Evaluation of canthinone alkaloids as cerebral protective agents

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A B S T R A C T

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), eucrycomine 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.

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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. 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

Cerebral ischemia was induced with bilateral carotid ligation in Mongolian gerbils (Meriones unguiculatus).3 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.

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 down 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.

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.3 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), eucrycomine 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...
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.7 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 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.
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. Thus, administration of kainic acid 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. Thus, the effects of compound 2 on tissue damage outside the hippocampus were investigated by $^{45}$Ca autoradiography. 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).

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 cerebral 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. 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.
45Ca autoradiography was performed according to the method of Kirino et al.1 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.

Kainic acid injection: Kainic acid injection was performed according to the method of Kirino et al.1 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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