined
1,2-dehydroapateline	15.9 ± 2.9***	not determined
Gasabiimine	13.4 ± 7.2***	not determined
Galanthamine	~ 100	0.53 ± 0.10

Values are mean ± SD from three experiments performed in triplicate. $^{**}P < 0.01$; $^{***}P < 0.001$ vs galanthamine (Kruskal–Wallis test followed by post-hoc comparison).

Discussion

False-positive Ellman's reaction on TLC and microplate assay have previously been observed with aldehydes and amines.[21] Some of these interferents (4-dimethylaminobenzaldehyde, 3-ethoxy-4-hydoxybenzaldehyde, di- and triethylamine, triethanolamine, tyramine) show real enzyme inhibition; others (heptanal, decanal, cinnamaldehyde, anisaldehyde, benzaldehyde, hexylamine and tryptamine) show non-specific chemical inhibition. In all cases, inhibitory concentrations were higher by 10^2–10^3 (TLC) or 10^3 (microplate) than for the reference compound galanthamine. In our experiments, the alkaloid nature of the compounds positive on TLC Ellman's reaction was verified by spraying a similar chromatogram with Dragendorff's reagent and detecting the typical colour of the alkaloid salt. To the best of our knowledge, no interference has been described so far for alkaloids.

The small number of molecules tested does not allow comparison of the three groups with regard to their structure–activity relationships. Nevertheless, our results indicate that the stereochemistry, the number of bridges and the type and position of substituents play an important role in AChE activity. Ogino *et al.*,[13] investigating a series of two-bridged BBIQs, have shown that fangchinoline-type compounds with a (1-*S*, 1′-*S*) configuration and bearing a hydroxyl group at the 7-position displayed inhibitory activity in the micromolar range; compounds with configurations (1-*S*, 1′-*R*) and (1-*R*, 1′-*S*) were inactive. The two-bridged BBIQ tested in our study, phaeanthine, despite its (1-*R*, 1′-*R*) configuration and the absence of a phenol function, exhibited a higher activity than the three-bridged analogues.

Within the three-bridged group, the oxidation effect, which is presumably identical in all groups, is clearly detrimental to the activity (% inhibition at 0.1 mg/ml): *N*-methylapateline (30%) > 1,2-dehydroapateline (16%) > gasabiimine (13%). The same effect has been reported previously for benzylisoquinoline monomers.[14] Considering *N*-methylapateline, the stereochemistry (1-*R*, 1′-*S*) might also be somewhat unfavourable for anti-AChE activity.

Lindoldhamine (Figure 1) is the first compound belonging to the one-bridged BBIQ group to be tested for anti-AChE activity. It showed a high potential (89% inhibition at 0.1 mg/ml) which can be a balance between two factors: on the one hand, a proton on each nitrogen and three hydroxyl groups at C-7, C-12 and C-7′ is favourable for high activity; on the other hand, the (1-*R*, 1′-*R*) configuration often leads to weak activity. As its measured inhibition is equivalent to that of the fraction from which it was isolated, lindoldhamine can be considered as the main active compound of the leaf tertiary alkaloid fraction.

Conclusion

The use of AChE inhibitors currently remains the main therapeutic option for the management of Alzheimer's disease. Since plants have yielded some valuable AChE inhibitors and some BBIQ alkaloids have shown AChE inhibitory activity, we have undertaken an investigation to check whether *T. sacleuxii* could be a source of AChE inhibitors. Interestingly, all alkaloid fractions exhibited anti-AChE activity. Isolated compounds showed weak-to-high AChE inhibitory activity, depending on their structural features; the highest activity was displayed by a one-bridged structure, lindoldhamine. Structure modification could provide interesting derivatives with enhanced anti-AChE activity.

Acknowledgements

We would like to thank all the people in Tanzania who helped us to obtain plant material, and particularly Dr F Mbago. The assistance of Mr P Callewaert, Dr E Couché, Professor J-C Braekman (Free University of Brussels/ULB) and Dr S Laurent (Mons-Hainaut University) is gratefully acknowledged.

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

Funding

We are grateful to the Coopération Universitaire au Développement (Belgium) for the financial support granted to SM.

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