Isolation of Adenosine and Cordysinin B from *Anredera cordifolia* that Stimulates CRE-Mediated Transcription in PC12 Cells

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ABSTRACT

Alzheimer's disease is a typical neurodegenerative disorder, and its prevention or treatment poses great concern in advanced countries. In our survey of numerous natural resources with neurotrophic activities, we found that *Anredera cordifolia* improved memory impairment and increased cyclic adenosine monophosphate (AMP) response element-mediated transcription, an important step in signal transduction for memory formation. The extracts of this food were dissolved in methanol and then partitioned with three organic solvents and water, separating into *n*-hexane, ethyl acetate, *n*-butanol, and water layers. The *n*-butanol layer with the strongest activity on cyclic AMP-response element-dependent transcription was fractionated using silica gel column chromatography and then the activity was monitored using preparative high-performance liquid chromatography to give adenosine and cordysinin B, respectively. Both compounds showed a concentration-dependent

increase in cyclic AMP-response element-mediated transcription activity. These results suggest that both adenosine and cordysinin B may participate in improving the action of *A. cordifolia* on memory impairment, and these actions, at least in part, result from the activation of adenosine A_1, A_{2A}, and A_{2B} receptors.

ABBREVIATIONS

A*β*	*β*-amyloid
AD	Alzheimer's disease
AMP	adenosine monophosphate
cAMP	cyclic AMP
CRE	cAMP-response element
CREB	cAMP-response element-binding protein
EtOAc	ethyl acetate
LTP	long-term potentiation
MEAC	methanol extract of *A. cordifolia*
BuOH	*n*-butanol
NMDA	*N*-methyl-D-aspartate
Nob	nobiletin

Introduction

AD is the most common neurodegenerative disorder and has become a severe social problem in advanced countries [1]. However, effective preventive and fundamental therapeutic methods for AD have not yet been established. Notably, A*β* peptide in AD patients' brains decreases CREB signaling pathway activation to inhibit hippocampal LTP formation [2], and A*β* oligomers inhibit CREB activation in hippocampal neurons [3]. Furthermore, the CREB/CRE pathway greatly contributes to LTP, a synaptic memory model, and memory formation *in vivo* [4–8].

Numerous natural resources have simultaneously provided useful pharmacological tools [9] and novel leading compounds for drug development [10]. We have reported that Nob, a polymethoxylated flavone from the peel of *Citrus depressa*, activates the CREB/CRE pathway in PC12D cells or cultured rat hippocampal neurons to exhibit memory-improving actions in various animal models of dementia [11–14].

There is considerable interest in identifying safe and effective compounds from natural resources that enhance the function of CREB transcription factor coupled with CRE-mediated transcription, which could improve memory deficits in AD.

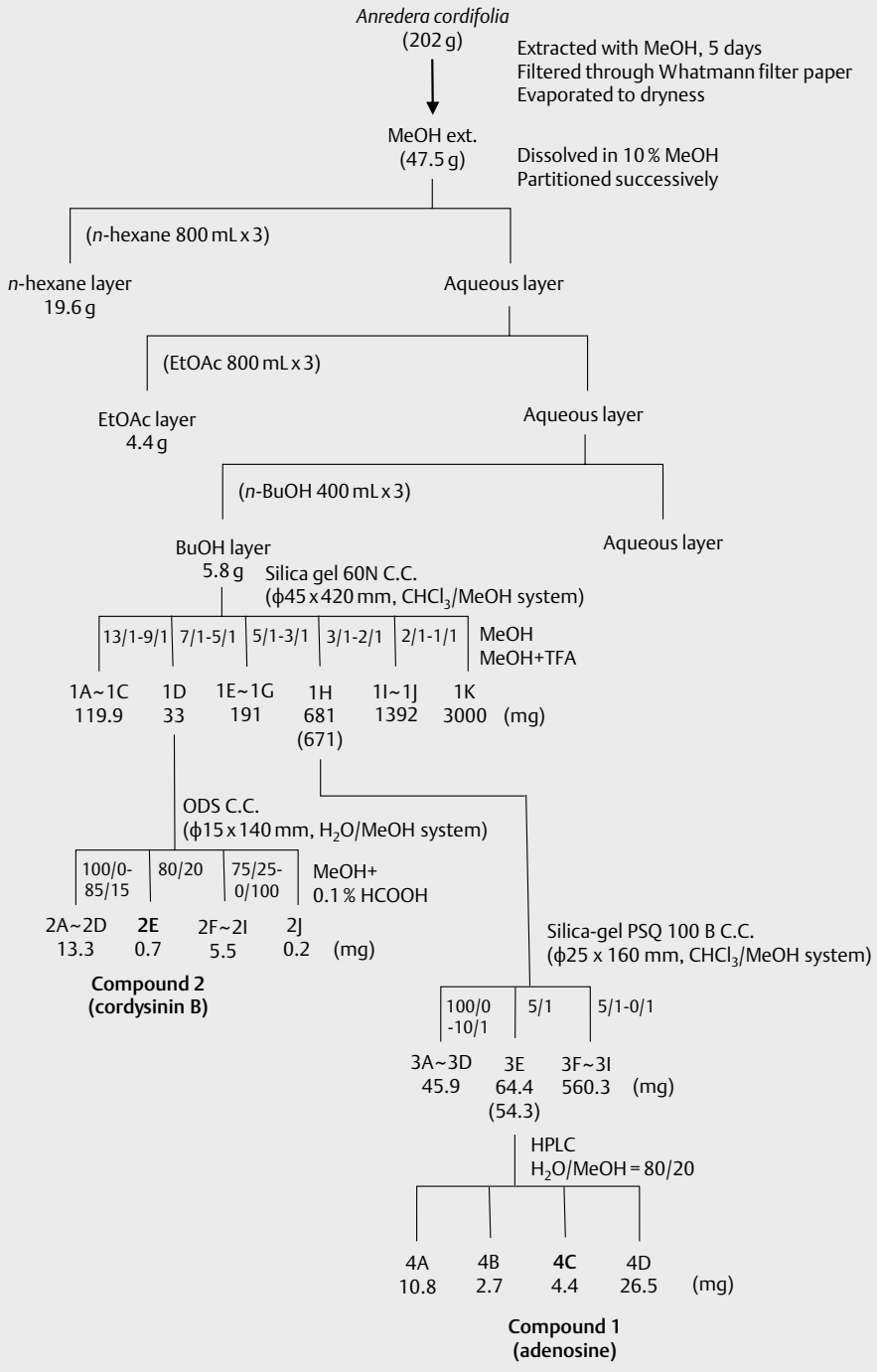
In our survey of natural resources having the increasing action of the CRE-mediated transcription activity, we found that *Anredera cordifolia*, like Nob, enhances this activity and reverses memory impairment caused by NMDA receptor antagonist MK-801 in mice [15]. Alternatively, *A. cordifolia* possesses pharmacologically interesting actions such as anti-obesity, anti-hyperlipidemia, antihypertensive, antidiabetic, antioxidant, and anti-inflammatory activities [16, 17]. Here, we unprecedentedly described that adenosine and cordysinin B are isolated as active ingredients from *A. cordifolia*, possessing increasing activity on CRE-mediated transcription. It is also suggested that these actions are caused by activating adenosine receptors.

Results and Discussion

AD is the most common neurodegenerative disorder showing progressive loss of memory and cognitive function. Notably, late LTP formation, a synaptic memory model, is blocked by the A*β* peptide by inhibiting the CREB signaling pathway [2]. Using a new strategy in our survey of numerous natural resources activating the CREB/CRE pathway, we found that *A. cordifolia*, like Nob from citrus peels, with activating actions on CRE-mediated transcription, improved MK-801-induced memory impairment [13–15, 18].

A. cordifolia leaves (202 g) were extracted using MeOH (0.4 L) for 5 days at room temperature in a stationary state to obtain the MeOH extract (47.5 g) by removing the solvent using a rotary evaporator. The MeOH extracts were fractionated by monitoring the CRE-mediated transcriptional activity using PC12 cells as illustrated in ▶ **Fig. 1**. The four layers partitioned with *n*-hexane, EtOAc, and BuOH had CRE-mediated transcriptional activities [12.2-fold activation (BuOH layer), 4.4-fold activation (EtOAc layer), 1.5-fold activation (*n*-hexane layer), and 1.3-fold activation (H_2O layer) at 30 µg/mL]. The BuOH layer (5.8 g) was subjected to silica gel 60 N column chromatography (φ45 × 420 mm, $CHCl_3$–MeOH system) to give fractions 1A ($CHCl_3$:MeOH = 13:1, 0.27 L; $CHCl_3$:MeOH = 9:1, 0.25 L), 1B ($CHCl_3$:MeOH = 9:1, 0.15 L), 1C ($CHCl_3$:MeOH = 9:1, 0.1 L; $CHCl_3$:MeOH = 7:1, 0.25 L), 1D ($CHCl_3$:MeOH = 7:1, 0.05 L; $CHCl_3$:MeOH = 5:1, 0.3 L), 1E ($CHCl_3$:MeOH = 5:1, 0.15 L; $CHCl_3$:MeOH = 3:1, 0.15 L), 1F ($CHCl_3$:MeOH = 3:1, 0.15 L), 1G ($CHCl_3$:MeOH = 3:1, 0.10 L), 1H ($CHCl_3$:MeOH = 3:1, 0.15 L; $CHCl_3$:MeOH = 2:1, 0.15 L), 1I ($CHCl_3$:MeOH = 2:1, 0.20 L; $CHCl_3$:MeOH = 1:1, 0.15 L), 1J ($CHCl_3$:MeOH = 1:1, 0.30 L), and 1K ($CHCl_3$:MeOH = 1:1, 0.05 L; MeOH, 0.15 L; MeOH + 0.1 % TFA, 0.15 L). Fraction 1D ($CHCl_3$:MeOH = 7:1–5:1, 33 mg) was suspended in 5 % MeOH and subjected to ODS open column chromatography (φ15 × 140 mm, MeOH–H_2O system) at once to give subfractions 2A (H_2O, 0.02 L), 2B (5 % MeOH, 0.02 L), 2C (10 % MeOH, 0.02 L), 2D (15 % MeOH, 0.02 L), 2E (20 % MeOH, 0.02 L), 2F (25 % MeOH, 0.02 L), 2G (40 % MeOH, 0.02 L), 2H (50 % MeOH, 0.02 L), 2I (MeOH, 0.02 L), and 2J (MeOH + 0.1 % HCOOH, 0.02 L). Subfraction 2E (20 % MeOH, 0.7 mg) was regarded as compound **2** (▶ **Fig. 2**). A part of fraction 1H ($CHCl_3$: MeOH = 3:1–2:1, 671 mg of 681 mg) was subjected to silica gel PSQ 100B column chromatography (φ25 × 160 mm, $CHCl_3$–MeOH system) to give subfractions 3A-3I. A part of subfraction 3E ($CHCl_3$: MeOH = 5:1, 54 mg of 64 mg) was subjected to HPLC [COSMOSIL Cholester (φ10.0 × 250 mm); eluent: 20 % MeOH; flow rate: 5.0 mL/min; UV detection: 254 nm] to give compound **1** (4.4 mg, t_R 4.7 min) (▶ **Fig. 2**).

DMSO and Nob served as the negative and positive controls, respectively, in measuring CRE-mediated transcriptional activity. As can be seen in ▶ **Fig. 3**, MEAC induces a powerful increasing action on CRE-mediated transcription in PC12 cells. Also, the BuOH layer showed the strongest activity among the four layers and then was chromatographed on silica gel to afford the active compounds **1** and **2**. The physicochemical properties of the active compounds **1** and **2** properly correspond to those of adenosine and cordysinin B, respectively (**Table S1 and S2**) [19, 20]. Cordysinin B was previously isolated from *Cordyceps sinensis*[20]. Therefore, it is concluded that the major active ingredients of *A. cordifolia* are adenosine and cordysinin B, respectively.



▶ **Fig. 1** Scheme for isolation of pharmacologically active compounds from *A. cordifolia*.

▶ **Fig. 4** indicates that introducing a methyl group into the OH group at the C-2′ position of adenosine decreases the CRE-mediated transcription activity approximately 100 times. Therefore, these results suggest that the OH group is important for developing the activity.

To demonstrate the mechanisms responsible for CRE-mediated transcription induction by adenosine and cordysinin B, we used adenosine A receptor antagonists CGS 15943 (a nonselective adenosine A receptor antagonist), DPCPX (an adenosine A_1 receptor antagonist), SCH 58261 (an adenosine A_{2A} receptor antagonist), MRS 1754 (an adenosine A_{2B} receptor antagonist), and MRS 1523 (an adenosine A_3 receptor antagonist). CRE-mediated transcription induced by adenosine and cordysinin B was blocked by pretreating with CGS 15943, DPCPX, SCH 58261, or MRS 1754, although

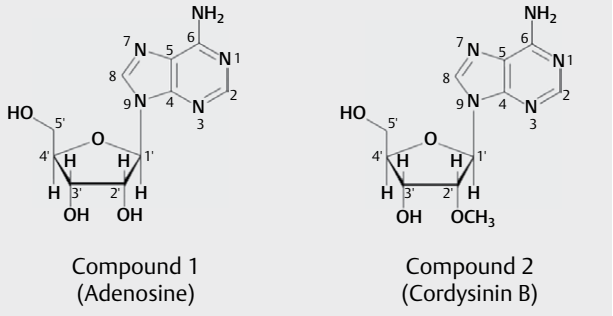
the effect of MRS 1754 on adenosine-induced CRE-mediated transcription was weak (▶ **Fig. 5**). In contrast, MRS 1523 did not affect CRE-mediated transcription induced by adenosine or cordysinin B. These results suggest that adenosine and cordysinin B induce CRE-mediated transcription, at least in part, by A_1, A_{2A}, and A_{2B} receptors.

Conclusively, adenosine and cordysinin B are isolated active ingredients from *A. cordifolia* and possess the activity to induce CRE-mediated transcription, an important event for memory formation. It is also suggested that these actions are induced, at least in part, by adenosine A_1, A_{2A}, and A_{2B} receptors, but not A_3 receptor.

Materials and Methods

Plant material

A. cordifolia leaves were collected from the hothouse of Sankyo Co., Ltd. in Fuji, Japan from May 2015 to July 2016. The plant material was identified by Dr. Koji Kajima. A voucher specimen was deposited at Sankyo Co., Ltd. (SS-U001). *A. cordifolia,* a perennial Basellaceae native to South America, has been used as a traditional medicine in China and Japan.



▶ **Fig. 2** Chemical structures of compounds **1** (adenosine) and **2** (cordysinin B) from the MEAC.

General experimental procedures in chemistry

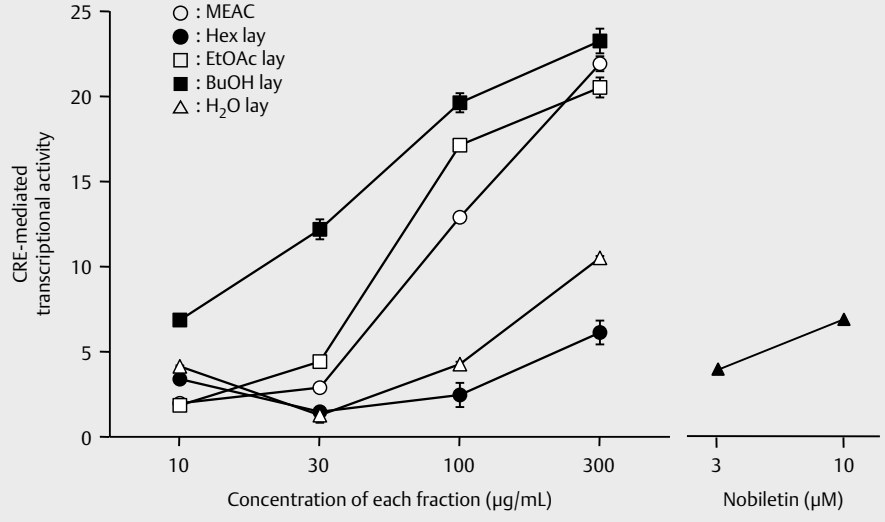
An ECZ-600 spectrometer (JEOL) was used for NMR spectroscopy and the chemical shift of the NMR solvent was used as an internal standard. An HPLC system (Shimadzu) comprising an SCL-10AVP system controller, LC-20AD pump, DGU-12A online degasser, SIL-20A, CTO-10ASVP column oven, SIL-20A autosampler, SPD-M20A PDA detector, and CLASS-VP software was used. The following adsorbents were used for purification: silica gel 60 F254 (0.25 mm; Merck) and silica gel 60 RP-8 F254 S (0.25 mm; Merck) for analytical TLC; Silica gel 60 N (Kanto), silica gel PSQ 100B, and Chromatrex ODS (Fuji Silysia chemical) for column chromatography; COSMOSIL Cholester (φ10.0 × 250 mm; Nacalai Tesque) for HPLC.

Reagents

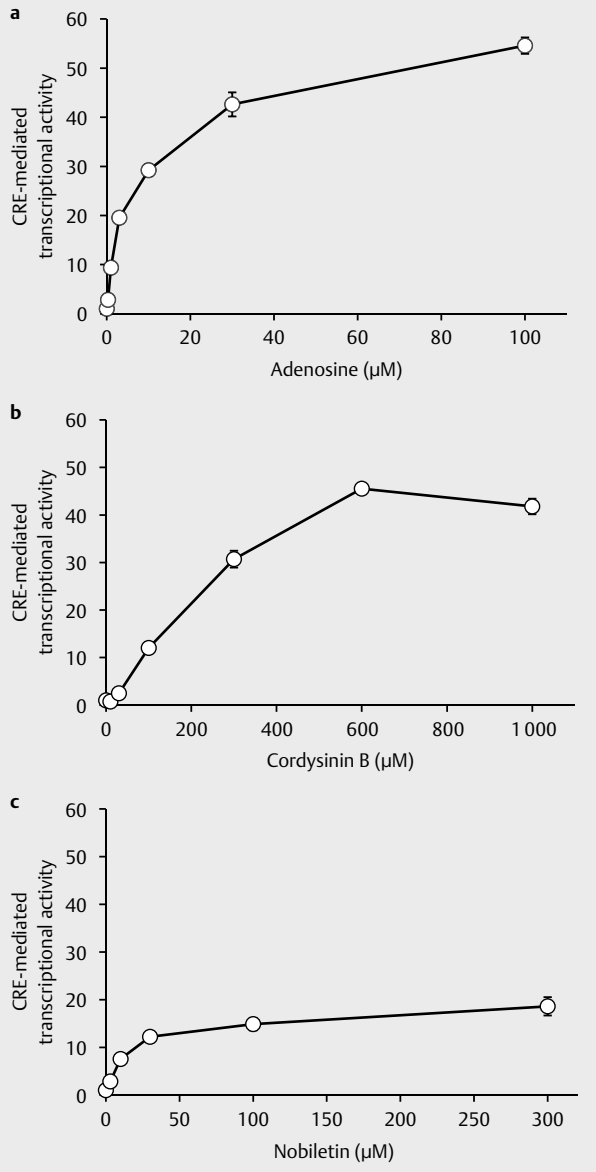
CGS 15943 was obtained from Cayman Chemical. DPCPX, SCH 5826, MRS 1754, and MRS 1523 were purchased from Abcam. Adenosine (purity ≥ 99 %) and cordysinin B (2'-O-Methyl Adenosine) (purity ≥ 98 %) were from Sigma-Aldrich and Toronto Research Chemicals, respectively. Nob was extracted and isolated from *C. depressa* peels as described previously [11, 12]. The purity of Nob was confirmed to be almost 100 %.

Culture of rat pheochromocytoma (PC12) cells

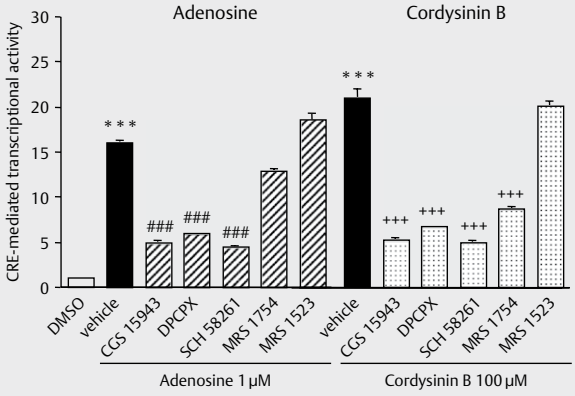
PC12 cells were grown in DMEM supplemented with 10 % heat-inactivated horse serum (Gibco by Life Technologies), 5 % heat-inactivated fetal bovine serum (Gibco), and 1 % penicillin/streptomycin (Gibco) at 37 °C in a humidified atmosphere of 95 % air and 5 % CO_2.



▶ **Fig. 3** Increasing activities of the MEAC and the layer of *n*-hexane (Hex), ethyl acetate (EtOAc), *n*-buthanol (BuOH), or water (H_2O) on CRE-mediated transcription in PC12 cells. Data are shown as the mean ± SEM (n = 4). For some data points, error bars are smaller than the symbol size.



▶ **Fig. 4** Concentration-dependent effects of adenosine **a**, cordysinin B **b**, and nobiletin **c** on CRE-mediated transcription in PC12 cells. Data are shown as the mean ± SEM (n = 4). For some data points, error bars are smaller than the symbol size.



▶ **Fig. 5** Effects of adenosine receptor antagonists on adenosine- and cordysinin B-induced enhancement of CRE-mediated transcription in PC12 cells. Data are shown as the mean ± SEM (n = 4). $^{***}P<0.001$ vs. DMSO-treated control; $^{###}p<0.001$ vs. adenosine alone-treated group; $^{+++}p<0.001$ vs. cordysinin B alone-treated group.

Measurements of cyclic AMP-response element-mediated transcriptional activity in PC12 cells

Transient transfection and the reporter gene assay were prepared as described previously [11]. PC12 cells cultured in 96-well plates (4×10^4/well) were transfected for 5 h with 0.2 mg of the reporter plasmid pCRE (Clontech) and 0.04 mg of the transfection efficiency *Renilla* luciferase phRG-TK plasmid (Promega) using LipofectAMINE (Invitrogen) according to the manufacturer's instructions. After transfection, the medium was replaced with a fresh medium containing advanced DMEM, 1 % horse serum (Gibco), and 1 % fetal bovine serum (Gibco), and the cells were incubated overnight. After incubation, the cells were stimulated for 5 h with MEAC, adenosine, or cordysinin B. In the reporter gene assay, commercially available adenosine standard (Sigma-Aldrich, catalog A4036) and cordysinin B standard (Toronto Research Chemicals, catalog M276150) were used. For experiments using adenosine receptor antagonists, the cells were preincubated with CGS 15943(3 μM), DPCPX (0.5 μM), SCH 58261 (3 nM), MRS 1754 (30 μM), or MRS 1523 (1 μM) for 30 min before stimulation and then stimulated for 5 h with adenosine or cordysinin B in the presence of an individual antagonist. Luminescence was measured using a Dual-Luciferase Reporter Assay System according to the manufacturer's instructions (Promega). All treatments were performed in quadruplicate on at least three independent cultures.

Statistical analysis

The results are expressed as the mean ± SEM. Data were analyzed using one-way analysis of variance (ANOVA), followed by Tukey-Kramer test, and $p < 0.05$ was considered statistically significant difference.

Supporting information

^{1}H NMR and MS spectra of compound 1, ^{1}H and ^{13}C NMR and MS spectra of compound 2, and tables for comparison of the physicochemical properties between compound 1 and adenosine and those between compound 2 and cordysinin B are available as Supporting information.

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Conflict of Interest

This work was supported by the research fund from Sankyo Co., Ltd.; however, this sponsor had no control over the interpretation, writing, or publication of this work.

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