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Novel Diterpenoid Acetylcholinesterase Inhibitors from *Salvia miltiorhiza*

Abstract

Acetylcholinesterase (AChE, EC 3.1.1.7) inhibitors are the only registered drugs used to treat Alzheimer's disease (AD). New AChE inhibitors may contribute to the design of new pharmaceuticals and supply information which will facilitate the understanding of the interaction between inhibitors and the enzyme. The dried root of *Salvia miltiorhiza* is called 'Danshen' in China, and has been used for the treatment of cerebrovascular disease and CNS deterioration in old age for over one thousand years. In this work, a modified Ellman method was used to guide the fractionation of the active AChE inhibitory compounds from an acetone extract. Four inhibitory compounds, dihydrotanshinone, cryptotanshinone, tanshinone I and tanshinone IIA were isolated, and the structures were identified by comparison of their spectral characteristics with previous reports. The inhibitory activities of dihydrotanshinone and cryptotanshinone were dose-dependent, their IC_{50} values being 1.0 μM and 7.0 μM, respectively. These two compounds were the major inhibitory compounds in the extract as judged by HPLC analysis, forming 0.054% w/w and 0.23% w/w in the dried root, respectively, and in mixture they appear to be less active than as isolated compounds. The clogP values of dihydrotanshinone, cryptotanshinone, tanshinone I and tanshinone IIA were calculated as 2.4, 3.4, 4.8 and 5.8, respectively, which indicate that these compounds have potential to penetrate the blood-brain barrier. This is the first example of diterpenoids as inhibitors of AChE.

Key words
Acetylcholinesterase inhibitors · *Salvia miltiorhiza* · Lamiaceae · diterpenoids · dihydrotanshinone · cryptotanshinone

Introduction

AChE is the principal cholinesterase in the brain which causes the hydrolysis of the endogenous neurotransmitter ACh [1]. AChE inhibitors are applied in the treatment of AD since a cholinergic deficit is characteristic of AD [2] and elevation of ACh levels leads to functional improvement of central cholinergic synapses, protection of neuronal degeneration, modification of amyloid precursor protein and regional enhanced synthesis of neurotrophic molecules [3].

A large number of plant extracts has been screened for activity against AChE and galantamine and huperzine A have been introduced as drugs against the early symptoms of AD. It is likely that novel natural product AChE inhibitors exist, especially from traditional remedies for cognitive dysfunction. The dried root of *Salvia miltiorhiza* Bunge is called 'Danshen' in China and is a good example of such a remedy used in Chinese folk medicine [4]. Previous investigations have shown that *S. miltiorhiza* root contains diterpene quinones, known as tanshinones, which have been shown to display several biological activities but no reports exist of their possessing anti-cholinesterase activity.

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Received July 7, 2003 · **Accepted** December 6, 2003

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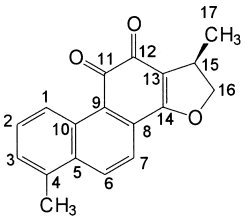
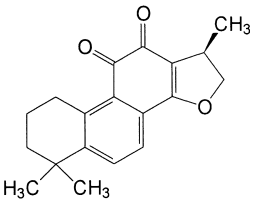
Planta Med 2004; 70: 201–204 · © Georg Thieme Verlag Stuttgart · New York · ISSN 0032-0943 · DOI 10.1055/s-2004-815535



1 Dihydrotanshinone



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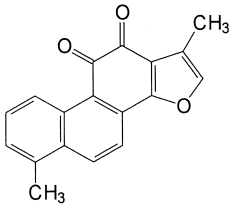
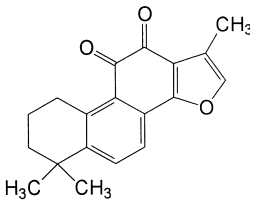


3 Tanshinone I



4 Tanshinone IIA

AChE possesses high catalytic power for the hydrolysis of acetylcholine [1], [5] although its interaction with agonists and antagonists remains somewhat unclear.

Materials and Methods

Materials

Bovine erythrocyte acetylcholinesterase (EC 3.1.1.7), 5,5-dithiobis-(2 nitrobenzoic acid) (DTNB), acetylthiocholine iodide and physostigmine hemisulphate (99% purity) were purchased from Sigma.

Plant material

The dried roots of *Salvia miltiorhiza* Bung were purchased from Tongrentang Ltd. (London) and authenticated by Christine Leon at the Royal Botanic Gardens, Kew. A sample voucher number Sa96R1 is deposited in the museum of the Pharmacy Department at King's College London.

Extraction and isolation

1500 g dried root of *Salvia miltiorhiza* were extracted by acetone at 55 °C for 4 h then reduced to dryness under reduced pressure to yield 58.8 g extract. VLC (Merck silica gel 60 PF_{254}, 40 g) was used on 20 g extract eluting with 100 mL aliquots of toluene 200 mL, toluene:CH_2Cl_2 1 : 1 200 mL, CH_2Cl_2 200 mL, EtOAc 200 mL, acetone 200mL and MeOH 200 mL, and monitoring by TLC, six fractions were obtained F1 (3.9 g) t_R between 100 and 200 mL, F2 (3.6 g) t_R between 200 and 350 mL, F3 (2.4 g) t_R between 400 and 550 mL, F4 (2.1 g) t_R between 600 and 750 mL, F5 (1.8 g) t_R between 850 and 950 mL and F6 (1.4 g) t_R between 1000 and 1100 mL. Only two (F1 and F2) of the six fractions indicated activity. The two active fractions were combined since they showed similar TLC profiles and were subjected to CC [silica gel (Merck) 200 g, CH_2Cl_2]. Four sub-fractions were obtained: A (551 mg) t_R between 50 and 95 mL, B (230 mg) t_R between 110 and 180 mL, C (548 mg) t_R between 220 and 310 mL and D (247 mg) t_R between 350 and 400 mL. Only two of the sub-fractions (A and C) showed activity and the major components were purified by preparative TLC [silica gel (Merck)/CH_2Cl_2] to yield A1 (58 mg; R_f 0.55 on silica gel/CH_2Cl_2) and A2 (101 mg; R_f 0.55 on silica gel/CH_2Cl_2) from A and C1 (30 mg; R_f 0.46 on silica gel/CH_2Cl_2:EtOAc, 4 : 1) and C2 (66 mg; R_f 0.48 on silica gel/CH_2Cl_2:EtOAc, 4 : 1) from C. The structures of compounds A1, A2, C1 and C2 were determined as dihydrotanshinone **1**, cryptotanshinone **2**, tanshinone I **3** and tanshinone IIA **4** by comparison with spectral data previously reported [6], [7]. The optical rotatory powers $[\alpha]_D^{20}$ values of **1** and **2** at 589 nm were found to be –82° and -47.8°, respectively, which agrees with previous reports [8].

Enzyme assay

The modified Ellman method was used [9]. This is a spectrophotometric method and is based on the reaction of released thiocholine to give a coloured product. Some reports have stated that the activity of the enzyme may be variable from different sources, and even from the same source between different batches, so the IC_{50} of physostigmine as a marker of AChE inhibitor was tested [9]. Acetylcholinesterase (40 μL of 0.86 U/mL buffer pH 8) and extract solution (20 μL) were added to 2.0 mL phosphate buffer (pH 8) and incubated at 4 °C for 20 min. The reaction was started by adding DTNB (20 μL of 0.05 mM buffer pH 7) and acetylthiocholine (20 μL of 0.06 mM buffer pH 7) in phosphate buffer pH7; 20 μL at 37 °C water for 20 min. The reaction was halted by placing the assay solution tubes at 4 °C and adding physostigmine (20 μL 0.018 mM in buffer pH 7). The production of the yellow anion was recorded for 10 min on a Shimadzu UV/VIS 2101 double beam spectrophotometer at 412 nm. A positive control was set up by adding physostigmine (20 μL, 0.018 mM buffer pH 7) in order to control the non-enzyme hydrolysis of acetylthiocholine. Blanks were used of reagents without extract and a positive control was set up which was the same as the blank except that physostigmine (20 μL, 0.018 mM in buffer pH 7) was added. The inhibition rate (%) was calculated by the equation:

$$\text{Inhibition \%} = \frac{(\text{Blank} - \text{Blank positive control}) - (\text{Experiment} - \text{experiment control})}{\text{Blank} - \text{Blank positive control}}$$

The activity of the solvents used for diluting the inhibitors was also examined and indicated that water and acetone have no significant inhibitory activity at the concentration used.

Determination of compounds in plant extract by HPLC

The dried acetone extract was dissolved to 0.8 mg/mL in MeOH:H_2O, 8 : 2. Dihydrotanshinone was diluted to 1.13×10^{-6} M, 2.26×10^{-6} M, 4.52×10^{-6} M and 9.04×10^{-6} M in MeOH:H_2O, 8 : 2, and cryptotanshinone was diluted to 0.59×10^{-5} M, 1.18×10^{-5} M, 2.36×10^{-5} M and 4.72×10^{-5} M in the same solvent mixture. The solutions were examined by HPLC: column: ODS2 (250 × 4.6 mm I.D., 5 μm, Beckman, Fullerton, CA, USA); mobile phase: MeOH:H_2O, 8 : 2; flow rate: 1 mL/min; detection: 254 nm; injection: 20μL. The system was validated by mixing known amounts of each compound with *S. miltiorhiza* root, previously exhausted of compounds by extraction with acetone, which was then subjected to the above procedure.

Lipophilicity was calculated by the ClogP program of Chemoffice 6.0.

Statistical analysis

All values are expressed as mean ± standard deviation (SD). Data were analysed using one-way ANOVA and $p < 0.05$ was regarded as significant.

Optical rotation determination

The optical rotations were measured on a Perkin Elmer 141 polarimeter, calibrated with a solution of sucrose (water; $c = 10$) giving a rotation of + 65.5°. UV spectra were measured in MeOH.

Results and Discussion

Dose-dependent inhibition of acetylcholinesterase

The activity of physostigmine was measured as an IC_{50} value of 2.5×10^{-7} M. In addition the solvents used, 1% MeOH, 1% ethanol and 1% acetone (v/v) in water were found to have no significant inhibitory activity ($n = 3$) ($p > 0.05$) whilst 1% EtOAc (v/v) had 22.13% ($n = 3$) ($p < 0.05$) inhibitory activity on acetylcholinesterase.

A dose-dependent inhibition of acetylcholinesterase for the acetone extract of *Salvia miltiorhiza* was observed and the IC_{50} value was calculated to be 24.7 μg/mL. Dihydrotanshinone **1** and cryptotanshinone **2** were the most potent inhibitors of the enzyme, giving dose-dependent inhibitions with IC_{50} values of 1.0×10^{-6} M and 7×10^{-6} M, respectively, while tanshinone I **3** and tanshinone IIA **4** had only weak inhibitory effects of 24.7% at 5×10^{-5} M and 28.3% at 1.4×10^{-4} M, respectively.

The compounds isolated are the first diterpenoids to show activity against AChE. A number of monoterpenoids and two triterpenoids have been reported with activity against AChE, but their activities generally seem to be weak compared with those reported here [9], [10], [11] (Table **1**).

The structures of dihydrotanshinone **1** and tanshinone I **3** differ by only one double bond as dihydrotanshinone has a dihydrofuran ring while tanshinone I has a furan ring. Dihydrotanshinone **1** has a much greater activity than tanshinone I **3** (IC_{50} of **1** is 1.0×10^{-6} M while **3** did not reach not reach 50% inhibition at the highest concentration used). Cryptotanshinone **2** and tanshinone IIA **4** show a similar difference in activity so it appears that the dihydrofuran ring is crucial for acetylcholinesterase inhibitory activity. Compound **1** has a seven-fold higher activity than **2** which suggests that an aromatic A ring may contribute more to inhibitory activity than a hexane A ring. In addition there are a variety of tanshinone compounds, with open dihydrofuran rings, known as metabolic products, but their activity against AChE was not reported [12].

The clogP values of dihydrotanshinone, cryptotanshinone **1**, tanshinone I and tanshinone IIA were calculated as 2.4, 3.4, 4.8 and 5.8, respectively, which indicates that these compounds have potential to penetrate the blood-brain barrier.

Determination of active compounds in the extract

In the HPLC system used the retention time of dihydrotanshinone was 6.03 ± 0.02 minutes ($n = 13$), that of cryptotanshinone **2**, 8.74 ± 0.02 minutes ($n = 15$), respectively. Calculation curves of these two compounds were constructed using a range of concentrations between 1 and 150 μg/mL and each determination was carried out using two sets of four replicate injections carried out over two days. Linearities and other parameters relevant to validation are shown in Table **2**. A straight-line relationship was obtained for both compounds with $R^2 = 0.990$ for dihydrotanshinone and $R^2 = 0.980$ for cryptotanshinone. HPLC determination of the amounts present of the two compounds added to exhausted root gave values of 97.2 ± 0.2% for **1** and 96.8 ± 0.2% for **2** of the amount added. From the calibration line, cryptotanshinone and dihydrotanshinone were found to comprise 4.5 ± 0.2% w/w and 1.3 ± 0.08% w/w, respectively, of the extracts, and to constitute 0.23% w/w and 0.054% w/w, respectively, of the dried root. The concentration of cryptotanshinone is similar to that previously reported [13], while the concentration of dihydrotanshinone in the herb has not been previously reported although previous studies show that cryptotanshinone is in much higher concentrations than dihydrotanshinone [8], [14], [15].

From the IC_{50} values measured, it appears that dihydrotanshinone **1** has an activity 63 times greater than that of the acetone extract, of which it comprises 1.3% w/w, from the HPLC measurement. Based on these values, it can be calculated that **1** is responsible for 83% of the inhibitory activity of the whole extract. In the same way crypotanshinone **2** appears to be responsible for 62% of the inhibitory activity of the whole extract. Thus the combined

Table **1** IC_{50} values against acetylcholinesterase for diterpenes isolated and other terpenoids

Compound	*Type of terpenoid*	*IC_{50} (μM)*	*ClopP*	*Reference*
Dihydrotanshinone **1**	Abietane diterpene	1.0	2.4	
Cryptotanshinone **2**	Abietane diterpene	7.0	3.4	
Tanshinone I **3**	Abietane diterpene	> 50	4.8	
Tanshinone IIA **4**	Abietane diterpene	> 140	5.8	
Argentatin A	Triterpenoid	42.8	NR	[17]
1,8-Cineole	Monoterpene	670	NR	[9]
α-Pinene	Monoterpene	630	NR	[9]
Camphor	Monoterpene	4700	NR	[9]
Physostigmine	Indole alkaloid	0.25	NR	

NR = not reported.

Table **2** Linearities between peak areas and concentrations and detection limits of **1** and **2**

Compound	*Slope*	*Intercept*	*Correlation coefficient*	*Linear range (μg/mL)*	*Detection limit (μg/mL)*
Dihydrotanshinone **1**	1.2×10^3	–149.7	0.990	2 – 150	1
Cryptotanshinone **2**	6.3×10^2	–285.6	0.980	15 – 150	10

inhibitory activity of **1** and **2** is more than 100% of that displayed by the total extract. A possible explanation is that the two compounds compete at the same site of the enzyme and so thus may account for the decrease in inhibitory activity when they are together in the extract. Although several other related diterpenes have been reported from *Salvia miltiorhiza*, only **1** possesses the dihydrofuran ring, is an *ortho*-quinone and has an aromatic ring A ring. Other molecules with a dihydrofuran ring and an *ortho*-quinone ring occur, but, apart from **2**, they are in very low concentrations. This suggests that dihydrotanshinone **1** and cryptotanshinone **2** are the major constituents with AChE inhibitory activity in this medicinal plant.

Lipophilicity

There appears to be an inverse correlation between the inhibitory activity and the lipophilicity for the compounds isolated since the clogP value is inversely related to the cholinesterase inhibitory activity. However, the lipophilicity of the more active compounds is still sufficient to enable them to cross the blood-brain barrier. As stated above an aromatic A ring appears to confer a more potent inhibitory activity than a hexane ring. It is reported that aromatic hydrocarbons were in general more potent AChE inhibitors than chlorinated aliphatic hydrocarbons and alcohols [16] which is in agreement with our results as regards this relationship between lipophilicity and activity. It may be explained by the "anion subsite" and the peripheral site being rich in aromatic residues and therefore readily entering π-π interactions with ligands. An additional aspect to this consideration is that molecules with such lipophilic properties can readily cross the blood-brain barrier.

Dihydrotanshinone and cryptotanshinone are the first diterpenoids reported to be inhibitors of AChE. They are very different structurally from the classic alkaloid and organophosphorus compound cholinesterase inhibitors. These results also support the traditional application of this plant to alleviate cognitive dysfunction and could serve as interesting templates for the development of new drugs against AD.

Acknowledgements

Our grateful thanks to ORS and the Royal Pharmaceutical Society for financial support and to Dr. M. Hanna-Brown for her help with the HPLC.

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