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HPLC-Based Activity Profiling of *Salvia miltiorrhiza* for MAO A and iNOS Inhibitory Activities

Abstract

In the course of a screening of plant extracts for potential CNS and anti-inflammatory activities, a dichloromethane extract of *Salvia miltiorrhiza* showed a pronounced inhibitory effect on recombinant monoamine oxidase A (MAO A) and on inducible NO synthase (iNOS) induction in Raw 267.4 cells. With the aid of HPLC-based profiling techniques, activities could be linked, to a significant extent, to peaks of tanshinone-type diterpenoids, four of which were subsequently isolated. The IC_{50} of the most active compound, 15,16-dihydrotanshinone I, on human recombinant MAO A was at 23 μM, and 2.4 μM on lipopolysaccharide-mediated iNOS induction in Raw 267.4 cells.

Key words
Salvia miltiorrhiza · Lamiaceae · diterpenoids · tanshinones · HPLC-DAD-MS · MAO A inhibition · inhibition of iNOS induction · Raw 267.4 cells

Introduction

Disorders of the central nervous system (CNS) and inflammatory diseases affect a large number of patients worldwide. Antidepressants, neuroleptics, tranquilizers and drugs for the symptomatic alleviation of CNS disorders of old age play important roles in current pharmacotherapy. Anti-inflammatory drugs are among the most widely prescribed pharmaceuticals. Given the numerous side effects of current drugs used in these indications, there is a continuing need for new compounds with potential as drug leads, and for the scientific evaluation of medicinal plants and phytomedicines which could be useful to treat or alleviate milder forms of such afflictions. As part of an ongoing investigation of crude drugs and purified natural products acting on therapeutically relevant targets of CNS disorders and of inflammation [1], [2], [3], we screened *Salvia miltiorrhiza*. The rhizome and roots of *Salvia miltiorrhiza* Bunge (Lamiaceae), known as "Danshen", represent a well-known Chinese herbal drug. It is chiefly used in the treatment of various cardiovascular disorders such as angina pectoris, atherosclerosis and cardiac ischemia, and in some infectious and inflammatory diseases [4]. Recent pharmacological studies revealed protective effects in animal models of cerebral ischemia [5], [6].

A dichloromethane extract of the root displayed a marked inhibitory effect on rat liver monoamine oxidase (MAO) (IC_{50}: 80 μg/mL), with a preference for the MAO A isoenzyme [7], and on the inducible NO synthase (iNOS) induction in Raw 264.7 cells (IC_{50}: 7.5 μg/mL). MAO catalyzes the oxidative deamination of biogenic amines which are essential in neuronal function. MAO

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A inhibitors are clinically used as antidepressants, whereas inhibitors of MAO B appear useful in the treatment of some neurodegenerative disorders [8]. The inducible form of NO synthase (iNOS) is upregulated by chronic inflammation and infections and is known to be involved in the pathogenesis of many chronic diseases including multiple sclerosis, Parkinson's and Alzheimer's diseases [9], [10], [11]. Beside direct inhibition of enzymatic activity, the induction process via, e. g., activation of transcription factor nuclear factor κB (NF-κB) represents an attractive target for anti-inflammatory agents. Given the activities in the MAO and iNOS assays, we embarked on an HPLC-based profiling for MAO A and iNOS inhibitory activities of the dichlormethane extract.

Materials and Methods

General experimental procedures

NMR spectra were recorded in $CDCl_3$ at 300 K with a Bruker DRX 400 spectrometer operating at 400.13 MHz for ^1H and 100.63 MHz for ^{13}C. HPLC analysis and microfractionation for profiling was carried out with an HP 1100 series system (Agilent, Waldbronn, Germany) consisting of a binary high-pressure mixing pump, autosampler, column oven, diode array detector and HP workstation, and a Gilson FC 2004 fraction collector (Gilson, Middleton, USA). For LC-MS analysis, the HPLC effluent was directed to a API 165 single quadrupole mass spectrometer equipped with a turbo ion spray interface (Applied Biosystems, Langen, Germany) via a T-splitter (1 : 4). HPLC-grade methanol (Roth, Karlsruhe, Germany) and water were used for HPLC separations. Dichloromethane (Roth, Karlsruhe, Germany) was purified by distillation. All other solvents and chemicals were of analytical grade. Silica gel (40 – 63 μm, Macherey & Nagel, Düren, Germany) was used for column chromatography.

All cell culture media and supplements were obtained from Invitrogen GmbH (Karlsruhe, Germany). Fetal bovine serum was from PAA Laboratories GmbH (Cölbe, Germany). Lipopolysaccharides (LPS from *Escherichia coli*, serotype 0127:B8, purified by gel filtration) were purchased from Sigma-Aldrich Chemie GmbH (Taufkirchen, Germany).

Plant material and extraction

Authenticated root of *Salvia miltiorrhiza* Bunge (Lot no. 00 03 9 01) was purchased from the Institut für Chinesische Medizin und Chinesische Heilkräuter (Bochum, Germany). A voucher specimen (SM 01) is deposited at the Lehrstuhl für Pharmazeutische Biologie, University of Jena.

The dry roots were coarsely ground with a cutting mill, followed by milling with an SMZ 1000 ultracentrifugal mill (both Retsch, Haan, Germany). Extracts for the initial screening were prepared with an ASE 200 extraction system with solvent module (Dionex, Sunnyvale, USA) by consecutive extraction with dichloromethane (2 × 5 min) and methanol (2 × 5 min). Extraction pressure was 120 bar and temperature was set at 80 °C. For preparative isolation of tanshinones, 1000 g of powdered root were extracted by percolation at room temperature with dichloromethane (5 L, for 48 h). The solvent was removed at reduced pressure to afford 9.85 g of extract which was stored at 4 °C until use.

HPLC separation and fractionation for profiling

Separations were performed on a LiChrospher 100 RP-18 cartridge (5 μm, 125 × 4 mm i. d.; Merck, Darmstadt, Germany). The following gradient profile with methanol (solvent A) and water (solvent B) was used: 70% A (0 min) to 100% A (20 min), hold for 8 min. The flow rate was 1 mL/min, and the detection wavelength of the diode array detector was set at 230 nm. The injection volume was 20 μL.

For activity profiling, the dichloromethane extract was dissolved in methanol at a concentration of 10 mg/mL.

For profiling of MAO A activity, 4 × 20 μL (corresponding to 800 μg of extract) were injected. The column effluents of the time interval of 0 to 14 min were fractionated into vials to afford 13 fractions. The solvent was removed in a centrifugal evaporator (Evaporator Centrifuge RC 10.22, Jouan, Saint Herblain, France). The dry films were redissolved in DMSO (50 μL) for bioassay.

For profiling of iNOS activity, aliquots corresponding to 200 μg of extract were injected. The column effluents were collected into a 96-well microtiter plate (Greiner, Frickenhausen, Germany) over a time window of 28 min, each fraction corresponding to 0.3 min. The solvent was removed under laminar air flow in a clean bench. The dry films in each well were redissolved in 10 μL DMSO and diluted with 90 μL water. Aliquots of 10 μL were used for bioassay.

Isolation of tanshinones 1 – 4

A portion (6.5 g) of the dichloromethane extract was separated by chromatography on a silica gel column (75 × 5.5 cm i. d.) using a step gradient of ethyl acetate/petroleum ether (10 : 90, 4.7 L; 20 : 80, 2.3 L; 30 : 70 5.1 L) to 15 fractions. Gel chromatography on Sephadex LH 20 (50 × 1 cm i. d.) (methanol) of fraction 5 (elution volume 3.5 to 4.,7 L, 13 mg) afforded **1** (9 mg), and fraction 12 (9.75 to 10.2 L, 10 mg) gave **3** (9 mg). Fraction 15 (10.8 to 12.1 L, 80 mg) was separated into two fractions (15A and 15B) by low pressure chromatography on a Lobar LiChroprep RP-18 column (40 – 63 μm, 310 × 25 mm i. d.; Merck, Darmstadt, Germany) and methanol/water (65 : 35) as mobile phase. Compound **2** (10 mg) was obtained from fraction 15 A (elution volume 850 to 1000 mL) by gel chromatography on Sephadex LH 20 (methanol). Fraction 8 (elution volume 7.0 to 7.5 L, 127 mg) was separated by column chromatography on silica gel (53 × 3 cm i. d.) using a petroleum ether/dichloromethane/methanol step gradient (35 : 65 : 0, 400 mL; 35 : 65 : 5, 400 mL; 35 : 65 : 10, 400 mL; 35 : 65 : 20, 450 mL) in 4 fractions. Compound **4** (7 mg) was obtained by preparative HPLC of fraction 8 B (elution volume 300 to 400 mL, 42.5 mg) on a LiChrosorb RP-18 (7 μm, 250 × 25 mm i. d., Merck, Darmstadt, Germany) with methanol/water (75 : 25) followed by gel chromatography on Sephadex LH-20 (methanol). Compounds **1 – 4** (Fig. **1**)were identified by comparison of their physico-chemical data (ESI-MS, ^1H- and ^{13}C-NMR, UV-vis, m. p.) with published values [12], [13], [14], [15], [16]. Purity of the compounds was > 98% (HPLC, ^1H-NMR). Copies of original spectra are available from the corresponding author.

Assay for inhibition of MAO A

Recombinant human MAO A was obtained as reported [17]. The preparation with an activity of 4.26×10^{-8} kat/L was diluted with Soerensen phosphate buffer to a protein concentration of 50 μg/

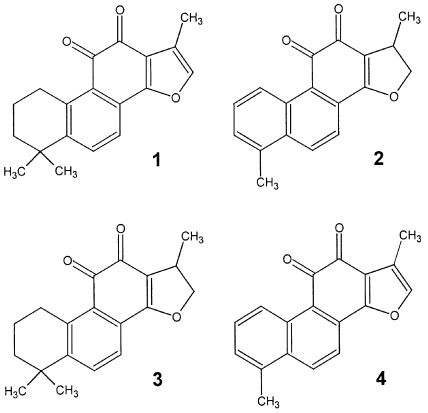

Fig. **1** Chemical structure of compounds **1 – 4**.

mL. Enzyme solution (495 μL) and test solution (5 μL) were incubated under shaking for 5 min at 30 °C. Enzyme activity in the assay was 7.1×10^{-10} kat/L. Aliquots (140 μL) of the incubation mix were transferred to a 96-well microtiter plate, and 10 μL of kynuramine dihydrobromide (7.5 mM) in Soerensen phosphate buffer were added (final concentration of kynuramine in the assay 500 μM). After brief shaking, the absorption measurement was carried out at 360 nm and 30 °C in a thermostatted plate reader (Fluostar Galaxy, BMG Lab Technologies, Offenburg, Germany) every 60 s over a period of 10 min. Inhibition (%) was calculated from the slope $\Delta E/\Delta t$, with control experiments without enzyme (0%) and without sample (100% activity). All assays were carried out as triplicates.

Inhibition of iNOS in murine macrophages

Raw 264.7 murine macrophages were obtained from the Tumorbank (German Cancer Research Center, Heidelberg, Germany) and maintained in DMEM containing 100 units/mL penicillin G sodium, 100 units/mL streptomycin sulfate and 250 ng/mL amphotericin B supplemented with 10% fetal bovine serum under endotoxin-free conditions at 37 °C in a 5% CO_2 atmosphere. Inhibition of LPS-mediated iNOS induction was determined as described previously with minor modifications [18]. Raw macrophages were plated at a density of 3.5×10^4 cells/well in 200 μL DMEM in 96-well plates and incubated overnight. Then, the medium was replaced by 90 μL fresh medium, and 10 μL of test compound solution (dissolved in DMSO and diluted 1 : 10 with water), fractionated HPLC effluent (prepared as described above) or 10% DMSO as solvent control were added to each well (0.5% final DMSO concentration). iNOS was stimulated by addition of 100 μL DMEM containing 100 ng/mL LPS (10 ng LPS/well), and cells were incubated for 24 h. NO production was determined via quantitation of nitrite levels in cell culture supernatants by means of the Griess reaction, and compared to a nitrite standard curve [19]. Effects on cell growth were estimated by sulforhodamin B staining [20]. IC_{50} values (half maximal inhibitory concentration) of LPS-induced nitrite production were generated from the results of two separate experiments testing 8 serial two-fold dilutions in duplicate.

Results and Discussion

Extracts for initial screening of *S. miltiorrhiza* root were obtained by accelerated solvent extraction (ASE) by consecutive treatment with dichloromethane and methanol. The dichloromethane extract inhibited rat liver MAO (MAO A and B) with an IC_{50} of 80 μg/mL [7], and human recombinant MAO A [17] with an IC_{50} of 6.2 μg/mL. Treatment of Raw macrophages with the extract caused a dose-dependent inhibition of LPS-induced NO production (measured via nitrite levels in cell culture supernatants by the Griess reaction) [18], [19] with an IC_{50} value of 7.5 μg/mL. For activity profiling, aliquots corresponding to 800 or 200 μg of extract, respectively, were separated by analytical HPLC with concomitant fractionation of the effluent. UV-vis and electrospray mass spectra (ESI-MS) were also recorded. Details of the activity profiling are given in the Material and Methods.

A representative chromatogram recorded at 230 nm is shown in Fig. **2**. Several distinct peaks were baseline separated, enabling a peak resolved collection of 13 microfractions for the MAO assay with human recombinant MAO A. The activity profile (Fig. **2**, top) revealed varying degrees of MAO A inhibition in these fractions. The most polar fractions I – III showed distinct activity which, however, was due to complex mixtures of compounds with low UV absorption. The HPLC peaks corresponding to fractions V – XII, in contrast, exhibited distinct UV-vis absorption spectra which suggested the presence of tanshinone-type diterpenoids [4]. When comparing the peak intensities in the chromatogram and the corresponding MAO inhibitory potential of these fractions, there seemed to be obvious differences with respect to the activity of individual compounds. Fraction VI, for example, contained a less prominent peak with the highest activity of all, whereas the major peak of fraction XII was only moderately active. The HPLC chromatogram of the dichloromethane extract and the corresponding profile for inhibition of NO synthesis in Raw cells is shown in Fig. **3**. Several peaks of activity were detected in the time window where tanshinone-type diterpenoids

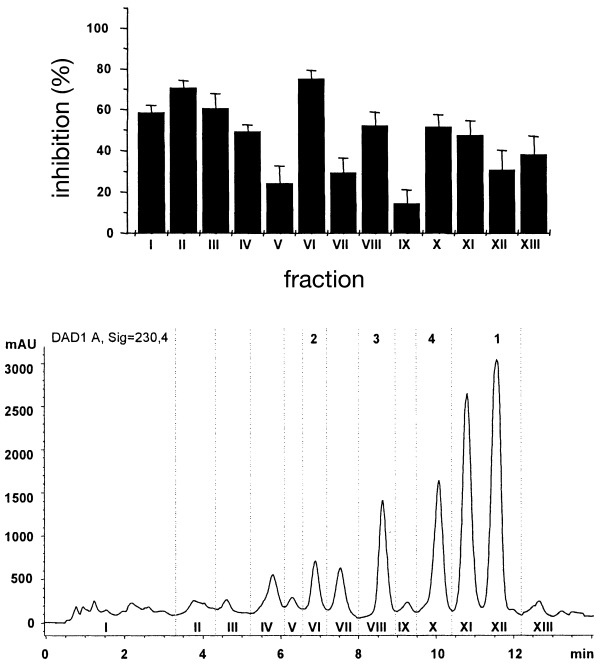

Fig. **2** Activity profile for MAO A inhibitors in *S. miltiorrhiza* extract. The HPLC fingerprint of a dichloromethane extract recorded at 230 nm with time segments for microfractionation (I – XIII) is shown below, and the inhibitory activity of microfractions I – XIII is given above. MAO A inhibition was determined in triplicate.

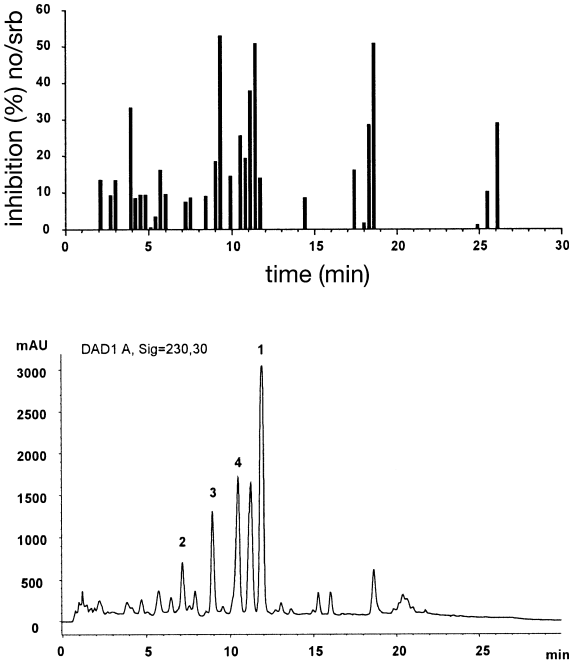

Fig. **3** Activity profile for inhibitors of iNOS induction in *S. miltiorrhiza* extract. The HPLC fingerprint of a dichloromethane extract recorded at 230 nm is shown below, and the inhibitory activity of microfractions (0.3 min each) on NO formation is shown above. No/srb indicates the ratio of OD values obtained for nitrite levels with the Griess reaction and OD values obtained with SRB staining (to correct for cytotoxic effects).

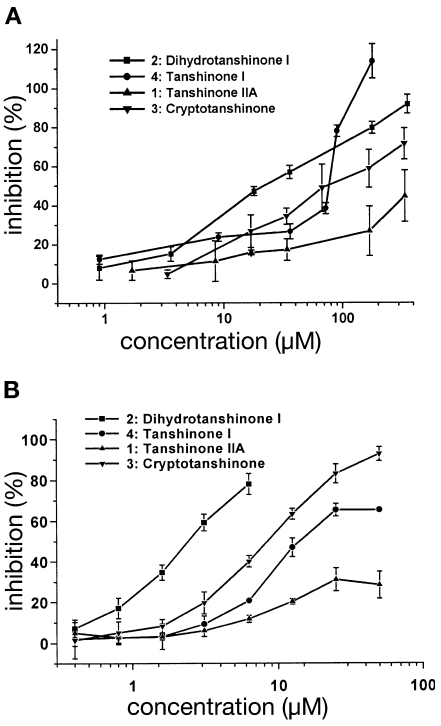

Fig. **4** **A** Dose inhibition curves for diterpenoids **1–4** in the MAO A assay. Each data point (± SD) was determined in triplicate. **B** Dose inhibition curves for **1–4** in the iNOS assay. Nitrite, as a measure of NO production was determined in cell culture supernatants (levels in unstimulated controls: 0.77 ± 0.06 nmol nitrite/mL; after LPS-stimulation: 5.54 ± 0.78 nmol nitrite/mL). Each datapoint (± SD) was determined in two separate experiments in duplicate.

eluted, i.e., between 6 and 12 min, along with some activity in the early part of the chromatogram, and in the late fractions which corresponded to the column wash.

The activity profiles strongly pointed towards tanshinones as compounds with dual inhibitory properties for MAO A activity and iNOS induction. A targeted preparative purification afforded compounds **1–4** which were subsequently identified on the basis of their spectral data and comparison with data from the literature as tanshinone IIA (**1**), 15,16-dihydrotanshinone I (**2**), cryptotanshinone (**3**) and tanshinone I (**4**) [12], [13], [14], [15], [16]. The compounds differed only in their degree of unsaturation in rings A and C and thus seemed of particular interest with respect to a preliminary understanding of structure-activity relationships in this class of compounds.

Dose-inhibition curves for tanshinones **1–4** are shown in Fig. **4**. In the MAO A assay, the IC_{50} values of **2**, **3** and **4** were 23, 80 and 84 μM, respectively. Tanshinone IIA (**1**) was only marginally active, as the inhibition remained below 50% even at 400 μM concentration. The data suggest that an aromatic ring A and a dihydrofuran ring were more favorable for inhibition of MAO A than a cyclohexene ring with geminal methyl groups and a tetrahydrofuran as ring D. In this assay, the IC_{50} of the selective MAO A inhibitor clorgyline was 0.014 μM, and that of the preferential MAO B inhibitor deprenyl was 20 μM. In the iNOS assay, the IC_{50} values for compounds **2**, **3** and **4** were 2.4, 8.4 and 13.5 μM, respectively, and > 50 μM for **1**. The positive control substance curcumin had an IC_{50} of 4.5 μM. The growth inhibitory potential of tanshinones was also determined, given that inhibition of NO synthesis could potentially also be related to cytotoxic or apoptotic properties of compounds in this cell based assay. The IC_{50} (Tox) values were 11.1 μM for dihydrotanshinone I (**2**), and > 50 μM for compounds **1**, **3** and **4**. Considering the relative potencies of **1–4** in the iNOS assay, the same structural features of tanshinones seem favorable for inhibition of MAO A and NO synthesis.

Recently, Jang et al. [21] reported on the inhibition of iNOS expression, TNF-α, IL-1β and IL-6 production in Raw cells by tanshinone IIA (**1**). Given that this compound was the least active in our series of diterpenoids, a more thorough testing of the anti-inflammatory potential of **2–4** is certainly warranted. While the MAO inhibition is via a direct interaction with the target enzyme, the mechanism involved with inhibition of NO production is presently not known. Indirect mechanisms seem to play a role, whereby interaction with NF-κB DNA binding as reported for various natural products [18], [22] would be most plausible. Our findings lend support to the earlier evidence for effectiveness of Danshen extracts in animal models of cerebral ischemia [5], [6]. Given the pronounced lipophilicity of these diterpenoids, passage through the blood-brain barrier should occur. The bioactivity of the extract, however, appears not solely linked to tanshinone-type diterpenoids. Pronounced activities were found by the HPLC profiling in some of the early eluting fractions and in the column wash. These fractions await further characterization.

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