A co-drug conjugate of naringenin and lipoic acid mediates neuroprotection in a rat model of oxidative stress

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Summary

Using our in vitro and in vivo models of oxidative stress, the current study was designed to determine the neuroprotective potential of naringenin, alone or in combination with lipoic acid. In our mixed neuronal culture exposed to hypoxia and subsequent reoxygenation, naringenin was shown to provide significant neuroprotection against cell death at a concentration of 2.5 μmol/L. Lipoic acid (LA) did not produce neuroprotection at any concentration tested (0.25–100 μmol/L). In contrast, when naringenin was covalently combined with LA, producing a novel compound named “VANL-100”, significant neuroprotection was observed at a concentration as low as 2×10⁻² μmol/L (100-fold more potent). An ELISA for antioxidant capacity demonstrated that naringenin and VANL-100 likely resulted in neuroprotection by increasing the free radical scavenging capacity of the neuronal cells. Pretreatment of rats with the above compounds prior to middle cerebral artery occlusion (MCAO) followed by reperfusion, showed similar results. Naringenin significantly reduced infarct volume at a dose of 10 mg/kg while VANL-100 produced significant neuroprotection at a dose as low as 1×10⁻⁴ mg/kg (10 000-fold more potent). This VANL-100-induced neuroprotection persisted even when administered 1 and 3 hours into the reperfusion time course. Taken together, these results suggest that our novel compound, VANL-100 is neuroprotective, likely via a mechanism that involves increasing the antioxidant capacity of neuronal cells. Our results also show that VANL-100 is 100-10 000-fold more potent than the parent compounds, which adds to the growing evidence in support of combination therapy targeting oxidative stress in neurodegenerative diseases.

KEYWORDS
antioxidant, co-drug, ischaemia, reperfusion injury, stroke

1 | INTRODUCTION

Stroke, or cerebral ischaemia, is a leading cause of death and disability,¹ with a pronounced risk being evident in higher income countries such as Canada.² In the United States, stroke is both the leading cause of disability and the fifth leading cause of death.³ In 2012, the American Heart Association estimated that every 4 minutes, a person dies from a stroke.⁴ While statistics suggest the worldwide mortality rate of stroke is decreasing, the long-term deficits in mental and physical performance in stroke survivors are significant and on the rise due to a combination of an aging population in the developed world and the lower mortality rate, ultimately producing additional strain on an already taxed health care system.⁵

There are a number of complex factors that result in injury during or following an ischaemic event. These include, but are not limited to, cerebral oedema and blood vessel leakage, cell death, reperfusion injury, autoimmunity, immune activation, and transcriptional reprogramming of the hypoxic cells.⁶ Reperfusion injury can be spontaneous or as a result of treatment with thrombolytic therapy, such as recombinant tissue plasminogen activator (r-TPA). Although
reperfusion returns oxygen to the hypoxic region, it can also cause injury that may continue to damage the brain cells. During reperfusion, the cells sensitized by a lack of O₂ and glucose perform increased aerobic respiration, which contributes to high levels of reactive oxygen species (ROS) such as superoxide (O₂⁻) and peroxynitrite (ONOO⁻). Several studies have also shown that platelets themselves generate ROS via a number of enzymes, e.g. NAD(P)H oxidase, eNOS, xanthine oxidase (XO), and phospholipase A₂. Caspase-3 activation is also affected by ROS.10 Caspase-3 plays a key role in the sequence of events that leads to reperfusion injury-induced neuronal apoptosis.

There has been significant evidence in support of free radical scavenging as an important mechanism to achieve neuroprotection following ischaemia/reperfusion.11 Compounds that are either antioxidant and/or anti-inflammatory in nature, seem to have potential as free radical scavengers, and have shown promise in several preclinical models of stroke.12 Over the last decade, research into combining antioxidants that may target different pathways in achieving neuroprotection, has increased as a potential therapeutic strategy. Our laboratory has synthesized several compounds by covalently linking two known antioxidants together. In particular, we have found that combining compounds with α-lipoic acid (LA) seems to produce synergistic neuroprotection when compared to the parent compounds, either alone or as a non-bound mixture. These covalently bound compounds, or co-drugs, are in the range of 100-1000 times more potent against neuronal cell death than the individual compounds.13–16 The key advantage to combining antioxidants and producing a novel chemical entity which provides comparable neuroprotection, but at several fold lower dose, is the avoidance of the pro-oxidant effects observed during high dose antioxidant therapy.17 This is particularly true for flavones and polyphenols such as retinoids, carotenoids, tocopherols, and ascorbic acid.17

Naringenin (4′,5,7-trihydroxyflavone), a naturally-occurring flavonoid found in fruit and vegetables, notably citrus varieties such as grapefruit and oranges, appears to afford significant protection. The key advantage to combining antioxidants and producing a novel chemical entity which provides comparable neuroprotection, but at several fold lower dose, is the avoidance of the pro-oxidant effects observed during high dose antioxidant therapy.17 This is particularly true for flavones and polyphenols such as retinoids, carotenoids, tocopherols, and ascorbic acid.17

Naringenin, a naturally-occurring bioflavonoid found in fruit and vegetables, notably citrus varieties such as grapefruit and oranges, appears to afford significant protection against ischaemic cell damage by providing antioxidant protection. Specifically, naringenin has been shown to be neuroprotective in a retinal model of ischaemia-reperfusion injury. In this study, naringenin was shown to reduce the amount of caspase-3 positive cells and thus attenuated apoptotic cell death.19 In another study investigating the effects of naringenin as well as other 5-hydroxy-substituted derivatives on a model of myocardial ischaemia-reperfusion, the data showed naringenin reduced ischaemic injury and restored contractile function, while other derivatives were ineffective.20 With its far-reaching and multifactorial effects, as well as its label as a "naturally occurring" polyphenol, naringenin holds great promise to become a potent player in the treatment strategies for preventing and treating stroke. However, cautious optimism must be maintained as concerns over potential toxic side effects of exogenous antioxidant/anti-inflammatory therapies come to light.

With the growing interest in naringenin as a dietary supplement to combat oxidative stress, our laboratory investigated the neuroprotective potential of naringenin, and compared it to the potential neuroprotection following covalent linkage with LA, to create a novel chemical called "VANL-100". This novel compound will be tested in both in vitro and in vivo models of ischaemia/reperfusion and assessed for the ability to increase endogenous antioxidant capacity of neurons. This study will also examine whether the new entity, VANL-100 maintains antioxidant activity and is more potent in comparison to either of the parent compounds.

2 | RESULTS

2.1 | In vitro oxygen-glucose deprivation (OGD) model validation

In order to demonstrate that our mixed neuronal culture model resulted in oxidative stress following OGD, we used superoxide dismutase (SOD), a known antioxidant, as a positive control. Following 24 hours of OGD and 24 hours of reoxygenation-refeeding (I/r), we observed a significant increase (approximately 2-fold) in cell death, as indicated by an increase in the concentration of lactate dehydrogenase (LDH) released into the media (Ps<.05; Figure 1A). We observed no interference from either vehicle tested in this protocol (ethanol and dimethyl sulfoxide (DMSO); Ps>.05; Figure 1A). Treating the cells with 1 μg/mL SOD during both the period of OGD as well as reoxygenation-refeeding prevented the OGD-induced cell death (Ps<.05; Figure 1A).

2.2 | The effect of naringenin, lipoic acid or VANL-100 on OGD-induced cell death in vitro

The effect of test drugs on cell death (% LDH release) following ischaemia/reoxygenation-refeeding (I/r) was examined using in vitro cell culture. Cells treated with LA were not protected from OGD-induced cell death at any concentration tested in our model (between 0.25 and 100 μmol/L; Ps>0.05 compared to vehicle; Figure 1B). Cells treated with naringenin at a single concentration of 2.5 μmol/L protected cells from I/r-induced cell death (Ps<0.05; n=3/dose; Figure 1C). However, treating cultures with VANL-100 resulted in significant neuroprotection from I/r-induced cell death at concentrations between 0.02 and 2.5 μmol/L (Ps<0.05; n=3/dose; Figure 1D).

2.3 | The effect of naringenin, lipoic acid or VANL-100 on intracellular antioxidant capacity

The effect of drug treatments on the total intracellular antioxidant capacity following I/r was examined using in vitro cell culture. Cells treated with lipoic acid at concentrations between 0.25 and 25 μmol/L had no effect (Ps>0.05 compared to vehicle; n=3/dose; Figure 2), while the higher concentrations of 125 and 1250 μmol/L resulted in a significant rise in intracellular antioxidant capacity (Ps<0.05 compared to vehicle; n=3/concentration; Figure 2). Naringenin demonstrated similar neuroprotective effects at the two highest doses (125 and 1250 μmol/L; Figure 2). However, several concentrations of VANL-100 in the range of 2.5–1250 μmol/L significantly increased...
intracellular antioxidant activity (P ≤ 0.05 compared to vehicle; n=3/concentration; Figure 2).

2.4 | The effect of pre-administration of naringenin versus VANL-100 on transient middle cerebral artery occlusion (tMCAO) in vivo

Pre-administration of naringenin in animals undergoing 30 minutes of vascular occlusion and 5.5 hours of reperfusion (tMCAO) resulted in a dose-dependent neuroprotection, with a dose of 10 mg/kg significantly decreasing infarct volume compared to the administration of vehicle (P ≤ 0.05; n=5/dose; Figure 3A). By comparison, the novel compound VANL-100 also resulted in dose-dependent decreases in infarct volume, but at 10 000-fold lower dose compared to naringenin (P ≤ 0.05; Figure 3B).

2.5 | The effect of delaying VANL-100 administration until after reperfusion

A more clinically relevant scenario for neuroprotective therapy would be to begin treatment at the time of, or after re-establishment of vascular flow. Thus, we next assessed the impact of VANL-100 administration in the tMCAO model that was delayed until either 1 or 3 hours into the reperfusion period. Statistical comparisons were made between the infarct volumes measured following VANL-100 administration at each time point and the infarct volume measured following vehicle (saline) administration 30 minutes prior to MCAO. Administration of VANL-100 (1×10⁻³ mg/kg) at either 1 or 3 hours following the start of vascular reperfusion resulted in approximately 70%-47% reduction respectively in infarct volume compared to vehicle administration (P ≤ 0.05 at each time point; Figure 3B).
2.6 | The effect of pre-administration of naringenin or VANL-100 on permanent middle cerebral artery occlusion (pMCAO)

Administration of naringenin 30 minutes prior to permanent occlusion did not demonstrate a significant effect on infarct volume following 6 hours of MCAO, using a dose shown to be protective in the tMCAO model (10 mg/kg; \( P \geq 0.05 \) compared to vehicle; Figure 4A). Similarly, pre-administration of VANL-100 at a dose shown to be effective in the tMCAO model (1×10\(^{-4}\) mg/kg), did not result in significant neuroprotection following 6 hours of pMCAO (\( P \geq 0.05 \) compared to vehicle; Figure 4A).

2.7 | The effect of naringenin and VANL-100 on intracellular antioxidant capacity following tMCAO

The effect of drug treatments on the total intracellular antioxidant capacity following tMCAO in vivo was examined. Cortical tissue from animals treated with naringenin (0.1 mg/kg) or VANL-100 (1×10\(^{-6}\) mg/kg) had no effect (\( P \geq 0.05 \) compared to vehicle; n=3/dose; Figure 4B), while the higher concentrations of 10 and 1×10\(^{-4}\) mg/kg respectively, resulted in a significant rise in intracellular antioxidant capacity (\( P \leq 0.05 \) compared to vehicle; n=3/dose; Figure 4B).

3 | DISCUSSION

Combinatorial drug therapy attempts to circumvent the deleterious side effects of higher dose treatments by chemically linking two or more biologically active molecules having separate or overlapping mechanisms in such a way that potency is optimized and toxicity is avoided. In the current study, the novel compound VANL-100 is comprised of naringenin and \( \alpha \)-lipoic acid chemically linked via an ester bond. This novel entity was shown to be neuroprotective in both in vivo and in vitro models of oxidative stress. Specifically, doses as low as 1×10\(^{-4}\) mg/kg given immediately prior to a 30 minute period...
of cerebral ischaemia followed by 5.5 hours of reperfusion in an anaesthetized rat, reduced infarct size compared to vehicle. Similar experiments conducted with naringenin alone, required a 10 000-fold higher dose (10 mg/kg) to achieve neuroprotection following ischaemia/reperfusion injury. As neuronal death associated with ischaemia/reperfusion injury is believed to be primarily the result of oxidative stress-induced apoptosis, we investigated the effect of both naringenin and VANL-100 on the antioxidant status of neuronal cells using an in vitro approach. Through a simulation of ischaemia/reperfusion using OGD followed by reoxygenation/refeeding, primary neuronal cells in culture are observed to display high levels of oxidative stress and apoptotic markers. Treating cells with α-lipoic acid or naringenin over a range of doses between 0.25 and 100 μmol/L, showed little to no neuroprotection. Naringenin was neuroprotective at a single dose (2.5 μmol/L) and ineffective at other doses tested. In contrast, VANL-100 displayed potent neuroprotective effects at several doses tested between 25 nmol/L and 25 μmol/L, demonstrating a significant shift in the therapeutic efficacy of this novel compound relative to the parent compounds. This effect of enhanced potency with the VANL-100 compound was also evident in the in vivo study as mentioned above with the additional finding that delayed treatment with VANL-100 could also produce significant neuroprotection in our model of transient ischaemia/reperfusion. Administration of VANL-100 at 1×10⁻³ mg/kg at either 1 or 3 hours post-MCAO significantly reduced infarct size following 5.5 hours of reperfusion. This is interesting since our previous work with LA demonstrated that neuroprotection was only observed when LA was administered prior to MCAO. Although our previous work with LA showed a similar mechanism of action in terms of increasing intracellular antioxidant capacity, specifically SOD2, it was not effective when administered post-MCAO. This implies that VANL-100 is likely to be involved in direct scavenging as well as increasing additional antioxidant systems.

Our in vitro findings further demonstrate a significant effect of VANL-100 on antioxidant capacity of neuronal cells in culture when exposed to oxidative stress. Specifically, endogenous antioxidant systems as measured by a commercially available assay, were shown to be significantly increased by micromolar doses (2.5-1250 μmol/L) of VANL-100 during a protocol of OGD/reoxygenation-refeeding. Antioxidant capacity was increased in cells treated with either of the parent compounds as well, although requiring higher doses (125 and 1250 μmol/L). As described earlier, higher doses are not without risk of pro-oxidant effects, which ultimately cause more harm than benefit.

A potent antioxidant, naringenin, suppresses oxidative stress due to reperfusion injury by quenching free radical production. Furthermore, naringenin has been shown to inhibit glutamate excitotoxicity, a fundamental component underlying the spreading neuronal damage observed during stroke, either through a direct effect at the receptor or indirectly by inhibiting the caspase-calpain pathway. In addition, its anti-inflammatory effects have been demonstrated to

![FIGURE 4](A) Representative images of vehicle (Veh; DMSO), naringenin (Nar; 10 mg/kg) and VANL-100-treated animals (1×10⁻⁴ mg/kg) following permanent occlusion of the middle cerebral artery (pMCAO). (B) Bar graph summarizing the lack of effect on infarct volume in animals treated with naringenin (mg/kg) or VANL-100 (mg/kg) following pMCAO. (C) Effect on total antioxidant capacity from cortical tissue homogenates taken from animals pretreated with naringenin or VANL-100 and following tMCAO. Asterisks (*) indicate significance from the vehicle (veh) as analyzed by one-way ANOVA (P ≤ .05)
prevent blood-brain barrier disruption and the cerebral oedema commonly associated with stroke. In this study by Bai and colleagues, they used doses of 50 and 100 mg/kg of naringenin administered orally prior to MCAO to observe a significant neuroprotective effect. Similarly, in a study by Raza and colleagues, neuroprotection with naringenin was only observed at 50 mg/kg administered daily for 21 days prior to ischaemia-reperfusion. In the present study, we observed neuroprotection in our tMCAO model at a dose of 10 mg/kg of naringenin administered 30 minutes prior to ischaemia. In comparison, VANL-100 was significantly more potent as a neuroprotectant compared to naringenin in our model, or compared to the studies following MCAO described above.

The enhanced antioxidant capacity of neurons in vitro and in vivo observed following I/R in animals pretreated with VANL-100 may provide insight into a potential mechanism by which this novel compound provides neuroprotection. This potent antioxidant capacity may be a reason why VANL-100 and naringenin were only effective in reperfusion injury versus permanent stroke model as observed in the current study. The extent of free radical production and apoptotic cell death seems to be a hallmark of reperfusion injury, versus the extent of necrotic cell death that contributes to the spreading depression and growth of the infarct following permanent occlusion. However, apoptotic cell death does occur in permanent occlusion, suggesting the antioxidant potential of VANL-100 may be the primary mechanism of neuroprotection.

The delicate balance between oxidative and antioxidative processes is believed to play a major role in maintaining human health. Our endogenous antioxidative system includes enzymes such as catalase, superoxide dismutase and glutathione peroxidise. Exogenous antioxidants which traditionally have been primarily dietary in nature, interact with endogenous antioxidants to maintain redox homeostasis. The current trend of sourcing exogenous antioxidants through dietary supplementation of highly purified and concentrated extracts is cause for concern as it could potentially shift the balance between antioxidant and pro-oxidant effects. Case in point, high dose EGCG supplementation, a key component in green tea, was shown to cause fatal hepatotoxicity in mice, while beta-carotene supplementation was demonstrated to increase cancer risk in smokers. With regard to naringenin, early studies have identified a pro-oxidant effect causing DNA strand breakage in the absence of transition metals, likely through a direct production of superoxide ion as well as an impairment of the endogenous glutathione antioxidant defense pathway. Indeed, high doses of naringenin (50 and 100 mg/kg) administered orally for 4 days prior to surgical permanent MCAO in rats, was shown to reduce infarct size and brain oedema. As the antioxidant properties are now being carefully delineated, the potential toxic side effects of chronic, high dose naringenin supplementation remains uncertain.

In summary, we present a novel chemical entity comprised of two “naturally-occurring” dietary compounds which individually have been shown to provide health benefits at physiologic doses. As a means of mitigating the potential toxicity associated with higher doses commonly employed to achieve therapeutic effects in many pathological animal models, our novel compound displays significant neuroprotection both in vivo and in vitro through a mechanism which includes in part, an enhancement of endogenous cellular antioxidant status. Future studies must necessarily examine additional mechanisms of action attributable to the primary effects observed herein given the multifactorial nature of the parent compounds. As well, secondary effects arising through as of yet unknown pathway(s), as well as via production of clearance metabolites will necessarily be essential in fully characterizing the potential application of this compound both therapeutically in clinic, as well as prophylactically as a dietary supplement.

4 MATERIALS AND METHODS

4.1 Ethics statement

All in vivo and in vitro experiments were carried out at the University of Prince Edward Island and approved by the Animal Care and Biosafety Committees, which adheres to the guidelines of the Canadian Council on Animal Care (protocol #14-041 and 15-028).

4.2 In vitro cell cultures

Mixed neocortical cultures, containing both neurons and glia, were prepared from fetal rats at 17-18 days gestation. Briefly, embryonic tissue was extracted from untimed pregnant Sprague-Dawley rats by caesarean section and transferred to ice cold Hanks Balanced Salt Solution (Gibco, Thermo Fisher Scientific, Waltham, MA, USA). Cortical brain tissue was carefully isolated by fine dissection using aseptic techniques. Cortices were minced in cold HBSS using a sterile razor blade prior to digestion with 0.012 5% trypsin. Dissociated cells were plated in poly-L-lysine (1 mg/mL; Sigma-Aldrich, St Louis, MO, USA) coated 96 well plates at a seeding density of 50 000 cells/ well in warm (37°C) Dulbecco’s Modified Eagle Medium (Gibco) containing 10% iron-supplemented bovine calf serum (Hyclone) and 1% antibiotic/antimycotic (Gibco). Cultures were allowed to adhere overnight in a humidified 37°C incubator having 5% CO2 and 1% O2 and the balance N2 for a period of 24 hours. Following OGD, an equal volume of normal supplemented Neurobasal A containing identical drug concentrations was added and cultures were transferred to normoxic conditions...
(5% CO₂ and atmospheric oxygen) for an additional 24 hours. Cell viability was assessed by measuring LDH release from damaged cells.

4.4 | Cytotoxicity assay

At the end of each experiment, cell injury was assessed by measuring lactate dehydrogenase (LDH) released into the culture medium from damaged cells (Cytoscan LDH Assay, G-Biosciences, St Louis, MO, USA). LDH measurements represent the average of four replicate treatments/dose over three separate experiments. LDH is expressed as the percent (%) of total LDH. Superoxide dismutase (SOD 1 μg/mL; Sigma-Aldrich) was used as a positive antioxidant control.

4.5 | Total antioxidant capacity assay

Separate groups of plates were exposed to OGD and reoxygenation-refeeding (l/r) as described above and subsequently processed for the measurement of total antioxidant capacity using a commercially available assay (Antioxidant Assay Kit, Sigma-Aldrich). Samples were run in triplicate in four separate experiments. At the end of the l/r protocol, the media was carefully removed from each well and replaced with 100 μL of 1× Assay Buffer provided in the kit. Plates were covered and wrapped in parafilm prior to being stored at −80°C overnight. Cells were then thawed at room temperature with gentle shaking on an orbital shaker for 30 minutes to aid in lysing. A 10 μL sample from each well was assayed for antioxidant capacity following the protocol provided in the kit. Results are expressed as mm Trolox equivalent.

4.6 | General surgical procedures for in vivo stroke surgery

All experiments were conducted on male Sprague-Dawley rats (300-350 g; Charles River, Montreal, PQ, CAN). For all animals, food and tap water were available ad libitum. Rats were anaesthetized with sodium thiobutabarbital (Inactin; 100 mg/kg i.p.; Sigma-Aldrich) and supplemented as needed. For intravenous drug administration, a polyethylene catheter (PE-10; Clay Adams, Parsippany, NJ, USA) was inserted into the right femoral vein. An endotracheal tube was inserted to facilitate breathing. Body temperature was monitored and maintained at 37±1°C using a feedback system (Physitemp Instruments; Clifton, NJ, USA).

4.7 | Transient and permanent middle cerebral artery occlusions (tMCAO and pMCAO)

We have previously published the detailed methodology for middle cerebral artery occlusion. Briefly, animals were placed in a David Kopf stereotaxic frame (Tujunga, CA, USA) and the right middle cerebral artery (MCA) approached through a rostra-caudal incision of the skin and frontalis muscle at the approximate level of bregma. Blood flow through the MCA was impeded by the placement of surgical suture under the MCA at three designated positions along the exposed vessel for 30 minutes (tMCAO model) or 6 hours (pMCAO model). The sutures were positioned so that the middle of each suture applied pressure to the underside of the MCA thereby impeding blood flow (ischaemia) which was confirmed using laser-Doppler based blood flow monitoring (OxyFlo Pro, Oxford-Optronix, Oxford, UK). This three-point placement of surgical sutures produced a highly reproducible ischaemic lesion localized to the prefrontal cerebral cortex. In the transient MCAO model (tMCAO), blood flow was returned to the area (reperfusion) for an additional 5.5 hours following the removal of the sutures.

In a separate set of experiments, the pre-administration of naringenin (0.1 or 10 mg/kg; n=3/dose), VANL-100 (1×10⁻⁶ or 1×10⁻⁵ mg/kg; n=3/dose) or vehicle (n=3; 35% DMSO) were made 30 minutes prior to MCAO. After 30 minutes of MCAO, the sutures were removed (reperfusion) for an additional 5.5 hours (tMCAO). Animals were then overdosed on Inactin, perfused transcardially with 200 mL of 0.1 mol/L phosphate buffered saline (pH 7.4), the brains removed and the ipsilateral cerebral cortex isolated by careful dissection. A biopsy needle (8 mm internal diameter) was used to collect a tissue sample from the region of the infarct.

The tissue was weighed and homogenized (20% w/v) in ice cold PBS. The homogenate was centrifuged 12 000×g for 15 minutes at 4°C. Aliquots of the supernatant were stored at −80°C until assayed for protein. Total antioxidant capacity was then quantified using the same ELISA kit (Antioxidant Assay Kit, Sigma-Aldrich) and procedures described above in Section 4.5.

4.8 | VANL-100 synthesis

Naringenin and α-lipoic acid were chemically linked via an ester bond using a simple synthetic route where the product was named VANL-100. To a solution of α-lipoic acid (2.0 mmol) and naringenin (2.0 mmol) in tetrahydrofuran (THF, 15 mL), dimethylaminopyridine (DMAP, 100. To a solution of 2.0 mmol) and dicyclohexylcarbodiimide (DCC, 2.0 mmol) were added. After stirring at room temperature for 24 hours, precipitated urea was filtered off and the filtrate was evaporated in vacuo. The residue was placed in dichloromethane and the solution was washed with 10% citric acid, water and brine, and dried over anhydrous sodium sulfate. The solvent was removed by evaporation and the crude product was purified by silica column chromatography (elucent, ethyl acetate:hexane (1:2)) to yield a pale yellow solid. Yield: 52%; mp 107-108°C; 1HNMR (400 MHz, CDCl₃) δ7.46 (d, 2H, 8.4 Hz), 7.15 (d, 2H, 8.4 Hz), 6.60 (s, 1H), 6.01-5.98 (m, 2H), 5.42 (dd, 1H, 12.9 Hz, 3.0 Hz), 3.61 (m, 1H), 3.15 (m, 2H), 3.03 (dd, 1H, 14.0 Hz, 12.0 Hz), 2.81 (dd, 1H, 14.0 Hz, 4.0 Hz), 2.60 (t, 2H, 7.50 Hz), 2.48-2.42 (m, 2H), 1.65-1.47(m, 4H), 1.47-1.50 (m, 2H); MS (EI): m/z 461.17 (M⁺+1); Anal. Calcd. For C₂₃H₂₆O₄S₂·C: C, 59.98; H, 5.25. Found: C, 59.81; H, 5.10.

4.9 | Drug injection protocol in tMCAO model

The sutures were left in place for 30 minutes (duration of ischaemia; MCAO), and then removed to allow reperfusion which lasted for 5.5 hours. Naringenin, VANL-100 or vehicle were administered (1 mL/kg; i.v.) immediately prior to the onset of reperfusion (time 0).
4.10 | Effect of naringenin on ischaemia-reperfusion (tMCAO model)

In the first experiment, naringenin (10, 1.0, 0.1 mg/kg) or vehicle (35% DMSO) was administered in a volume of 1 mL/kg (i.v.; n=5/group) immediately prior to the removal of the sutures and the onset of reperfusion (t=0).

4.11 | Effect of VANL-100 on ischaemia-reperfusion (tMCAO model)

In the second experiment, VANL-100 (1×10^{-3}, 1×10^{-4}, 1×10^{-5}, 1×10^{-6} mg/kg; n=6/group) or vehicle (0.5% DMSO; 1 mL/kg; i.v.; n=7) was administered in a volume of 1 mL/kg (i.v.) immediately prior to the removal of the sutures and the onset of reperfusion (t=0).

4.12 | Effect of VANL-100 on infarct volume when administered during reperfusion

To examine neuroprotection of VANL-100 when administered during the 5.5 hours of reperfusion, injections of an optimal dose of VANL-100 (1×10^{-3} mg/kg; 1 mL/kg; i.v.; n=6 per group) were made 1 or 3 hours following suture removal (start of reperfusion). In all cases, the experiments were terminated following 5.5 hours of reperfusion. The comparison group for statistical analysis was VANL-100 vehicle at time 0 (0.5% DMSO; n=7).

4.13 | Drug injection protocol in pMCAO model

The above compounds were also tested in a permanent model of middle cerebral artery occlusion. The sutures were left in place for the entire 6 hours (no reperfusion). Naringenin, VANL-100 or vehicle was administered 30 minutes prior to the beginning of the occlusion (1 mL/kg; i.v.). The doses of naringenin or VANL-100 that were effective in the tMCAO model were used to determine efficacy in the pMCAO model.

4.14 | Histological procedures

At the end of each experiment where infarct volume was measured, animals were perfused transcardially with 200 mL of 0.1 mol/L phosphate buffered saline. The brains were removed and sliced into 1 mm coronal sections using a rat brain matrix (Harvard Apparatus; Holliston, MA, USA). Sections were incubated in a 2% solution of 2,3,5-triphenyl tetrazolium chloride (TTC; Sigma-Aldrich) for 5 minutes. Infarct volumes were calculated with the use of scanned digital images of each brain section. The infarct area for opposing views of each brain section was calculated using computer software (Scion Corporation; Frederick, MD, USA), averaged and multiplied by section thickness (1 mm) to determine infarct volume for each section. The individual infarct volumes were added together to provide the infarct volume for each rat. Digital images were coded prior to being sent for analysis of infarct volume to allow the analyst to be blinded to treatment groups.

4.15 | Statistical analysis

Data were analyzed using a statistical software package (SigmaStat and SigmaPlot; Jandel Scientific, Tujunga, CA, USA). All data are presented as a mean±standard error of the mean (SEM). Differences were considered statistically significant if P<0.05 by an analysis of variance (ANOVA) followed by a Bonferroni post hoc analysis. When only two groups were being compared the Student’s t test was used.

DISCLOSURE

The authors declare that they have no conflict to disclose regarding the publication of this paper.

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