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A novel neuroprotective agent with antioxidant and nitric oxide synthase inhibitory action

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Abstract

N^{α}-vanillyl-N^{ω}-nitroarginine (**N − 1**) that combines the active functions of natural antioxidant and nitric oxide synthase inhibitor was developed for its neuroprotective properties. **N − 1** exhibited protective effects against hydrogen peroxide-induced cell damage and the inhibitory effect on nitric oxide 'NO' production induced by calcium ionophore in NG 108-15 cells. **N − 1** inhibited the constitutive NOS isolated from rat cerebellar in a greater extent than constitutive NOS from human endothelial cells. Low binding energy (− 10.2 kcal/mol) obtained from docking **N − 1** to nNOS supported the additional mode of action of **N − 1** as an nNOS inhibitor. The *in vivo* neuroprotective effect on kainic acid-induced nitric oxide production and neuronal cell death in rat brain was investigated via microdialysis. Rats were injected intra-peritonially with **N − 1** at 75 μmol/kg before kainic acid injection (10 mg/kg). The significant suppression effect on kainic acid-induced NO and significant increase in surviving cells were observed in the hippocampus at 40 min after the induction.

Keywords: *Nitric oxide synthase, neuroprotection, cell culture, NG108-15, microdialysis, nitro arginine*

Introduction

Overproduction of nitric oxide 'NO' by neuronal nitric oxide synthase (nNOS) has been implicated in a number of clinical disorders including acute (stroke) and chronic neurodegenerative diseases such as schizophrenia, Alzheimer's, Parkinson's and dementia, convulsion and pain. In the central nervous system, excess $^{\cdot}$NO by induction of iNOS and nNOS caused neuronal dysfunction and subsequent neuronal death. However, acute toxicity, such as that observed after a stroke or trauma, seems to require the production of superoxide anion concomitantly with $^{\cdot}$NO. In the presence of superoxide anion, $^{\cdot}$NO becomes a potent neurotoxin because of the formation of the potent oxidant peroxynitrite ($ONOO^{-}$). $ONOO^{-}$ is a lipid-permeable molecule with a wider range of chemical targets than $^{\cdot}$NO, it can oxidize proteins, lipids, RNA and DNA. Because $^{\cdot}$NO and reactive oxygen species (ROS) act independently, as well as cooperatively, to induce neuronal death in neurological disorders, inhibition of nNOS and scavenging of radicals have been proposed as neuroprotective strategies. The combination of the two actions was found to be synergistic in reducing neuronal damage [1–4] leading to better neuroprotective efficacy.

Hence, N^{ω}-nitro-L-arginine, the potent inhibitor of NOS, was selected to combine with the hydroxyl

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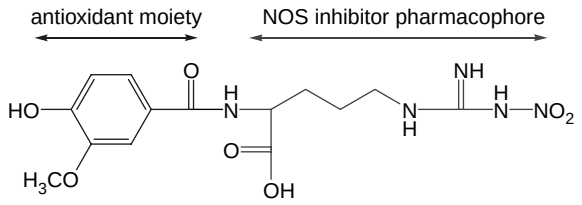

Figure 1. The structure of N^{α}-vanillyl-N^{ω}-nitroarginine (**N − 1**).

containing acid, vanillic acid, to obtain a new neuroprotective agent with dual action, N^{α}-vanillyl-N^{ω}-nitroarginine (**N − 1**). The structure of **N − 1** is composed of natural antioxidant, vanillic acid linking to the NOS inhibitor pharmacophore (Figure 1). The antioxidant moiety in vanillic acid is the hydroxyl group, while the pharmacophore for NOS inhibitory action is nitro-arginine. From a QSAR study reported by Vajragupta et al. [5], the hydroxyl group was found to be the active function that reacted with the radical directly. The increase and stabilization of the negative charge on hydroxyl function by connecting to the system of conjugated double bond or aromatic leading to the increase in the scavenging reactivity. Based on the finding, in addition to several reports on the radical scavenging activity of vanillin *in vitro* [6,7], vanillyl moiety was chosen as the function for scavenging radicals in **N − 1**. Nitroarginine was incorporated in the molecule of **N − 1** to bind and inhibit NOS. Arginine, with the nitro group, binds to the NOS at the active binding site. However, it does so in a different orientation and mode of binding due to the steric nitro group so that NOS is not able to catalyst the production of ˙NO. To possess a dual action of NOS inhibitor and radial scavenger, and, to increase brain accessibility, the structures between the NOS inhibitor and radical scavenger were combined. **N − 1** was investigated both *in vitro* and *in vivo* for its neuroprotective properties. For *in vitro* radical scavenging ability, **N − 1** had a protective effect against hydrogen peroxide-induced cell damage and an inhibitory effect on ˙NO production in NG108-15 cells. Constitutive NOS from rat cerebellar and constitutive NOS from human endothelial cells in the presence of **N − 1** were assayed for activity to demonstrate the NOS inhibitory action. *In vivo*, the neuroprotective effect on the levels of 'NO' from brain microdialysate of kainic acid-induced ˙NO production was evaluated in rats. The effect of '**N − 1**' on hippocampal neuronal cell damage induced by KA was also histologically elucidated. The interaction between **N − 1** and nNOS was studied using molecular modeling techniques to determine the binding energy and binding mode in order to support the nNOS inhibitory action. In addition, to prove that **N − 1** is responsible for scavenging 'NO' *in vivo*, the accessibility of **N − 1** to the brain of mice was evaluated by HPLC method.

Materials and methods

Chemicals

Unless stated otherwise, all reagents were purchased from Sigma-Aldrich Chemical Company (Saint Louis, MO, USA). Wang resin (100–200 mesh), Fmoc-nitro-L-arginine, N-hydroxy benzotriazole were purchased from Calbiochem (San Diego, CA, USA). Kainic acid was from Tocris, (Ellis, MO, USA). Nitro-L-arginine, sulfanilamide, N-naphthylethylenediamine, sodium nitrite and sodium nitrate were purchased from Wako Pure Chemicals Industries (Osaka, Japan).

Synthesis

N^{α}-vanillyl-N^{ω}-nitroarginine (**N − 1**) was prepared by solid phase synthesis method which involved the coupling of an amino acid to a Wang resin. Diisopropylcarbodiimide (DIC) was used as a coupling agent. In the first step of synthesis, Fmoc-amino acid was attached to the polystyrene based Wang resin via a DIC/HOBt/DMAP coupling reaction. In the second step, Fmoc protecting group was deprotected by treatment with 20% piperidine in DMF for 30 min. In the next step, unprotected amine of amino acid was coupled with the carboxylic acid to form amide bond via a DIC/HOBt coupling reaction. For cleavage of the completed product from resin, the resin-bound product was treated with 25% trifluoroacetic acid (TFA) in DCM at room temperature for 30 min. The structures of compounds were identified by their melting points, ^{1}H NMR, FAB MS (M − 1), LC MS (M + 1) and elemental analyses of C, H, N.

Attachment of carboxylic group of Fmoc-nitro-L-arginine to Wang resin (resin A). Wang resin (0.2273 g, 0.25 mmol) was placed in a dry flask, and suspended in 9:1 (v/v) dichloromethane (DCM)/dimethylformamide (DMF) (2–3 ml) for 30 min. In a separate flask, Fmoc-nitro-L-arginine (0.2207 g, 0.5 mmol, 2 equivalents relative to resin loading) was dissolved in minimum amount of DMF. Then, N-hydroxybenzotriazole (HOBt 0.0676 g, 0.5 mmol) was added. This solution was added to the resin. In a separate flask, 4-(dimethylamino)pyridine (DMAP 0.0031 g, 0.025 mmol, 0.1 equivalents relative to the resin loading) was dissolved in a minimum amount of DMF. 1,3-Diisopropylcarbodiimide (DIC 80 µl, 0.5 mmol) was added drop wise to the resin mixture then the DMAP solution was added. The flasks were equipped with drying tubes. The reaction mixture was agitated with a mechanical shaker for 24 h at room temperature. The resin (resin A) was filtered using a fine porosity sintered glass funnel and washed 3 times with DMF, then 3 times with DCM and finally 3 times

with methanol. Enough solvent (1–2 ml) to slurry the resin was used in each washing.

Removal of Fmoc protecting group (resin B). N-terminal Fmoc group in the resin A was removed by adding 20% piperidine in DMF (3–5 ml) to resin A. The reaction mixture was shaken for 30 min at room temperature. The resin was transferred to a sintered glass funnel with fine porosity and washed 3 times with DMF and finally 3 times with DCM.

*N^{α}-vanillyl-N^{ω}-nitroarginine (**N − 1**)*. Resin B was suspended in 9:1 (v/v) DCM/DMF (2–3 ml) for 30 min. In a separate flask, vanillic acid (0.0847 g, 0.5 mmol) was dissolved in minimum amount of DMF (1 ml). Then, HOBt (0.0676 g, 0.5 mmol) was added. This solution was added to the resin B. DIC (80 μl, 0.5 mmol) was added drop wise to the resin mixture. The flask was equipped with a drying tube. The reaction mixture was shaken with a mechanical shaker for 12 h at room temperature. The resin was filtered in a fine porosity sintered glass funnel and washed 3 times with DMF (3 × 2 ml), then 3 times with DCM (3 × 2 ml), and finally 3 times with methanol (3 × 2 ml). The resin was slurred in a 1:3 mixture of TFA/DCM (3 ml) for a minimum of 30 min to cleave **N − 1** from resin. The resin was filtered in a fine sintered glass funnel and washed with a small portion of the cleavage cocktail (1:3 TFA/DCM). The filtrate was added with 8–10 times the volume of cold ether to precipitate the product. The precipitate was washed several times with cold ether to remove TFA. The obtained precipitate was 0.0436 g (47.23% yields) of **N − 1** as a white powder, m.p. 187–188°C. 1H NMR(CD_3OD): δ 2.25–1.30 (m, 8H), 3.91 (s, 3H), 4.60 (m, 1H), 6.85 (d, 1H), 7.41–7.39 (dd, 1H), 7.46 (d, 1H). FAB MS (M − 1): 368.13. LC MS (M + 1): 370. TLC: stationary phase-silicagel GF_{254}, mobile phase: *n*-butanol/glacial acetic acid/water (3:1:1): R_f of **N − 1** = 0.59. *Anal. Calcd*. for $C_{14}H_{19}N_5O_7$ (M.W. 369.3): C, 45.53; H, 5.19 ; N, 18.69. Found: C, 44.98; H, 4.81; N, 18.32.

Effect on H_2O_2-induced oxidative cell damage in NG108–15 cells [8]

NG108–15 cells were cultured in Dulbecco's Modified Eagle Medium (pH 7.5) containing 100 μM hypoxanthine, 16 μM thymidine, 1 μM aminopterin, 4% fetal bovine serum and minocycline (1 μg/ml) at 37°C, 5% CO_2 and 95% relative humidity. The medium was changed every 2–3 days. The cells were plated on 96-well plate and used for testing after 4–5 days incubation. In the assay, the cell culture was treated with **N − 1** for 2 hours and washed with phosphate buffer saline (PBS) to remove the unabsorbed test compounds, next, oxidative stress was induced by H_2O_2. After 2 hours incubation, cell viability was determined by staining the cells with 0.5 mg/ml of MTT. MTT is a water soluble tetrazolium salt that is reduced by metabolic viable cells to blue colored, water-insoluble formazan. It is solubilized with DMSO and becomes formazan dye that will be measured by a well plate reader at 560 nm.

Nitric oxide scavenging activity in NG108–15 cells [9–10]

NG108–15 neuroblastoma- glioma hybrid cells were cultured as described before. The cultured cells were treated with 0 to 20 μM of calcium ionophore (A23187) to determine the concentration to induce significant increase in ·NO level. The cells were plated on 6-well plate and incubated until confluent. After that, cell medium was excluded. The cells were washed with phosphate buffer and then stimulated by treatment with A23187 in the presence or absence of **N − 1** in DMEM without phenol red. Cell media was harvested 24 hours later and assayed for nitrite (NO_2^-) production by spectrophotometric assay based on the Griess reaction. To determine the amounts of nitrite, the supernatant collected from the NG108–15 cells culture was mixed with Griess reagent in equal portion. The reaction products were colorimetrically quantitated at 540 nm. The concentration of NO_2^- was determined on the basis of the standard regression line for sodium nitrite.

Effects on the activity of constitutive NOS assay

Effects constitutive nNOS. The activity of the enzyme was assayed by measuring the formation of citrulline from arginine using an enzyme constitutively expressed in the rat cerebellum. The enzyme was isolated according to the method reported by Bredt and Snyder. [11] **N − 1** was added to a buffer containing 40 mM Tris-HCl (pH 7.8), 0.5 mM NADPH, 4 μM FAD, 12 μM BH_4, 3 mM DTT, 10 nM calmodulin, 2 mM $CaCl_2$, 2 mM EGTA, 400 nM L-arginine and 28 nM [^{3}H]L-arginine. Thereafter, the reaction is initiated by addition of the enzyme (20 μg) and the mixture is incubated for 30 min at 37°C. For control basal measurements, the enzyme is omitted from the reaction mixture. Following incubation, the reaction is stopped by adding an ice-cold buffer containing 20 mM Tris-HCl (pH 5.0), 2 mM EDTA and 2 mM EGTA. [^{3}H] citruline is separated from [^{3}H] arginine using an ion exchanger Dowex column. Collected [^{3}H] citruline is quantified in a scintillation counter (LS series, Beckman) using a scintillation cocktail (Formula 989, Packard). The standard inhibitory reference compound, L-NMMA, was tested concurrently.

The results are expressed as the percent inhibition of the control enzyme activity.

Effects on eNOS. The activity of the enzyme was assayed by measuring the formation of citrulline from arginine using an enzyme constitutively expressed in the human umbilical vein endothelial cells (HUVEC). The enzyme was isolated according to the method reported by Pollock et al. [12] The test compound, reference compound, or water (control) are added to a buffer containing 40 mM Tris-HCl (pH 7.8), 0.5 mM NADPH, 4 μM FAD, 12 μM BH_4, 3 mM DTT, 10 nM calmodulin, 2 mM $CaCl_2$ and 28 nM [^{3}H]L-arginine and 50 nM arginine. Thereafter, the reaction is initiated by addition of the enzyme (5 μg) and the mixture is incubated for 30 min at 37°C. For control basal measurements, the enzyme is omitted from the reaction mixture. Following incubation, the reaction is stopped by adding an ice-cold buffer containing 20 mM Tris-HCl (pH 5.0), 2 mM EDTA and 2 mM EGTA. [^{3}H]citruline is separated from [^{3}H]arginine using an ion exchanger Dowex column. Collected [^{3}H]citruline is quantified in a scintillation counter (LS series, Beckman) using a scintillation cocktail (Formula 989, Packard). The results are expressed as the percent of inhibition of the control enzyme activity.

Brain accessibility of N − 1

Swiss albino mice weighing 25 − 35 g were kept in a temperature-controlled room on a 12/12-h cycle. The mice were divided into 4 groups of 5 animals in each. One group served as a control group receiving vehicle (15%Tween 80) only. The other 3 groups were treated with nitro-arginine, vanillic acid and **N − 1**. After subcutaneous injection of test compounds (100 mg/kg) or vehicle for 4 hours, the mice were killed and the brains were removed for the assay of penetrated test compounds.

Chromatographic system. The essential parts of HPLC consisted of column oven, UV detector set at 260 nm and a 150 × 4.6 mm i.d. column. The stationary phase of column was Hypersil C18 (5-μm particle size). The mobile phase system for the determination of **N − 1** and vanillic acid was acetonitrile: water (15:85, v/v) (adjusted to pH 3.0 using phosphoric acid), and the mobile phase system for the determination of nitro-arginine was acetonitrile: 1% formic acid in water (60:40, v/v). The mobile phase was delivered at a flow rate of 0.8 ml/min.

Sample preparation. Four hours after injection of the test compounds, the mice's brains were removed. The brain was homogenized in methanol (10 ml) and then centrifuged at 3500 rpm for 15 min. The methanol extract was filtered by syringe with nylon filter unit (0.45 μm pore size and 13 mm i.d.). The collected methanol extracts were dried by evaporation. The residue was dissolved in 5 ml methanol and the sample preparation was then injected into the chromatographic system.

Effect on kainic acid-induced nitric oxide overproduction [13–14]

Animal preparation. The animals used in this study were standard male Wistar rats (10–12 weeks old; SLC, Japan) weighing 280–330 g. All animals were housed in a temperature controlled room (25°C) and humidity on a 12 h light-dark cycle (light on between 07:30–19:30 h) and had free access to food and water. All experimental procedures were performed in accordance with the standards established by the Guide for the care and use of Laboratory Animals of Toyama Medical and Pharmaceutical University. After handling for one week, animals underwent implantation of a dialysis probe. The rats were anesthesized with pentobarbital (50 mg/kg, i.p.) and placed in a stereotaxic apparatus. The skull was exposed; a burr hole was made with a dental drill. Microdialysis tubes were inserted transversally into the hippocampus, following the procedure described by Giovannini et al. [15]. The microdialysis tube was covered with super-epoxy glue along its entire length except for the regions corresponding to the hippocampus (two sections of 3 mm separated by a glued central zone 2 mm long). The stereotaxic coordinates used for the transversal implantation of the microdialysis tubing were following Paxinos and Watson [16]: AP-3.3 mm and horizontal -3.3 mm. All coordinates were refereed to bregma, with bregma and lambda on the horizontal plane. After the operation, each animal was kept in a separate cage and was allowed at least 48 hours for recovery time.

In vivo microdialysis. The dialysis fiber was connected via polyethylene tubing to a microsyringe driven by a microinjection pump (CMA/100). Artificial cerebrospinal fluid (118 mM NaCl, 3.4 mM KCl, 1.26 mM $CaCl_2$, 1.15 mM $MgCl_2$, 20 mM $NaHCO_3$, 1.2 mM Na_2HPO_4 and 10 mM glucose) was driven through the system at a flow rate of 2 μl/min. Perfusates were collected from the outlet of the probe via polyethylene tubing. Three hours after beginning of dialysis, perfusate collection was started and carried out every 20 min (in fractions of 40 μl). The first three perfusates were collected to determine their baseline release. In the saline and kainic acid group, one hour after the start of sample collections, rats were injected intraperitoneal with vehicle. Forty

minutes after vehicle injection, saline or 10 mg/kg kainic acid (Tocris, USA) was injected and then the perfusates were collected for 180 min. In the drug treatment group, 75 μmole/kg of **N − 1** was injected i.p. 1 hour after the start of sample collection. Forty min after the pre-treatment, 10 mg/kg of kainic acid was injected i.p. and perfusates were collected for 180 min. Each dialysate fraction served for determination of NO_2^- and NO_3^- as follows. At the end of the perfusion period, each animal was decapitated, its brain removed, and the accuracy of the probe placements in the hippocampus were verified histologically.

Measurement of nitrite and nitrate. The method for measuring NO_2^- and NO_3^- levels was as followes; 10 μl of dialysate fraction obtained by in vivo microdialysis was injected into NO-detector-HPLC system (ENO-20, Eicom). NO_2^- and NO_3^- in the dialysate were separated by a reverse-phase separation column packed with polystyrene polymer (NO-PAK, 4.6 × 50 mm, Eicom) and 'NO'$_3$- was reduced to 'NO'$_2$- in a reduction column packed with copper-plated cadmium filing (NO-RED, Eicom). NO_2^- was reacted with Griess reagent to form a purple azo dye in a reaction coil. The separation and reduction column and the reduction coil were placed in a column oven that was set at 35°C. The absorbance of the color of the product dye at 540 nm was measured by a flow-through spectrophotometer. The mobile phase which was delivered by a pump at a rate of 0.33 ml/min, were purchased from Eicom, Japan. The Griess reagent, which was 1.25% HCl containing 5 g/L sulfanilamide with 0.25 g/L N-naphthylethylenediamine, was delivered at a rate of 0.1 ml/min. the contamination of NO_2^- and NO_3^- in artificial cerebrospinal fluid and the reliability of the reduction column were examined in each experiment. Sodium nitrite and sodium nitrate were used as standards. NO_{2-} and NO_{3-} were eluted with a retention time of 4.6 and 7.05 min, respectively. The correlation between six nitrite and nitrate concentrations ranged from 10^{-12} to 10^{-10} mole and the integrated area of their peaks was linear ($r^2 = 0.9778–0.9972$). The amount of NO_2^- and NO_3^- in the perfusate samples were calculated from a linear regression plot between concentrations of standard sodium nitrite and sodium nitrate with peak areas. The integration value of the artificial cerebrospinal fluid used for perfusion was subtracted from that for the perfusate. The resulting values were converted into percentages, taking the value for samples just before the kainic acid or saline injection as 100%.

Histological assessment. Three days after KA treatment, rats brains were removed under pentobarbital anesthesia, frozen on dry ice powder, and stored at −80°C until the histological examination. Coronal sections (10 μm) of hippocampus were prepared and stained with 0.1% (w/v) of cresyl violet. The numbers of surviving neurons in CA1 and CA3 subfields were counted within a 0.09 mm^2 area selected by an investigator unaware of the treatment that the rat had received.

Statistical analysis. The results are expressed as mean ± SEM of percentage values of basal level (n = 5–7). Statistical significance was determined by Student's t-test (two group comparison). A p value of < 0.05 was regarded as significant.

Molecular modeling simulations

The nNOS template constructed from a crystallographic structure of nNOS bound with the selective nNOS inhibitor, L-N$^{\omega}$-nitroarginine-(4R)-amino-L-proline amide, 1P6J and validated by crystal structures of three nNOS inhibitors (1P6H, 1RS7 and 1K2R) was used in docking study. AutoDock 3.0.5 [17] was employed to perform the docking calculation. To prepare **N − 1** to dock to the nNOS template, all hydrogens were added and Gasteiger charges were assigned.[18] The AutoDockTools (ADT) was used to merge nonpolar hydrogens and define which bonds were rotatable. **N − 1** was docked to the nNOS template by using a Lamarkian genetic algorithm search. Due to the stochastic nature of genetic search algorithms, each ligand was docked in 100 trials. Each docking trial for ligand was initiated with a randomly generated population of 150 ligand orientations and the highest affinity orientation was selected after 5 million energy evaluations had been performed. Standard AutoDock parameters were used for the genetic algorithm: 2% point mutation; 80% cross over rate; 6% local search rate.[19] The resulting ligand orientations from 100 trials with a 2.0 Å RMSD tolerance of each other were grouped together as clusters. The final docked structure, docked energy and predicted free energy of binding were used for the analysis of its interaction with the active site.

Results and discussion

N − 1 was prepared by solid phase synthesis with a percentage yield of 47.23 and showed a single spot on TLC. All spectral data (IR, NMR, MS, LC-MS) were compatible with the assigned structure. The melting point of **N − 1** was 187 – 188 °C. The results of the elemental analyses were within 0.4% of the calculated values. The investigation of the neuroprotective effect was accomplished *in silico, in vitro* and *in vivo*.

Effect on H_2O_2-induced oxidative cell damage in NG108–15 cells

NG108–15 cells were treated with 0 to 1 mM concentrations of H_2O_2 in order to determine the concentration of H_2O_2 which kills 50% of cells. The results showed that H_2O_2 reduced the viability of NG108–15 cells in a concentration dependent manner, the IC_{50} was found to be 250 μM after treatment with H_2O_2 for 2 hours. At this concentration, the cell viability was about 50% of the control viability. Therefore, for subsequent protection experiment, H_2O_2 at a concentration of 250 μM was used to treat the cells for 2 hours. Trolox was included as a reference compound. Figure 2 showed that **N − 1** increased the viability of NG108–15 cells. **N − 1** and vanillic acid, starting material showed a significant effect at the dose of 0. 1 μM and 1 μM, respectively with a *p*-value less than 0.01 when compared with the H_2O_2 only treated control. At the concentration of 1 μM, **N − 1** was more potent than trolox in increasing cell viability, with % cell viability of 69.07 and 62.54, respectively. **N − 1** was more potent than vanillic acid in the reduction of cell death due to the additional mode of action of **N − 1** as NOS inhibitor. This result indicates that the protective action against oxidative stress was enhanced by the NOS inhibitory action resulting from the added nitro-arginine function in **N − 1** structure.

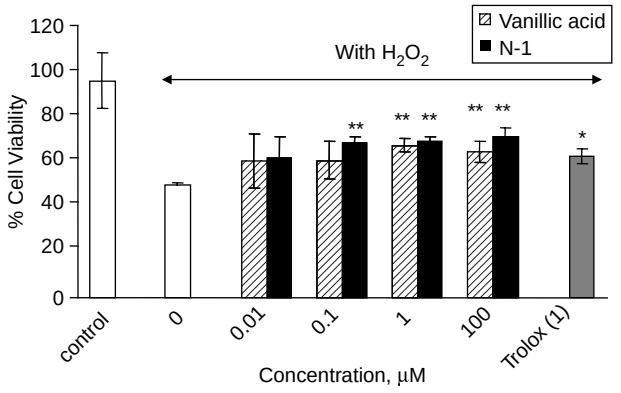

Figure 2. Effect of **N − 1** on H_2O_2-induced oxidative cell damage in NG108-15 cells. NG108-15 cells were pretreated with **N − 1**, trolox and starting material, vanillic acid for 2 hrs and subsequently washed with phosphase buffered saline before the addition of 250 μM H_2O_2. After 2 hrs of incubation, cell viability was measured by MMT assay. Data are means + SD (n = 3) $*p < 0.05$ and $**p < 0.01$ compared to the H_2O_2-treated control group.

Nitric oxide scavenging activity in NG108–15 cells

N − 1 was examined for its inhibitory effect on ˙NO production in NG 108–15 cells. The cultured NG 108–15 cells were stimulated to produce ˙NO by treating the cells with calcium ionophore (A23187). To determine the concentration of A23187 which increase 'NO' level significantly, the cells were treated with various concentration of A23187. The result showed that A23187 significantly increased ˙NO production in a concentration dependent A concentration of 15 μM was chosen to induce NO production of NG 108–15 cells in the subsequent experiment. In this test, L-NAME at 10 μM was used as a reference compound which significantly decreased ˙NO production in NG108–15 cells with *p*-value less than 0.05 compared with the calcium ionophore only treated group. Because of poor solubility, nitro-arginine, a NOS targeting pharmacophore, was not included in this protocol. However, a comparison of NOS inhibitory action between nitro-arginine and **N − 1** was investigated *in vivo* and *in silico*. Figure 3 showed the effect of **N − 1** and vanillic acid, starting material, on ˙NO production in cell culture. The result showed that **N − 1** significantly decreased ˙NO production in NG108–15 cells compared with the calcium ionophore only treated group. **N − 1** was more potent than vanillic acid as it started showing a significant effect on reducing ˙NO production at 100 μM, while vanillic acid did not show a significant effect until it reached a concentration of 1000 μM. The increase in potency of

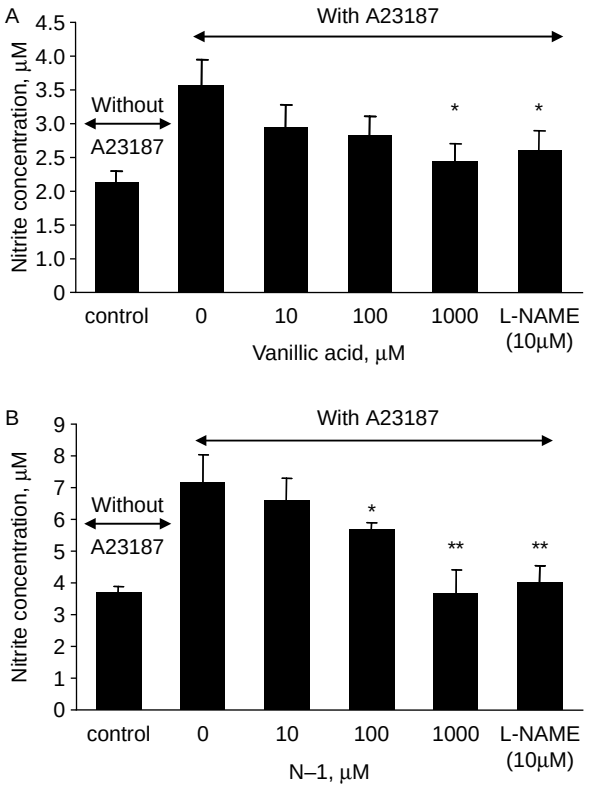

Figure 3. Effect on ˙NO production in cell culture. NG 108-15 cells were incubated with 15 μM of A23187 together with various concentrations of (A) vanillic acid or (B) **N − 1** for 24 hrs in DMEM without phenol red, L-NAME was included as the positive control. Cell media was harvested 24 hours later and assayed for nitrite (NO_2^-) production by spectrophotometric assay based on the Griess reaction. Data results are the means + SD (n = 3). $*p < 0.05$ and $**p < 0.01$ compared with A 23187-treated group.

N − 1 supported the NOS inhibitory action which resulted in the reduction of the increased ˙NO induced by sodium ionophore.

Effect on NOS

Evaluation of the effects of **N − 1** on the activity of the cerebellar constitutive NOS expressed in the rat cerebellum and the constitutive NOS expressed in the human umbilical vein endothelial cells (HUVEC) was determined by measuring the formation of [^{3}H]citrulline from [^{3}H] arginine. The predominant NOS isoform in the rat cerebellar and in the HUVEC is nNOS and eNOS, respectively. In the presence of **N − 1** at the concentration of 10^{-5}M, the activity was only 12.1 ± 0.8% of the control values for nNOS and 25.4 ± 2.2% for eNOS. The activity of nNOS and eNOS decreased significantly from the control. In terms of selectivity, **N − 1** inhibited nNOS to a greater extent than eNOS, 88% inhibition vs. 75% inhibition ($p < 0.05$). However, the selectivity of **N − 1** from the enzyme assay was not good enough to be categorized as a selective nNOS inhibitor.

Investigation of nNOS interaction by docking study

The docking results using AutoDock 3.0.5 indicated that nitro-arginine, starting material, interacted with nNOS through 5 H-bonds at GLN478, TYR588, GLU592, TRP587 and Hem750 with binding energy of −9.78. **N − 1** showed stronger interaction with nNOS than nitro-arginine with 6 H-bond interaction at GLN478, TYR588, GLU592, TRP587and Hem750 and lower binding energy of −10.2 cal/mol (Figure 4). According to Flinspach et al[21], the structure of nNOS-selective inhibitors adopt a curled conformation in nNOS but extended conformation in eNOS. The α-amino group of nNOS inhibitors should be in position to form a direct hydrogen bond with GLU592 of nNOS. The orientation of the docked **N − 1** can not provide a direct hydrogen bond with GLU592 so **N − 1** was not considered as a selective nNOS inhibitor according to the results from the enzyme assay. The docking study showed a stronger capacity of **N − 1** in binding with the active binding site of nNOS. The interaction was found to be tight with low binding energy. The *in silico* result supported the additional mode of action of the designed **N − 1** as nNOS inhibitor which is in accordance with the *in vitro* result in the enzyme assay.

Brain accesibility of N − 1

In order to prove that **N − 1** is responsible for scavenging NO *in vivo*, the accessibility of **N − 1** to the brain was evaluated. Figure 5 showed typical chromatograms of the brain extract from the control

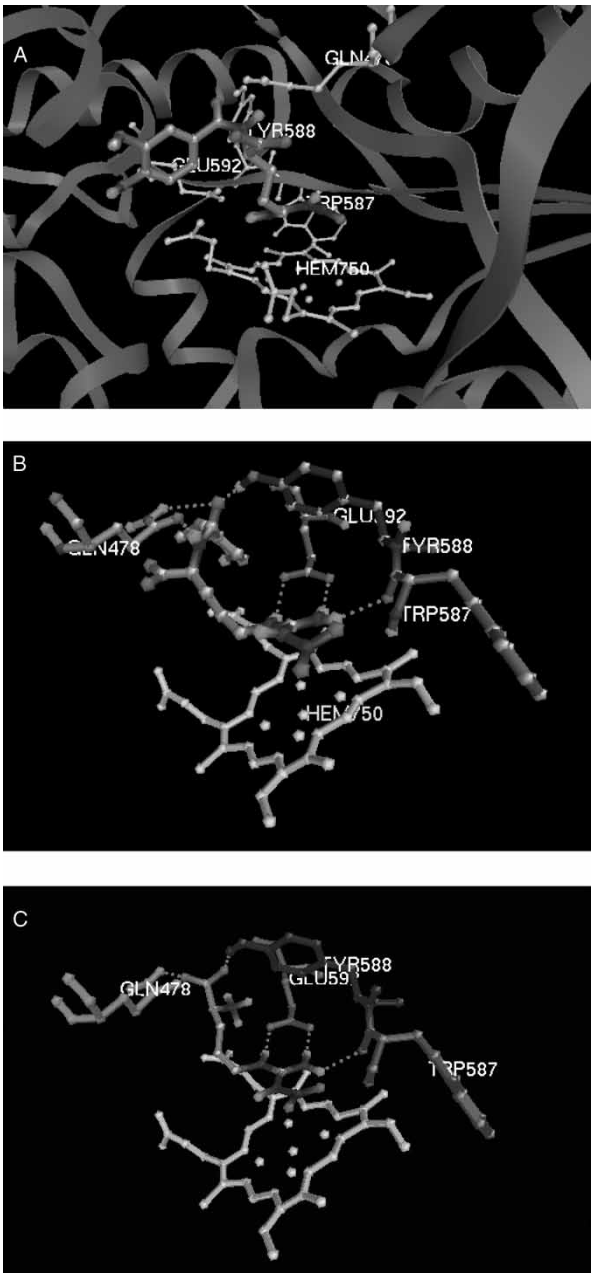

Figure 4. The active site structure (A), interaction between **N − 1** and enzyme nNOS (B) and interaction between nitro-arginine and enzyme nNOS (C).

group, the brain extract from **N − 1**, vanillic acid and nitro-arginine treated mice. The mobile phase system of acetonitrile:water (15:85, v/v) adjusted to pH 3.0 using phosphoric acid) was used to monitor **N − 1** and vanillic acid. The retention times were found to be 6.49 min for **N − 1** and 8.97 min for vanillic acid. This mobile phase system can not be used to identify nitro-arginine in brain extract because the interfering peaks from the brain components appeared in the same area as the nitro-arginine peak, at a retention time of 3.56 min. Therefore, the mobile phase system of acetonitrile: 1% formic acid in water (60:40, v/v) was used for the determination of nitro-arginine in brain

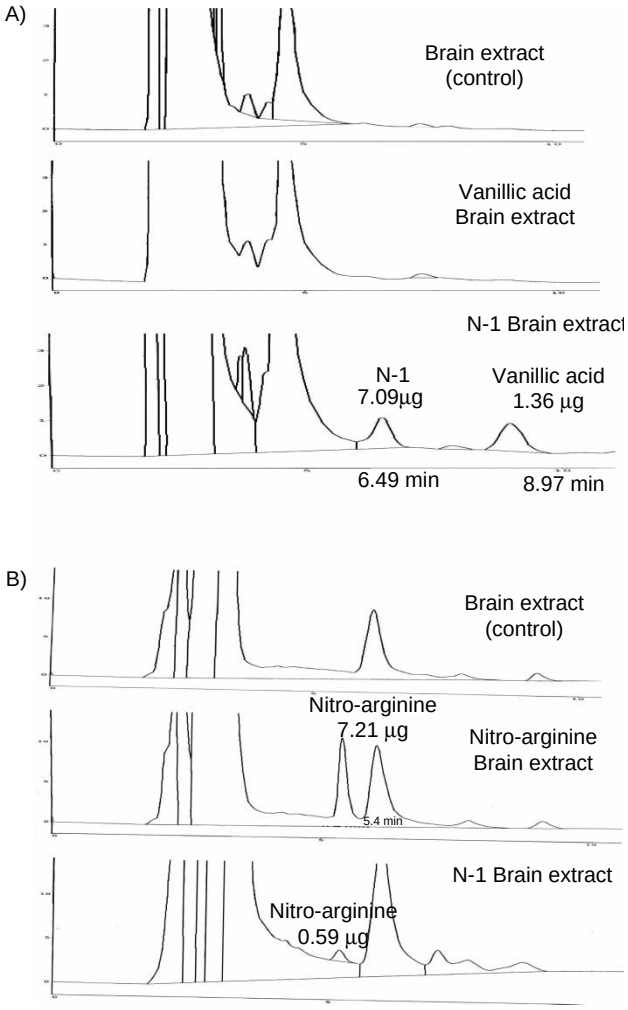

Figure 5. Chromatogram of brain extract of a mouse taken 4 hours after the subcutaneous injection of the test compounds in mobile phase system of (A) acetonitrile: water (15:85, v/v) (adjusted to pH 3.0 using phosphoric acid) and (B) acetonitrile:1% formic acid in water (60: 40, v/v).

extract. Figure 5B showed the typical chromatograms of nitro-arginine when using the mobile phase system of acetonitrile: 1% formic acid in water (60: 40, v/v). The retention time of nitro-arginine in this system was 5.4 min and resolved from the peaks of brain components. The calibration curves of the peak-area versus the concentrations of **N − 1**, vanillic acid and nitro-arginine were linear with $r^2 = 0.9962$, 0.9972 and 0.9883, respectively.

After 4 hour subcutaneous injection of vanillic acid, nitro- arginine and **N − 1**, nitro-arginine was detected in the brains of nitro-arginine treated mice with the total amount of 7.21 μg. For **N − 1** treated mice, **N − 1**, vanillic acid and nitro-arginine were detected the total amount of 7.08 μg, 1.36 μg and 0.59 μg, respectively, whereas vanillic acid was not detected in the brains of vanillic acid treated mice. It is apparent that **N − 1** crossed the blood brain barrier to exert its effects. Thus, **N − 1** carried the antioxidant component into the brain and the radical scavenging action of **N − 1** was the effect of vanillic moiety on **N − 1**.

In vivo effect on kainic acid-induced ˙NO overproduction

The results from NOS enzyme assay and cell culture models indicated that **N − 1** possesses the dual action capacities of radical scavenger and NOS inhibitor. The further neuroprotective activity of **N − 1** was conducted *in vivo* in rats. **N − 1** and nitro-arginine were evaluated for their effect on ˙NO level in rat brain using a microdialysis technique coupled with the combination of a post-derivatization to diazo compound on HPLC system. Vanillic acid, the antioxidant component, was not included in this study because its brain accessibility was poor according to the brain accessibility result. The overproduction of ˙NO level in the brain was induced by ˙NO-inducers, kainic acid. The quantitative analysis of ˙NO production in the hippocampus of conscious rats under freely moving conditions were performed by measuring the level of ˙NO metabolites, nitrite (NO_2^-) and nitrate (NO_3^-) simultaneously in the dialysate. The extracellular levels of nitrite and nitrate in the hippocampus of rats treated with kainic acid are shown in Figure 6A and Figure 6B. The concentration of NO_2^- and NO_3^- in brain perfusates were calculated from the linear regression plot. The significant change in NO_2^- and NO_3^- concentrations were observed after 40 min following kinic acid injection. NO_2^- and NO_3^- level in dialysates were increased and remained elevated throughout the experiments until the time at 160 min ($p < 0.05$). Statistical analysis indicated that the increase in NO_2^- and NO_3^- levels was prominent in animals treated with kainic acid. The effect of **N − 1** and nitro-arginine on kainic acid-induced increases in nitrite and nitrate level was determined by pre-treatment with **N − 1** and nitro-arginine (75 μmole/kg, ip.) 40 min before kainic acid injection (10 mg/kg). The result showed that the animal treated with nitro-arginine died after 1 hour of kainic injection with a pale body. This result was possibly due to the non-selective inhibition of nitro-arginine against NOS, the inhibition of eNOS resulted in reduction of cerebral blood flow. **N − 1** did not cause animal death and was able to suppress the increased nitrite and nitrate level significantly when compared with untreated group (p value < 0.05) after 40 min following kainic acid administration.

Figures 6A and Figure 6B showed that kainic acid enhanced hippocampal ˙NO generation in the conscious rats. KA is a potent neuroexcitatory and neurotoxic analog of glutamate and caused a widespread pattern of brain damage when administered to adult rats. [20] The hippocampus brain region was

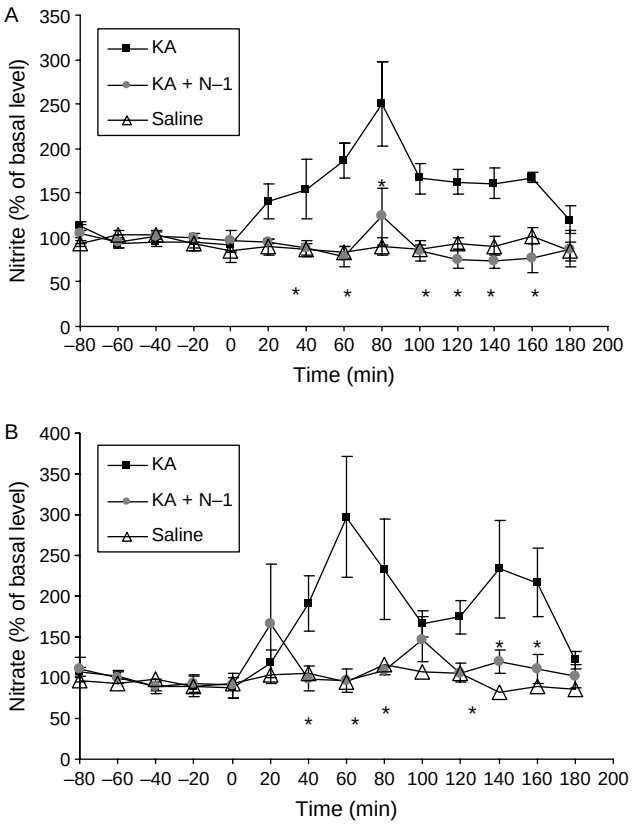

Figure 6. Temporal changes in extracellular nitrite (A) and nitrate (B) levels in the rat hippocampus after injection of kainic acid with or without pre-treatment of **N − 1**. The hippocampus was perfused with artificial CSF at flow rate of 2 μl/min and perfusates were collected every 20 min. Animals were treated i.p. with 10 mg kainic acid (KA; ■, $n = 7$ rats) or saline (control; Δ, $n = 5$). Five animals were pre-treated with 75 μmole/kg compound **N − 1** (○, $n = 5$) i.p. followed by 10 mg/kg kainic acid i.p. 40 min later. Data are expressed as means ± S.E.M of percentage changes in nitrite and nitrate concentrations in perfusates. The values for samples obtained during a 20 min period just before injection of saline or kainic acid were calculated as 100%. The increase in NO_2^- and NO_3^- level was prominent in kainate-treated animals ($p < 0.05$) when compare with that of saline group.

among the most consistently and severely damaged. The result demonstrated that injection of kainate into the hippocampus induced ·NO synthesis in the hippocampus and that responses are attenuated by the **N − 1**. **N − 1** with the dual action of combined pharmacophore of radical scavenging activity and NOS inhibitor was able to suppress ·NO overproduction *in vivo*. The cell death analysis confirmed that **N − 1** is neuroprotective *in vivo*.

KA was found to cause severe neuronal disruption and neuronal loss in the CA1 and CA3 subfields of the hippocampus as shown in Figure 7. Forty minutes of pretreatment with **N − 1** significantly attenuated KA-induced neuronal cell death in the CA1 and CA3 cell layers. The quantitative analysis of the histological damage in Figure 8 revealed that **N − 1** increased the number of surviving cells significantly when compared with KA-treated group (p value < 0.05).

The results from NOS enzyme assay, cell culture and *in silico* models indicated that **N − 1** possesses two modes of actions: radical scavenger and nitric oxide synthase inhibitor. Interestingly, **N − 1** was less toxic in the *in vivo* model. This demonstrated that the addition of vanillyl function, that directly scavenges free radicals, may be the cause of the less toxic effect of **N − 1** as well. The overall results clearly demonstrated that **N − 1** was able to protect neurodegeneration mediated by overproduction of radicals i.e. the oxidative stress condition and the ·NO overproduction from NOS stimulation. The *in vitro* and *in silico* results

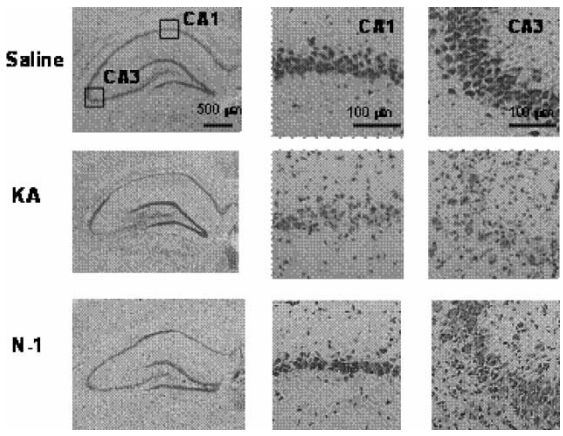

Figure 7. Micrographs of 10-μm coronal sections of hippocampus stained with 0.1% (W/V)cresyl violet.

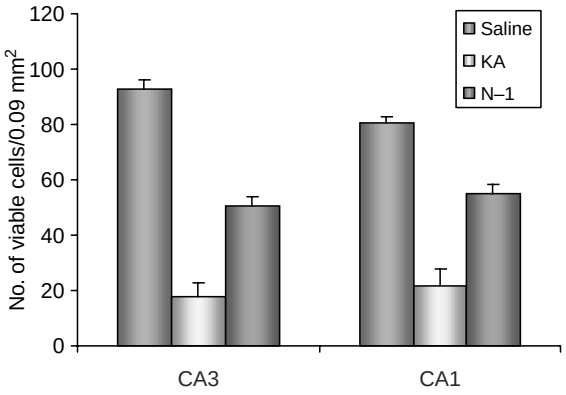

Figure 8. The surviving neurons in CA1 and CA3 subfields.

supported the molecular mechanism of the exerted neuroprotective action which is from the dual action of NOS inhibition and antioxidative action. **N − 1** inhibits nNOS to synthesize ·NO and directly scavenges nitric oxide radical and other radicals including the cascaded radicals by the radical scavenging property.

Conclusion

N − 1 was designed to enhance neuroprotective efficacy through strategy of dual action. Molecular structure of **N − 1** composes of antioxidant pharmacophore and the moiety of NOS substrate which are vanillic acid, the natural antioxidant and N^{Ω}-nitro-L-arginine (NNA), respectively. The structural design aimed at improving the suppression of radicals, ·NO and ROS, and, enhancing brain accessibility. The neuroprotectant, **N − 1** suppressed the radicals by directly scavenging and inhibiting the ·NO production. From this study, it was shown that the combined structure between nitro-arginine and vanillic acid enabled the vanillic antioxidant component to be available in the brain to exert additional action as a direct radical scavenger. This supports the dual action of nNOS from nitro-arginine and radical scavenger from vanillic function resulted in improved neuroprotective efficacy. Although the incorporated nitro-arginine in the structure of natural antioxidant was able to exert nNOS inhibitory action, the selectivity on nNOS was not substantial. A structural modification to increase nNOS selectivity is currently being furthered.

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