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(–)-Phenserine inhibits neuronal apoptosis following ischemia/reperfusion injury

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Abstract

Stroke commonly leads to adult disability and death worldwide. Its major symptoms are spastic hemiplegia and discordant motion, consequent to neuronal cell death induced by brain vessel occlusion. Acetylcholinesterase (AChE) is upregulated and allied with inflammation and apoptosis after stroke. Recent studies suggest that AChE inhibition ameliorates ischemia-reperfusion injury and has neuroprotective properties. (–)-Phenserine, a reversible AChE inhibitor, has a broad range of actions independent of its AChE properties, including neuroprotective ones. However, its protective effects and detailed mechanism of action in the rat middle cerebral artery occlusion

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model (MCAO) remain to be elucidated. This study investigated the therapeutic effects of (–)-phenserine for stroke in the rat focal cerebral ischemia model and oxygen-glucose deprivation/reperfusion (OGD/RP) damage model in SH-SY5Y neuronal cultures. (–)-Phenserine mitigated OGD/RP-induced SH-SY5Y cell death, providing an inverted U-shaped dose-response relationship between concentration and survival. In MCAO challenged rats, (–)-phenserine reduced infarction volume, cell death and improved body asymmetry, a behavioral measure of stroke impact. In both cellular and animal studies, (–)-phenserine elevated brain-derived neurotrophic factor (BDNF) and B-cell lymphoma 2 (Bcl-2) levels, and decreased activated-caspase 3, amyloid precursor protein (APP) and glial fibrillary acidic protein (GFAP) expression, potentially mediated through the ERK-1/2 signaling pathway. These actions mitigated neuronal apoptosis in the stroke penumbra, and decreased matrix metalloproteinase-9 (MMP-9) expression. In synopsis, (–)-phenserine significantly reduced neuronal damage induced by ischemia/reperfusion injury in a rat model of MCAO and cellular model of OGD/RP, demonstrating that its neuroprotective/neurotrophic cholinergic and non-cholinergic properties warrant further evaluation in conditions of brain injury.

Keywords

(–)-Phenserine; ischemia/reperfusion injury; stroke; middle cerebral artery occlusion (MCAO); amyloid precursor protein (APP); brain-derived neurotrophic factor (BDNF); B-cell lymphoma 2 (Bcl-2) expression; activated-caspase 3; glial fibrillary acidic protein (GFAP); ERK-1/2 signaling pathway; metalloproteinase-9 (MMP-9)

Introduction:

Cardiovascular disease, including cerebrovascular disease (CVD) and coronary artery disease (CAD), is a major cause of death internationally. In addition, stroke not only results in disability and death but also reduces life quality, wreaks a heavy burden on the economy, and has an incidence of approximately 150-200 in 100,000 in Taiwan and North America [1, 2] – annually afflicting some 15 million people worldwide and leading to 6 million fatalities. Cell damage and death induced by ischemia reperfusion arises, in part, from depolarization-induced calcium entry, intracellular nitric oxide, and mitochondrial dysfunction by free radical generation, especially in brain. In recent years, therapeutic strategies for stroke have focused on antioxidants for decreasing the size of ischemic injury and neural regeneration for rescuing dying cells early after injury initiation [3, 4]. Therapeutic interventions that target endogenous systems to mitigate and/or repair stroke-induced injury may broaden the window of treatment after stroke onset, and are urgently needed. Among potential intervention strategies, some studies have suggested that the expression of secreted amyloid precursor protein (sAPP) is upregulated after cerebral ischemia [5, 6] and may be a potential therapeutic target for stroke management.

APP is a type 1 transmembrane protein, and alternative splicing of the APP gene, located on chromosome 21, generates three isoforms (APP695, APP751 and APP770) with the former found nearly exclusively in neurons and the latter two being expressed almost ubiquitously [7,8]. Mature APP can be processed by different membrane-associated proteolytic enzymes, initially by γ -secretase with subsequent cleavage by α - or β -secretases, to generate different

APP fragments such as secreted (s)-APP α implicated in synapse formation, neural plasticity, iron transport, and the differentiation of neural stem cells to generate cells of either neuronal or glial lineage [7-11], and A β , implicated in Alzheimer's disease (AD) neuropathology and associated with synaptic loss, neuronal cell death and neuroinflammation [7-14]. APP has additionally been reported to be a stress-related protein whose neuronal expression can be rapidly up and down regulated transcriptionally and post-transcriptionally at the level of translation, in response to changes in the microenvironment consequent to oxidative stress or inflammation [7-11]. Global or focal cerebral ischemia up regulates APP expression in neurons and glia, as does hypoxia in vitro [14-18]. Although the biological functions of APP remain to be fully elucidated, increasing evidence suggests that the level of APP expression correlates with injury size in the ischemic brain. Animals overexpressing APP have larger infarct areas than their wild type littermates [19, 20], and neurotoxic A β peptide has been found to accumulate in vulnerable neurons in the post-ischemic hippocampus [21]. Recent studies on patients with stroke and cerebral infarction suggest a high risk for developing AD [22, 23], and thus therapeutic approaches that target the lowering of APP and its proteolytic product, A β , may have utility.

Treatment strategies whose molecular cascades augment neuroprotective and regenerative actions, likewise have been highlighted as potentially beneficial for stroke therapy [24,25]. A well tolerated experimental drug that reliably combines neuroprotective/regenerative activities with APP/A β lowering actions is (–)-phenserine [24]; thereby making it an attractive candidate to evaluate as a new treatment for stroke. (–)-Phenserine, an analog of physostigmine and an acetylcholinesterase (AChE) inhibitor, has been shown to improve cognition in AD animal models and patients [26-29]. Longer acting and better tolerated than physostigmine, (–)-phenserine has a greater brain uptake following parental administration (brain plasma ratio (–)-phenserine 8:1, versus physostigmine 1:1) [26, 30]. In the light of physostigmine's reported action to increase the survival time of mice subjected to hypoxia [31, 32] in addition to a range of non-cholinergic actions of (–)-phenserine to decrease A β levels by reducing APP synthesis at the level of its mRNA translation in neuronal cells [33, 34], as well as to provide neuroprotective and neurotrophic properties [35], we herein evaluated the action of (–)-phenserine in cellular and animal models of hypoxia. Utilizing a focal cerebral ischemia model in rat and an oxygen-glucose deprivation/reperfusion (OGD/RP) damage model in human SH-SY5Y neuronal cells in culture, we demonstrate that (–)-phenserine mitigates neuronal cell death in ischemia/reperfusion injury at a clinically translatable dose.

Results:

Loss of cell viability after OGD-Hypoxia is attenuated by (–)-phenserine:

The addition of (–)-phenserine to SH-SY5Y neuronal cultures proved well tolerated at concentrations up to 30 μ M, as appraised by measuring cell viability by MTT assay under normoxic conditions. At a concentration of 100 μ M, however, (–)-phenserine induced substantial cell death (Fig. 1a). The OGD-Hypoxia cell culture condition was used to model ischemia/reperfusion (I/R) injury in vitro. A time-dependent evaluation of this condition, versus normoxia, resulted in a 53% loss in SH-SY5Y cell viability at 4 hr exposure that rose

to in excess of 74% cell death following 8 h exposure, with 24 h exposure inducing no greater effect (Fig. 1b). A concentration-dependent evaluation of (–)-phenserine (0 to 100 μ M) to mitigate SH-SY5Y cell death-induced by OGD-Hypoxia 4 h exposure is illustrated in Figure 1C. An inverted U-shaped dose-response relationship between (–)-phenserine concentration and improved cellular survival was evident, with 10 μ M providing optimal protection.

Selecting the optimal concentration of (–)-phenserine (10 μ M) and OGD-Hypoxia 4 h exposure, an evaluation of mechanisms underpinning the neuroprotective action of (–)-phenserine was performed. In the light of the demonstration that (–)-phenserine lowers the rate of synthesis of APP and elevates the expression of phosphorylated extracellular signal-regulated kinase (p-ERK-1/2) [33] in neuronal cells, levels of these, together with the housekeeping protein β -actin, were time-dependently quantified by Western blot in SH-SY5Y cells following (–)-phenserine (10 μ M) administration during normoxia. As illustrated in Figure 2A and B, (–)-phenserine (10 μ M) induced a reduction in cellular APP levels, achieving a maximal decline of 47% by 24 hr. In contrast, (–)-phenserine (10 μ M) rapidly increased p-ERK-1/2 levels, achieving a maximal rise of 3.8-fold by 1 h that declined to control levels within 12 h.

Consequent to the different time-dependent responses of APP and p-ERK-1/2 to (–)-phenserine (10 μ M) treatment, the reaction of cells challenged with OGD-hypoxia in the presence and absence of (–)-phenserine (10 μ M) was evaluated following 6 h exposure; a time selected as physiological responses occurred for both proteins. As shown in Figure 2C and D, 6 h treatment with (–)-phenserine (10 μ M) of normoxic SH-SY5Y cells recapitulated the previously observed 1.6-fold rise in p-ERK-1/2 expression. In contrast, OGD-hypoxia induced a decline (52%) in p-ERK-1/2 expression that was mitigated by the addition of (–)-phenserine (10 μ M). Notably, (–)-phenserine (10 μ M) administration dramatically elevated levels of the neurotrophin BDNF (2.9-fold), which remained significantly elevated during the condition of OGD-hypoxia in the absence and presence of (–)-phenserine. OGD-hypoxia additionally lowered and elevated the anti- and pro-apoptotic proteins Bcl-2 (69% decrease) and activated caspase-3 (99% increase), changes that were mitigated in part by (–)-phenserine (10 μ M) treatment. Finally, with regard to APP (Fig. 2E) and in accord with Figure 2A and B, (–)-phenserine (10 μ M) reduced APP levels during normoxia (25% reduction, $p < 0.05$) and mitigated the OGD-hypoxia induced elevation in APP levels.

(–)-Phenserine reduces ischemia-reperfusion injury in the MCAO rodent model by inhibiting neuronal apoptosis and mitigates behavioral impairment:

To appraise whether neuroprotective properties of (–)-phenserine evident in neuronal SH-SY5Y cultures translate in vivo, we investigated the agent's action in rats challenged with MCAO-induced hypoxia [36]. Infarction size was quantitatively evaluated by triphenyltetrazolium chloride (TTC) staining after 24 h, as illustrated in Figure 3A. Administration of (–)-phenserine (1 mg/kg, i.p.) prior to MCAO significantly reduced infarction volume (Fig. 3A). The physiological relevance of this decline was gauged behaviorally by measuring body asymmetry at 24 h following ischemia-reperfusion injury [37]; the severity of which was significantly ameliorated by (–)-phenserine (Fig. 3C). As

evident in Figure 4A and evaluated within the penumbra of the stroke, apoptotic neuron cell death was induced by ischemia-reperfusion injury, as assessed by both TUNEL and NeuN immunohistochemical staining, and was substantially mitigated by (–)-phenserine (Fig. 4 B-D). Specifically, within the stroke penumbra there were 246% more NeuN stained cells and 27% fewer TUNEL stained cells per field evaluated; leading to a (–)-phenserine induced 70% reduction in the TUNEL/NeuN cell ratio (ratio of cells per field, vehicle: 1.45 ± 0.03 ; (–)-phenserine: 0.43 ± 0.01). Likewise notable and in line with (–)-phenserine's reported action to lower APP, elevated levels of APP immunohistochemical staining were evident throughout the stroke region, as illustrated in Figure 5A, and these were dramatically lowered by (–)-phenserine.

Samples of penumbra were evaluated by Western blot analysis to probe for neuroprotective mechanisms underpinning the in vivo actions of (–)-phenserine. Cross-validating the ability of (–)-phenserine to lower MCAO-induced elevations in APP levels evident by immunohistochemistry (Fig. 5A), a 4.2-fold MCAO-induced rise in APP levels was evident by Western blot, and was substantially inhibited by (–)-phenserine (Fig. 4C and D). Notably, levels of GFAP, a marker of astrogliosis whose rapid up regulation is a hallmark of stroke [38], were increased (20%) within the stroke penumbra and this was normalized by (–)-phenserine. In contrast, levels of BDNF were reduced (10%) in stroke penumbra and were elevated by (–)-phenserine (89% in unaffected brain and by 20% in stroke penumbra). The expression of the pro- and anti-apoptotic proteins, activated-caspase-3 and Bcl-2, were up-regulated (+256%) and down-regulated (–36%), respectively, by MCAO; actions that were fully reversed by (–)-phenserine treatment (Fig. 4C and D).

Consequent to an increasing body of evidence implicating the involvement of the matrix metalloproteinase (MMP)-9 in not only the pathogenesis of blood-brain barrier breakdown and subsequent development of vasogenic edema that occurs after stroke [39-41], but also the development of hemorrhagic transformation [42], we evaluated the expression of MMP-9 within the stroke penumbra and found it elevated by 1.4-fold ($p < 0.05$) (Fig. 4E and F). Of note, (–)-phenserine significantly mitigated this increase (reducing the elevation by 53%, $p < 0.01$).

Discussion:

With some 15 million people worldwide suffering a stroke annually, which leads to nearly 6 million deaths and the development of permanent disabilities in 5 million, stroke is the second leading cause of disability and death for those above the age of 60 years [43]. Here we demonstrate that the anticholinesterase, (–)-phenserine, ameliorates key aspects of neuronal cell death in acute cellular and in vivo models of ischemic stroke. Importantly, these models emulate specific cardinal characteristics of ischemic stroke in humans, and highlight the complex processes that lead to apoptotic cell death that time-dependently occurs within the stroke penumbra. Our data indicate that such processes can be halted, and add to the extensive literature on mechanisms underpinning neuronal cell death following an ischemic event [44-46]. This mitigation of cell death was achieved in the current study by the administration of (–)-phenserine to cultured neuronal (SH-SY5Y) cells challenged with OGD-hypoxia and to rats subjected to ischemia/reperfusion injury. In a concentration-

dependent manner, (–)-phenserine ameliorated OGD-hypoxia-induced cell death in cultured neuronal cells in accord with prior studies demonstrating neuroprotection against oxidative stress and glutamate excitotoxicity in both SH-SY5Y cells and rat primary cortical cultures [34, 47]. To evaluate translational relevance, (–)-phenserine was administered to rats that then were challenged with MCAO, and it substantially reduced apoptosis within the stroke penumbra – leading to a dramatic 70% reduction in the TUNEL/NeuN stained cell ratio ($p<0.001$). This (–)-phenserine dose (1 mg/kg) was selected based on its clinical relevance [48], as it is equivalent to 10 mg in a 65 kg human (following normalization between species based on body surface area [48]), which proved to be well tolerated in both healthy volunteers and patients with Alzheimer’s disease [28, 29, 49]. This neuroprotective action of (–)-phenserine resulted in a reduction of stroke volume and a physiologically relevant decrease in neurological deficits, as evaluated by measuring body asymmetry [37]; thereby providing anti-apoptotic actions in line with (–)-phenserine’s ability to inhibit neuronal cell death and improve the survival of rats exposed to the organophosphorus nerve gas, soman [50].

In the light of the use of anticholinesterases as cognitive enhancers and their reported widespread improvements in domains of attention and arousal [51, 52], many of the prior studies evaluating the actions of this drug class in ischemic stroke have focused on their potential to improve rehabilitation outcomes by augmenting cognitive functioning and decreasing apathy to, thereby, facilitate participation in and capacity to learn from rehabilitation [53-55]. In addition to these studies, there are evaluations of cholinesterase inhibitors in rodent models of ischemic stroke. As a positive example, systemic administration of neostigmine (40 ug/kg) has been reported to reduce infarct size and resulting neurological deficits in rodents [56], albeit this agent possesses a quaternary amine that limits its brain uptake across the normal blood-brain barrier, but that likely is compromised within the stroke area. Pretreatment with donepezil (12 mg/kg but not 6 or 3 mg/kg) has similarly demonstrated the ability to reduce infarct volume in rats subjected to MCAO [57]; although the effective dose in this donepezil study is above the clinically translatable range. In both these studies, neuroprotection was considered mediated, at least in part, by the cholinergic nicotinic system and primarily via the $\alpha 7$ nicotinic acetylcholine receptor. By contrast, the anticholinesterase methanesulfonyl chloride mitigated simple learning and memory deficits induced by MCAO in rats, but did so without reducing the size of the cerebral infarction [58]. Furthermore, galantamine (2.5 mg/kg administered pre- and post the induction of cortical photothrombosis), an anticholinesterase that allosterically modulates nicotinic acetylcholine receptors, was found to lack activity on histological and functional outcomes of experimental stroke in rats [59].

A caveat of these as well as our own study is that drug was administered prior to the occurrence of stroke; thereby limiting clinical relevance. Such a study design, however, provides a valuable drug development go/no go decision checkpoint. In this scenario beneficial actions support further evaluation of a compound’s time-dependent window of activity when administered following MCAO, and research on inactive compounds can be brought to closure.

To aid the differentiation between potential symptomatic actions on cognition and arousal from anti-apoptotic actions, and to understand underlying mechanisms, we evaluated the actions of (–)-phenserine in cell culture. SH-SY5Y cells have been reported to express moderate levels of dopaminergic phenotypic markers, epitomized by dopamine β -hydroxylase [60], but relatively negligible levels of cholinergic markers epitomized by choline acetyl-transferase, acetylcholinesterase or butyrylcholinesterase [61]. In this light, the observed neuroprotective effects of (–)-phenserine to OGD-hypoxia challenge, in addition to potential cholinergic mediated actions, almost certainly involve non-cholinergic processes. Numerous studies have reported that hypoxia elevates APP expression levels in affected brain areas as well as in neuronal cultures [8], which our studies concur with (Fig. 2A/B, 4C/D). The biological actions of APP following an injury ultimately depend on the level and the activity of the secretases involved with its processing [7-11]. Whereas processing along the amyloidogenic route would favor generation of toxic products that can ultimately lead to neurodegeneration, that along the non-amyloidogenic pathway would potentially provide neuroprotection [7–11]. The exposure of brains or neuronal cells to hypoxia or ischemia has been reported to activate β - and γ -secretase cleavage of APP and, thereby, drive amyloidogenic APP processing; stimulating A β production and the accumulation of amyloid plaques [62-64]. Notably, and in accord with our previous studies [34], (–)-phenserine elevated pERK-1/2 and lowered APP levels in neuronal cultures. The time-dependence of these two actions is evident in Figure 2B and suggests that they are independent from one another, which is supported by (–)-phenserine-induced reductions in APP occurring in the absence of p-ERK-1/2 activity following its inhibition in our previous studies [33]. In light of accumulating evidence that the level of APP expression correlates with injury size in the ischemic brain, that animals overexpressing APP have larger infarct areas than their wild type littermates [15, 16], and that A β accumulates in vulnerable neurons in the post-ischemic hippocampus [17] with a higher risk for developing AD [18, 19], the ability of (–)-phenserine to dramatically lower APP in cellular and animal models of ischemia may be of clinical benefit.

Further prior studies of ours indicate that selective inhibition of the protein kinase C and ERK pathways results in the loss of (–)-phenserine-mediated neuroprotective and neurotrophic actions in SH-SY5Y cells [34]. ERK-1/2 has been described as critical for the characteristic cell proliferation, differentiation and programmed cell death observed in brain injury [65], and hence has been considered as a potential target for stroke treatment [66]. Precisely how ERK-1/2 impacts brain injury remains to be fully elucidated, as it has been reported to both protect and exacerbate neuronal injury following stroke [67]. ERK-1/2 kinase activity is distinguished by its capacity to phosphorylate substrates, particularly nuclear transcriptional factors, other protein kinases, cytoskeletal proteins and membrane receptors [68]. To achieve this ERK-1/2 must become phosphorylated to be active. Our cellular studies indicate that OGD/hypoxia reduces pERK-1/2 levels and that (–)-phenserine normalizes them; an action in accord with neuroprotectants, such as growth factors and ischemic preconditioning that similarly elevate pERK-1/2 levels [69-75].

To further evaluate the neuroprotection-associated and (–)-phenserine-mediated elevation in pERK-1/2, levels of the key apoptotic proteins Bcl-2 and activated-caspase 3 were quantified in our cellular and in vivo models. Bcl-2 is localized to the outer membrane of mitochondria,

where it has a key role in promoting cellular survival by inhibiting the actions of pro-apoptotic proteins. Bcl-2 overexpression protects against neuron loss within the ischemic margin of experimental stroke and inhibits cytochrome c translocation and caspase-3 activity [76]. Among the 14 distinct caspase family members that play a role in programmed cell death and neuroinflammation, caspase 3 is not only the most abundantly expressed in adult rodent brain, chiefly in neurons [77], but its activation is classically detected in ischemic tissue; whereupon it degrades multiple substrates within the cytoplasm and nucleus, and induces cell death [78]. Mice over-expressing human caspase 3 have demonstrated increased apoptosis and larger lesion volumes following an ischemic event [78]. In contrast, caspase 3 knockout mice have smaller infarct volumes than their wild type littermates [78]. Concurring with these findings, levels of Bcl-2 were decreased and activated caspase 3 elevated in both our cellular and in vivo ischemia models (Fig. 2C and D, Fig. 5B and C). (–)-Phenserine dramatically mitigated these ischemia-induced changes, in accord with treatments that block caspase 3 [79]. Also notable, (–)-phenserine substantially increased BDNF levels in SH-SY5Y cells during normoxia and hypoxia, which translated to raised brain BDNF levels in ischemic challenged animals (Fig. 5B and C). Elevating brain BDNF levels, whether by pharmacological or physiological means [80, 81], has become a therapeutic strategy in the treatment of neurodegenerative disorders [82] consequent to its reduced levels in brain across multiple neurodegenerative and neuropsychiatric disease [83]. Our studies with (–)-phenserine cross-validate elevations in brain BDNF achieved following (+)-phenserine administration to rodents [35, 84] and, importantly, demonstrate that this action is not dependent on the enantiomeric form of the compound.

MMPs are a family of zinc and calcium-dependent endopeptidases that can efficiently degrade the components of the extracellular matrix and, of these, MMP-9, in particular, has been extensively investigated for its role in blood-brain barrier disruption and subsequent vasogenic edema formation following stroke [85], as well as in the development of hemorrhage after thrombolytic (tissue plasminogen activator (tPA)) therapy [86]. Numerous clinical and experimental studies have demonstrated a rise in serum MMP-9 following stroke, likely deriving from circulating neutrophils [85], and this rise correlates with poorer outcome measures [87]. As blood-brain barrier breakdown is a key event in the secondary injury cascade following stroke that leads to neuroinflammation and exacerbates brain injury, mechanisms that lower MMP-9, as achieved by (–)-phenserine, are considered potentially valuable therapeutic strategies [88]. In support of this viewpoint, MMP-9 knockout provides substantial protection against increases in blood-brain barrier permeability following cerebral hypoxia-ischemia [89], suggesting that MMP-9 is the dominant protease causing barrier breakdown following ischemic stroke ([90].

In synopsis, our studies demonstrate that (–)-phenserine is neuroprotective in cellular and in vivo models of ischemia/reperfusion injury, providing anti-apoptotic actions at a clinically translatable dose that result in an improved behavioral outcome. The mechanisms underpinning this action appear to be non-cholinergically as well as, likely, cholinergically mediated and involve the suppression of apoptosis via the ERK-1/2 signaling pathway to augment Bcl-2 levels and decrease caspase-3 activation, with additional beneficial modulatory actions on BDNF, APP and MMP-9. This anti-apoptotic action of (–)-phenserine was achieved with a study design, like other studies [57-59], involving the pre-

administration of the agent. These beneficial actions are in accord with prior research demonstrating the efficacy of (–)-phenserine in animal models of traumatic brain injury and soman-induced neurotoxicity at clinically translatable doses [47, 50], and support the further evaluation of (–)-phenserine as an anti-apoptotic agent in acute and chronic neurodegenerative disorders.

Conclusion:

These studies establish that (–)-phenserine possesses anti-apoptotic neuroprotective actions in cellular and in vivo studies of ischemia-induced neuronal cell death at a clinically translatable dose, and additionally demonstrated that it can mitigate pathologically associated elevations in APP and augment levels of the neurotrophic protein BDNF.

Experimental procedures:

Cell culture:

Human neuroblastoma SH-SY5Y cells were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA), cultured in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F-12) containing fetal bovine serum (10%) and penicillin/streptomycin (1%) (Invitrogen, Carlsbad, CA) and maintained in a humidified incubator with 5% CO₂ at 37 °C. Neuronal cultures, to mimic ischemia/reperfusion injury, were incubated in oxygen-glucose deprivation and hypoxia (OGD-Hypoxia). In brief, the culture medium was exchanged with serum and glucose-free media. The cultures were then put into an air-tight box, and a 95% N₂ and 5% CO₂ mixed gas was continually aerated into the box for 5 min to completely replace the air and then returned to 5% CO₂ at 37 °C.

Animals:

Animals were anesthetized before MCAO surgery in accord with a Taipei Medical University Laboratory Animal protocol. Animal studies (LAC-101-0147) were approved by the Institutional Animal Care and Use Committee (IACUC) of the Taipei Medical University. Adult male Sprague–Dawley rats (10-12 weeks, 250-300 g) were used in this study and anesthetized with chloral hydrate (400 mg/kg, i.p. initially and 100 mg/kg every hour).

(–)-Phenserine:

(–)-Phenserine was obtained from Tocris Bioscience (Bristol, UK). HPLC analysis demonstrated 99% purity, with structural confirmation undertaken by ¹H NMR and mass spectrum analyses. The agent was prepared freshly prior to use, initially dissolved within a small volume of 100% DMSO, which was then appropriately diluted with either culture media for cellular studies or with physiological saline for animal studies. The amount of DMSO associated with each (–)-phenserine treatment was determined, and kept constant across treatment groups (including vehicle controls – that contained an alike concentration of DMSO but no (–)-phenserine). Final solutions were carefully checked to ensure the solubility of the drug.

Cell viability:

(–)-Phenserine was added to cells 24 hours before OGD-Hypoxia incubation. Cell viability was measured by determining the reduction of 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma, St Louis, MO). SH-SY5Y cells were cultured in 24-well plates. After treatment, MTT (0.5mg/mL) was added into media followed by incubation for 2 to 4 h at 37°C in a humidified incubator. 200µL DMSO was then added to each well to dissolve the blue formazan crystals. The absorbance of the solution was read at 570 nm wavelength against a 1 : 1 mixture of acid propanol and media as a blank.

Western blotting:

Cells were lysed by adding RIPA lysis buffer containing 0.5M Tris-HCl, pH 7.4, 1.5M NaCl, 2.5% deoxycholic acid, 10% NP-40, 10mM EDTA and protease inhibitor cocktails, and stored at –70 °C for further measurements. Proteins were separated by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE). After electrophoresis, proteins were electrotransferred onto a polyvinylidene difluoride (PVDF) membrane. The PVDF membrane was washed once with Tris-buffered saline (TBS) and twice with TBS plus 0.1% Tween 20 (TBST), blocked with blocking solution containing 5% non-fat dry milk in TBST for 1 h at room temperature, and blotted with primary antibodies APP (Abcam, Cambridge, U.K.), active caspase-3 (Abcam, Cambridge, U.K.), β-actin (Abcam, Cambridge, U.K.), p-ERK-1/2 (Cell Signaling Technology, MA, USA), Bcl-2 (Santa Cruz, CA, USA), BDNF (EBD Millipore, Livingston, U.K.), GFAP (BD Biosciences, CA, USA) in the blocking buffer. The PVDF membrane was incubated with peroxidase-linked anti-mouse or anti-rabbit immunoglobulin G (IgG) antibodies (Cell Signaling Technology, MA, USA) for 1 h and then developed by an enhanced chemiluminescence (ECL) plus detection kit (Amersham Life Sciences, Piscataway, NJ, USA).

MCAO (Animal model of middle cerebral artery occlusion):

Adult male Sprague–Dawley rats, after anesthesia with chloral hydrate, were ligated on the right MCA with a 10-0 suture. The ligature was removed after 60-min ischemia to generate reperfusion injury. Rat body temperature was monitored with a thermistor probe and maintained at 37 °C with a heating pad during anesthesia. After recovery from the anesthesia, body temperature was maintained at 37 °C using a temperature-controlled incubator. Using this animal model, our collaborative group has generated consistent ischemic damage in rodents as demonstrated by brain infarction visualization and behavioral analysis [36]. Rats were treated with either (–)-phenserine (1mg/kg, p.) or vehicle for 24 h before the MCAO surgery. (–)-Phenserine was prepared at an initial concentration of 10 mg/mL in DMSO immediately prior to use, and was diluted with saline to a final concentration of 1 mg/mL for administration, ensuring that drug remained in solution. Animals administered vehicle received saline/DMSO without the addition of (–)-phenserine.

Gelatin Zymography:

Gelatinolytic zymography was used to detect gelatinase, such as from matrix metalloproteinases (MMP)-2 and -9 derived from tissue. The collected tissue lysates (50 µg) after MCAO were loaded onto 10% SDS–PAGE containing 0.1% gelatin and subjected to

electrophoresis. After electrophoresis, to remove the SDS, the gel was washed twice with 2.5% Triton X-100 solution for 30 min. Gelatinase activation of the gel was incubated in buffer (0.05 M pH 8.0-Tris-HCl buffer, 5 mM CaCl_2 , and 5 mM ZnCl_2) at 37°C over 16 h. Then, the gel was stained with a solution of 0.25% Coomassie blue R250 dissolved in 40% methanol and 10% acetic acid for 2 h at room temperature. The lysis bands were observed after de-staining in 40% methanol and 10% acetic acid solution.

TTC Staining:

For histological evaluation of stroke damage, the size of infarction was evaluated by TTC (Sigma, St Louis, MO) staining. Two days after MCA ligation, some animals were euthanized and perfused intracardially with saline. The brain tissue was then removed, immersed in cold saline for 5 min, and sliced into 2.0 mm thick sections. The brain slices were incubated in 2% TTC, dissolved in normal saline for 10 min at room temperature, and then transferred into a 5% formaldehyde solution for fixation. The area of infarction on each brain slice was measured under observer blinded conditions using a digital scanner and the Image Tools program (University of Texas Health Sciences Center, San Antonio, TX). The total infarction volume in each animal was obtained from the product of average slice thickness (2 mm) and sum of the area of infarctions in all brain slices.

Immunocytochemistry and TUNEL staining:

Animals were anesthetized and perfused transcardially with saline followed by 4% paraformaldehyde (PFA) in phosphate buffer saline (PBS; 0.1 M; pH 7.2). The rat brains were serially transferred to 20 and 30 % sucrose in PBS overnight. Brains were then sectioned at 10-12 μm thickness by cryostat and kept at -30°C. The sections were immersed in PBS for 30 min and then incubated in PBS containing 0.2% Triton X-100(PBST) for 15 min at room temperature. The brain sections were then incubated with 3% PBS after removing the endogenous peroxidase activity by 3% H_2O_2 in PBS for 10 min and, thereafter, incubated overnight at 4°C with 10% normal goat serum-PBST. After blocking in goat serum-PBST, sections were immersed in primary antibody, APP (Abcam, Cambridge, UK) and NeuN (EBD Millipore, Livingston, UK) diluted to 1:100 with 10% normal goat serum-PBST. The sections were then rinsed with PBS (3 $\times$ 5 min per time) and incubated with secondary antibody linked with polymer-horseradish peroxidase (HRP) for 1 h. After the secondary antibody incubation and washing in PBST (3 $\times$ 5 min), the sections were incubated for 10 min in 0.04 mg of 3,3'-diaminobenzidine (DAB in 200 mL distilled water; DAKO Corporation, Hamburg, Germany). The sections were then rinsed with PBS (3 $\times$ 10 min) to halt the chromogen reaction, wet-mounted onto gelatin/chromium-coated slides, and allowed to air-dry overnight. TUNEL staining was performed using a Roche assay kit (In situ Cell Death Detection Kit; Roche, USA), according to the manufacturer's protocol.

Body Asymmetry:

Behavioral changes were evaluated by body asymmetry. Body asymmetry was quantitatively calculated by using the elevated body swing test; rats were evaluated by raising the animals by their tails above a horizontal surface [37]. The number of turns of the head or upper body contralateral to the ischemic side was counted in 20 consecutive trials and was normalized as follows: % recovery $[1-(\text{lateral turns in 20 trials}-10)/10]\times 100\%$.

Statistical Analysis:

Evaluations were undertaken in an observer blinded manner. Statistical analyses were performed with a Student's t-test and one-way or two-way ANOVA. Additionally, a Dunnett's or Bonferonni test was performed to evaluate multiple comparisons against a single or multiple comparator groups, respectively. Significance was inferred at $p < 0.05$ or less, and is noted within each Figure legend. Data are presented as mean $\pm$ SEM values throughout.

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- Pretreatment with a clinically translatable dose of (–)-phenserine, a drug originally developed for and evaluated in Alzheimer's disease, reduced infarct volume in a rat middle cerebral artery occlusion (MCAO) model of ischemia/reperfusion injury.
- This (–)-phenserine-induced reduction in infarct volume was associated with reduced behavioral impairment.
- The mechanisms underpinning the neuroprotective action of (–)-phenserine were evaluated within the penumbra of the stroke area of rats challenged with MCAO ischemia/reperfusion injury and in human SH-SY5Y cells challenged with oxygen-glucose deprivation/reperfusion.
- (–)-Phenserine significantly inhibited ischemia-induced neuronal apoptosis, elevating anti-apoptotic Bcl-2 and lowering pro-apoptotic activated-caspase 3 protein levels - mediated via the ERK-1/2 signaling pathway.
- (–)-Phenserine additionally elevated BDNF levels and lowered ischemia-induced elevations in amyloid precursor protein (APP) and matrix metalloproteinase-9 (MMP-9) protein levels.
- These studies demonstrate that (–)-phenserine possesses anti-apoptotic neuroprotective actions and support the compound's further evaluation in conditions of brain injury.

Figure 1:

OGD-Hypoxia versus normoxia conditions induce a loss in SH-SY5Y cell viability that is mitigated by (-)-phenserine. **(A)** Dose-dependent action of (-)-phenserine (1 to 100 μM) on the viability of normoxic SH-SY5Y cells. **(B)** Time-dependent action OGD-Hypoxia versus normoxia on the viability of SH-SY5Y cells. **(C)** Concentration-dependent action of (-)-phenserine (1 to 100 μM) to mitigate OGD-Hypoxia-induced loss of viability of SH-SY5Y cells (OGD-Hypoxia was induced over 4 h). # Indicates comparison versus the normoxia group, where ### $p < 0.001$. * Indicates comparison versus the hypoxia alone group, where ** $p < 0.01$, and *** $p < 0.001$ (N=4 per group for all cellular studies).

Figure 2:

(-)-Phenserine regulates the protein expression levels of APP, Bcl-2 and active caspase-3, as well as phosphorylation levels of ERK-1/2 in SH-SY5Y cells. **(A)** (-)-Phenserine (10 μ M) regulated the protein levels of APP and phosphorylation levels of ERK-1/2 in a time-dependent manner. **(C)** (-)-Phenserine increased BDNF and Bcl-2 expression as well as the phosphorylation level of ERK-1/2, and lowered activated caspase-3 protein levels in SH-SY5Y cells exposed to OGD-hypoxia. **(B)** and **(D)**: Bar graphs demonstrating quantitative densitometry to provide a ratio of protein expression relative to the normoxia value (values were normalized in relation to β -actin). **(E)** (-)-Phenserine (10 μ M) reduced APP levels during normoxia and OGD-hypoxia. N = 3 per group for all cellular studies. For **(B)**: * indicates comparison to zero time (0 h), where * p 0.05, ** p 0.01 and *** p 0.001. For **(D)**: * indicates comparison to normoxia group, where * p 0.05, ** p 0.01 and *** p 0.001. In contrast, # indicates comparison to OGD/Hypoxia group, where ## p 0.01.

Figure 3:

The efficacy of (-)-phenserine in the middle cerebral artery occlusion (MCAO) model in rat. **(A)** (-)-Phenserine decreased infarction volume following MCAO. **(B)** The bar graph shows semi-quantified densitometry, providing an infarction volume (mm³). **(C)** (-)-Phenserine improved body asymmetry after MCAO. * Indicates comparison between MCAO groups administered either vehicle or (-)-phenserine (1 mg/kg), where * $p < 0.05$ ($N = 6$ each group for TTC staining and body asymmetry test).

Figure 4:

The neuronal apoptosis induced within the penumbra of a MCAO-induced stroke is mitigated by (-)-phenserine. **(A)** (-)-Phenserine (1 mg/kg) decreased apoptosis and neuronal cell death in the cortex after MCAO (representative sections demonstrating TUNEL staining and NeuN immunohistochemistry). Quantitative evaluation of **(B)** NeuN expressing cells, **(C)** TUNEL stained cells, and **(D)** TUNNEL/NeuN stained cell ratio across similar size fields within the penumbra of MCAO-induced stroke animals treated with vehicle or (-)-phenserine (1 mg/kg). (N=3 per group, * Indicates comparison between vehicle and (-)-phenserine treated groups, where * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$).

Figure 5:

The protective actions of (-)-phenserine may be regulated via the ERK-1/2 signaling pathway following a MCAO challenge in the rat. (A) The expression of APP was examined by immunohistochemical staining in the rat brain cortex and was found dramatically elevated within the infarction area of vehicle but not (-)-phenserine-treated animals (representative sections (N=3 per group)). (B) (-)-Phenserine increased Bcl-2 and BDNF expression and attenuated rises in GFAP and activated caspase-3 after MCAO challenge in rats. (D) (-)-Phenserine decreased active MMP-9 after MCAO. (C) and (E) bar graphs showing semi-quantified densitometry of protein expression levels (relative to normoxia, as

assessed in left cortex of vehicle treated animals). N = 3 per group for Western blotting analysis studies. For (C) and (E): * Indicates comparison to normoxia (left cortex – vehicle) group, where * p < 0.05, ** p < 0.01 and *** p < 0.001. In contrast, # indicates comparison to right cortex – vehicle (stroke area) group, where ## p < 0.01.