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Evaluation of canthinone alkaloids as cerebral protective agents

Tatsunori Sasaki, Wei Li *, Taichi Ohmoto †, Kazuo Koike *

Faculty of Pharmaceutical Sciences, Toho University, Miyama 2-2-1, Funabashi, Chiba 274-8510, Japan

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ABSTRACT

Considerable attention has been paid to cerebral protective drugs as a potential therapy for dementia. Screening of a natural compound library here resulted in identification of five canthinone alkaloids, viz., picrasidine L (**1**), picrasidine O (**2**), eurycomine E (**3**), 3-ethyl-canthin-5,6-dione (**4**), and 3-ethyl-4-methoxy-canthin-5,6-dione (**5**), as novel cerebral protective agents. The structure–activity relationship indicated that C-4, C-9, and N-3 substitutions greatly affected their cerebral protective effect. Among these, compound **2** exhibited a cerebral protective effect through suppressing neuronal hyperexcitability due to an increase in the excitatory neurotransmitter glutamic acid. Furthermore, compound **2** did not affect heart rate and mean systolic blood pressure. This investigation suggests that compound **2** has potential for further development as a cerebral protective drug.

Dementia is a clinical syndrome characterized by a cluster of symptoms and signs manifested by memory difficulties, language disturbances, psychological and psychiatric changes, and impairment in the activities of daily living. Alzheimer's disease is the most common type of dementia, followed by vascular dementia, and Lewy body dementia.[1] Clinical drugs for the treatment of dementia include acetylcholinesterase inhibitors, such as donepezil, galantamine, rivastigmine, and an *N*-methyl-D-aspartate (NMDA) receptor antagonist, memantine.[2]

Picrasma quassioides and other plants in the *Picrasma* genus of the Simaroubaceae family have been used as bitter stomachics for gastritis, loss of appetite, and indigestion in Chinese and Japanese traditional medicine. From these plants, a number of β-carboline and canthinone type alkaloids have been isolated, which have been reported to exert a variety of biological activities, including PTP1B-inhibition, anti-inflammatory activity, 3′,5′-cyclic adenosine monophosphate phosphodiesterase inhibition, and cytotoxicity.[3] In the present study, we report five canthinone alkaloids (**1**–**5**) that are potential new cerebral protective agents (Fig. 1).

Natural or chemical synthetic canthinone alkaloids (**1**–**16**) and β-carboline alkaloids (**17**–**22**) were screened for their cerebral protective effects at 100 mg/kg (p.o.) in ischemic animal, which provided a disease model of dementia. Measurement of the latency time in a step-down passive avoidance test and the density of surviving neurons of these animals were used to assess the cerebral protective effects. The experimental scheme is summarized in Figure 2.[4]

Cerebral ischemia was induced with bilateral carotid ligation in Mongolian gerbils (*Meriones unguiculatus*).[5] Gerbils were divided into groups: (1) Normal group, a group of gerbils that underwent no treatment; (2) Control group, a group of gerbils in which cerebral ischemia was induced, but which was given no compound; (3) Compound groups, groups of gerbils that underwent cerebral ischemia and were given compounds **1**–**22**, individually; and (4) the vinpocetine group, a group of gerbils that underwent cerebral ischemia and were given vinpocetine.[6]

A step-down passive avoidance test is widely used as a standard test for evaluation of learning/memory in gerbils. In this test, electrical stimulation was provided when the gerbils stepped downed from the platform. The step-down latency time, which was defined as the length of time that gerbils stayed on the platform, was used as a parameter for accessing learning and memory ability.[7]

Cerebral ischemia led to selective necrosis of neurons in specific brain regions. The CA1 subfield of the hippocampus is a brain region that is particularly sensitive to ischemia.[8] Thus, in this study, the density of surviving neurons in the CA1 subfield of the hippocampus was measured to examine the cerebral protective effect.

We identified five canthin-5,6-dione alkaloids, namely, picrasidine L (**1**), picrasidine O (**2**), eurycomine E (**3**), 3-ethyl-canthin-5,6-dione (**4**), and 3-ethyl-4-methoxy-canthin-5,6-dione (**5**), which resulted in a longer step-down latency time and greater density of surviving neurons than in the control animals (Figs. 3 and 4). Notably, picrasidine L (**1**) and picrasidine O (**2**) treatment resulted in virtually the same results as the normal group (Figs. 3 and 4). How-

* Corresponding authors. Tel.: +81 47 4721161; fax: +81 47 4721404.
E-mail addresses: liwei@phar.toho-u.ac.jp (W. Li), koike@phar.toho-u.ac.jp (K. Koike).
† Deceased.

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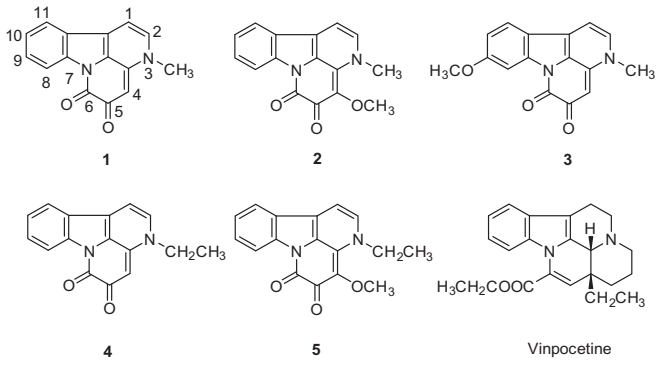

Figure 1. Five canthinone alkaloids and vinpocetine.

ever, the other 11 canthinone alkaloids (**6**–**16**) and six β-carboline alkaloids (**17**–**22**) showed no cerebral protective effects in either measurements (Supporting information). Vinpocetine, a structurally related carboline alkaloid, which is currently prescribed for the treatment of disorders arising from cerebrovascular and cerebral neurodegenerative diseases that ultimately lead to dementia in the elderly,[6] showed a much weaker effect than compounds **1**–**5** in this assay model.

In terms of step-down latency time, compounds containing an *N*-3-methyl moiety were approximately twice as potent as those containing an *N*-3-ethyl moiety (**1** vs **4**, and **2** vs **5**). Compounds containing a C-9-methoxy moiety showed a remarkably reduced activity (**3** vs **1**) (Fig. 3). In terms of the measurement of density of surviving neurons, a C-4-methoxy moiety decreased the cerebral protective activity [**1** vs **2** (p <0.05), and **4** vs **5**] (Fig. 4).

The mechanisms underlying the cerebral protective effects of compound **2** were investigated further. Benzodiazepines have been reported to have a cerebral protective effect.[9] β-carbolines, such as β-carboline-carboxyl-ethylester and harmane, were reported to bind to rat brain benzodiazepine receptor.[10] Compound **2** had β-carboline backbone skeleton in the molecular, and the cerebral protective activity of **2** was predicted to show by binding with benzodiazepine receptors. Thus, the ability of compound **2** to bind to the benzodiazepine receptor was investigated using an in silico docking study.[11] However, compound **2** showed much weaker

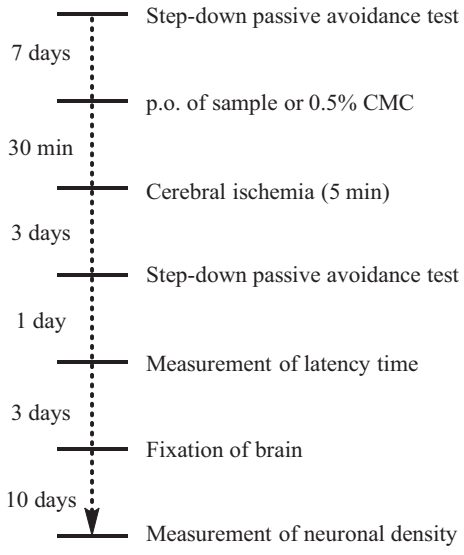

Figure 2. Experimental scheme of measurement of step-down latency time and neuronal density.

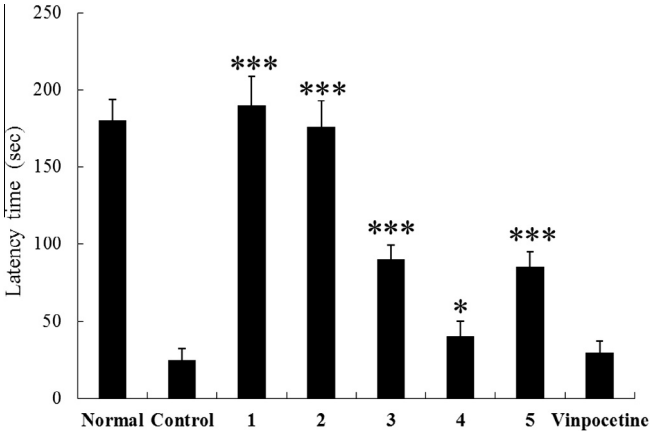

Figure 3. Step-down latency time (s) in ischemic gerbils (n = 10) treated with vehicle or with either of five canthinone alkaloids or vinpocetine administered before bilateral carotid ligation. Results are presented as the mean ± standard error of the mean. Normal: Normal group; Control: Control group; **1**, **2**, **3**, **4**, **5**: groups administered compound **1**, **2**, **3**, **4**, or **5**; Vinpocetine: group administered vinpocetine. One asterisk (*) indicates a p-value smaller than 0.05 (p <0.05), compared to the control group, three asterisks (***) indicate a p-value smaller than 0.001 (p <0.001), compared to the control group.

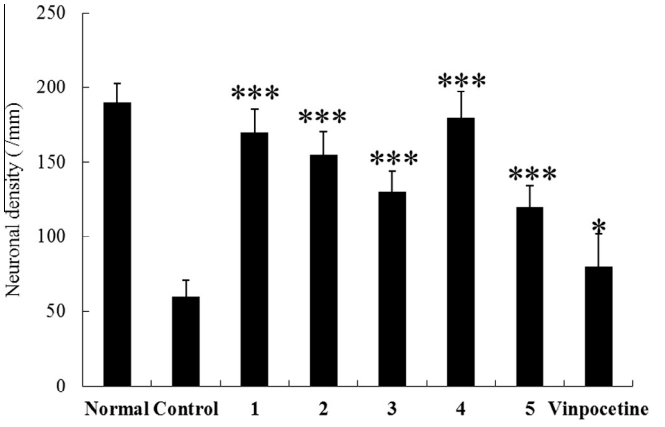

Figure 4. The density (/mm) of surviving neurons in the CA1 subfields of the hippocampus in ischemic gerbils (n = 10) treated with vehicle or either of five canthinone alkaloids or vinpocetine, administered before bilateral carotid ligation. Results are presented as the mean ± standard error of the mean. Normal: Normal group; Control: Control group; **1**, **2**, **3**, **4**, **5**: groups administered compound **1**, **2**, **3**, **4**, or **5**; Vinpocetine: group administered vinpocetine. One asterisk (*) indicates a p-value smaller than 0.05 (p <0.05), compared to the control group, three asterisks (***) indicate a p-value smaller than 0.001 (p <0.001), compared to the control group.

binding (−11.90 kcal/mol) than diazepam (binding energy: −72.64 kcal/mol), suggesting that it exerts its cerebral protective effect by other mechanisms.

Glutamic acid is the principal excitatory neurotransmitter in the brain. Endogenous glutamic acid may contribute to acute brain damage occurring after status epilepticus, cerebral ischemia, or traumatic brain injury, by activating NMDA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid, or metabotropic glutamate receptor 1 receptors.[12] Canthinone alkaloids might exert a cerebral protective effect through suppression of neuronal cell death due to the hyperexcitability caused by a cerebral ischemia-induced elevation in the glutamic acid concentration. To test this mechanism, kainic acid was administered peripherally to gerbils to provoke over-excitement and neuronal cell death, and the gerbils were then given compound **2** orally.

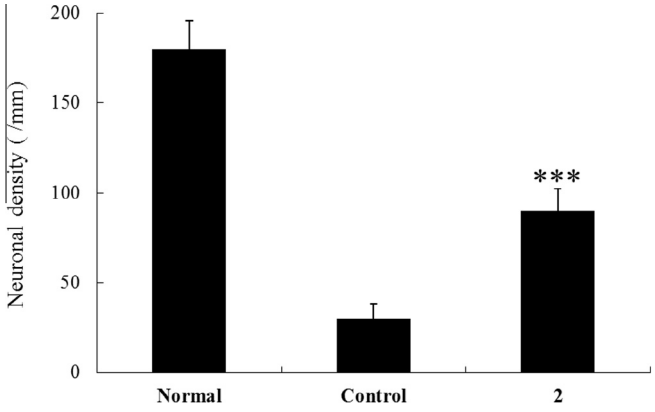

Figure 5. The density (/mm) of survival neurons in the CA1 subfields of hippocampus in ischemic gerbils ($n = 10$) treated vehicle or compound **2** administered after neuronal cell death induced by kainic acid injection. Results are presented as the mean ± standard error of the mean. Normal: Normal group; Control: Control group; **2**: group administered compound **2**. Three asterisks (***) indicate a p-value smaller than 0.001 ($p < 0.001$), compared to the control group.

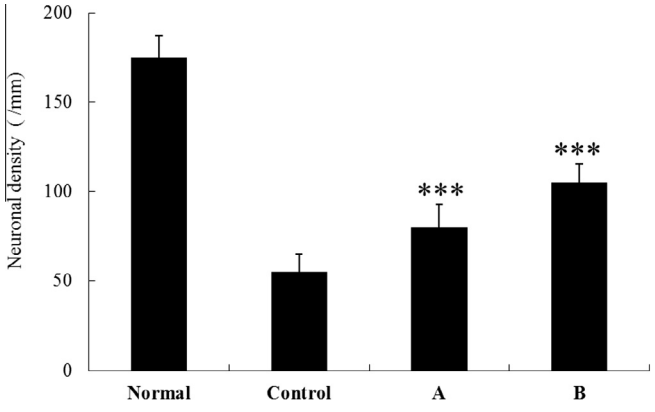

Figure 7. The density (/mm) of survival neurons in the CA1 subfields of the hippocampus in ischemic gerbils ($n = 10$) treated with vehicle or compound **2** administered after bilateral carotid ligation. Results are presented as the mean ± standard error of the mean. Normal: Normal group; Control: Control group; A: Orally administered compound **2** (at 30 mg/kg) 3 h after the cerebral ischemia; B: Orally administered compound **2** (at 30 mg/kg) 3 h and 6 h after cerebral ischemia. Three asterisks (***) indicate a p-value smaller than 0.001 ($p < 0.001$), compared to the control group.

Kainic acid is a potent agonist of the glutamate receptor. In gerbils, injections of kainic acid results in recurrent seizures, behavioral changes, and subsequent degeneration of selective populations of neurons in the brain.[13] Thus, administration of kainic acid[14] has been widely used as a model for studying the mechanisms underlying neurodegenerative pathways induced by excitatory neurotransmitters. In this study, kainic acid-induced neuronal necrosis was significantly improved in the compound **2**-treated group compared to the control group (Fig. 5). This suggested that the mechanism underlying the cerebral protective effect of compound **2** involved the suppression of over-excitation of neuronal cells, caused by abnormal glutamatergic signaling.

As mentioned above, cerebral ischemia significantly increases extracellular glutamate levels. Activation of the glutamate receptor enhances the influx of calcium ions into the cell, resulting in damage to nerve cells.[15] Thus, the effects of compound **2** on tissue damage outside the hippocampus were investigated by ^{45}Ca-autoradiography.[16] In the control group, after subjecting the animals to cerebral ischemia for 15 min, accumulation of $^{45}Ca^{2+}$ was measured in the cerebral cortex, striatum, and optic vesicle, in addition to the hippocampus. The levels of $^{45}Ca^{2+}$ accumulation were reduced in the group that received compound **2** at 30 mg/kg orally, suggesting that compound **2** resulted in significant improvement in extent of brain damage (Fig. 6).

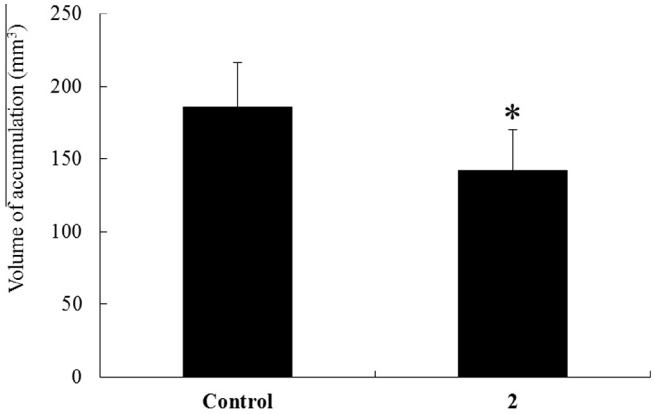

Figure 6. The volume of ^{45}Ca accumulation (mm^3) in gerbils ($n = 10$). Results are presented as the mean ± standard error of the mean. Control: Control group; **2**: group administered compound **2**. One asterisk (*) indicates a p-value smaller than 0.05 ($p < 0.05$), compared to the control group.

To investigate the administration schedule for using compound **2** as an acute-phase cerebral protection drug candidate, the effect of compound **2**, orally administered at 3 h and/or 6 h after cerebral ischemia, on the density of surviving neuronal cells was examined. Neuronal cell death was reduced more when compound **2** (at 30 mg/kg) was administered twice, at 3 h and 6 h after ischemia, than when only administered once at 3 h (Fig. 7).

The use of antihypertensive drugs in acute ischemic stroke must be considered carefully, as the drugs decrease cerebral blood flow and can exacerbate ischemic injuries.[18] Effects of compound **2** on heart rate and mean systolic blood pressure were therefore tested; neither was affected by compound **2**, even at a dose 3-fold higher than that required for pharmacological effect (30 and 100 mg/kg). These results indicated that brain damage would not be aggravated by administration of compound **2**. Furthermore, compound **2** showed no effect on body temperature, and showed a weak locomotion-reducing effect.

In conclusion, the main effects of compound **2** were (i) improving learning and memory performance, (ii) inhibiting delayed neuronal cell death induced by cerebral ischemia, (iii) reducing neuronal cell death induced by the excitatory neurotransmitter, glutamic acid and the excitotoxin, kainic acid, and (iv) decreasing the extent of brain tissue damage. Moreover, compound **2** was shown to have no effect on heart rate or mean blood pressure.

These effects suggest that compound **2** has a cerebral protective effect, preventing the progression of various intractable neurological diseases that are accompanied by neuronal cell death. More detailed investigations are required for development of picrasidine O (**2**) as a cerebral protective drug candidate.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2016.09.006.

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4. The protective effect of compounds on delayed neuronal death after transient cerebral ischemia was assessed. Animals: Male Mongolian gerbils were purchased from Seiwa Experimental Animals Ltd. (Oita Japan). They were maintained with ad libitum access to water and chow diet (Funabashi Farm-F2, Chiba, Japan) on a 12-h light–dark cycle, in a temperature- and humidity-controlled room, until the experiment, at which time they weighed 53–85 g. Assessment of cerebral protective effect of compounds: Five to 11 animals were used for each experimental group, and the effect of 22 alkaloids and vinpocetine were assessed. First, the animals underwent a step-down passive avoidance test for about 1 week, and were then orally administered 100 mg/kg per 3.0 mL of samples dissolved or suspended in 0.5% carboxymethyl cellulose solution. Thirty minutes after administration, the cervical region was opened via a midline incision under anesthesia with 2.5% halothane/N 20(70)/02(30), and the common carotid arteries were revealed. After cessation of anesthesia, cerebral ischemia was induced in animals for 5 min by bilateral ligation of the common carotid arteries, using Yasargil temporal clips (AESCULAP Co. Ltd., Tuttlingen Germany), after which the blood flow was resumed. Three days thereafter, the animals underwent the step-down passive avoidance test. After 1 day, the step-down latency time was measured. After an additional 3 days, the animals were again anesthetized by intraperitoneal injection of 50 mg/kg of sodium pentobarbital (Abott Co. Ltd., Newark, UK). Their brains were perfusion-fixed using 10% formalin neutral buffer solution (Wako Pure Chemical Industries Ltd., Osaka Japan). The brains were removed from the skulls, embedded in paraffin, and the coronal sections were sliced into 5.0-μm-thick sections. The density of surviving neurons in the CA1 subfields of the hippocampus was determined by microscopy after hematoxylin–eosin staining. Statistical analysis: Experimental data were statistically assessed using Student's *t*-test.
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11. Molecular docking simulation: Molecular docking simulation was performed against the GABA(A)-receptor carried out using the CDOCKER docking protocol of Discovery studio 4.5 (Dassault Systemes Biovia K.K., Vélizy-Villacoublay, France). CDOCKER (CHARMm-based DOCKER) is a molecular dynamics-based docking algorithm. It uses the CHARMm family of force fields and offers full flexibility for ligands, including dihedrals, angles, and bonds. Docking helps to predict the best binding compounds based on various scoring functions. The high-resolution crystal structure of the GABA(A)-receptor-associated protein, GABARAP was obtained from the Protein Data Bank (PDB-ID: 1kjt) (http://www.rcsb.org). Missing amino acid sequences were complemented, and all co-crystalized ligands and water data were removed. The starting 3D conformations of compound **2** and diazepam were prepared with Dock Ligands Fit. The calculated binding score of all docking positions were evaluated. The best ranked position from each of the binding sites was determined by the lowest calculated binding energy.
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14. Kainic acid injection: Kainic acid injection was performed according to the method of Kirino et al.[8] Male Mongolian gerbils ($n = 10$) were deprived of food overnight. On the following morning, they were injected with atropine sulphate (5.0 μg/animal) and anesthetized with pentobarbital (40 mg/kg, ip). They were then fixed on a stereotaxic apparatus. A small burr hole was made in the skull at 1.6 mm posterior to the bregma and 3.0 mm right of the midline. An unbeveled stainless steel needle (external caliber = 100 μm) was lowered 1.5 mm from the dural contact. The tip of the needle was thus located in the CA3 sector of the right hippocampus. Kainic acid (Sigma-Aldrich, St Louis, MO, USA) was dissolved in saline and its pH adjusted to 7.4. Using a microinfusion pump, 0.2 μL of the kainate solution (1.0 μg/μL) was injected into this sector over a period of 4.0 min. The needle was left undisturbed for 10 min and then withdrawn. Four days after this procedure, animals were perfusion-fixed. Five animals were fixed with 3.5% formaldehyde in 0.1 M phosphate buffer. These specimens were silver impregnated for localization of degenerating presynaptic terminals. The remaining animals were perfusion-fixed and processed for electron microscopy as described above. For comparison, male Mongolian gerbils were perfusion-fixed 1 month after ischemia, without kainate injection, and the specimens were processed using the silver impregnation method.
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16. Ca autoradiography: ^{45}Ca autoradiography was performed according to the method of Sakamoto et al.[17] Seven days after ischemia, the animals were anesthetized with 2% halothane in a mixture of 30% O_2 and 70% N_2O. A bolus of 100 μCi of $^{45}CaC1_2$ (Amersham, Little Chalfont, UK; spec. act. 10–40 mCi/mg) was injected intravenously (iv). The animals were anesthetized with ether at 5 h after the injection of $CaCl_2$; their brains were then rapidly removed and frozen in powdered dry ice. Serial coronal sections, 20-μm-thick, were cut from the frozen brains in a cryostat at −20 °C, and mounted and dried at 60 °C on cover glass. For the ^{45}Ca autoradiographic study, the sections were placed in contact with Kodak NMC-film and exposed for 2 weeks.
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