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Original Articles

Neuroprotection of Hydroxynicotinyl Amide 18 against Lipid Peroxidation and Memory Impairment

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Abstract - Hydroxynicotinyl amide **18**, a novel radical-scavenging agent, was developed for neuroprotection. **18** showed substantial *ex vivo* inhibitory action against lipid peroxidation in mice brain regardless of low *in vitro* inhibition. These indicated that **18** effectively reached the brain, the target site. Electron paramagnetic resonance (EPR) verified the scavenging ability of **18** by showing suppression of the $HO^{\bullet}$ and $O_2^{\bullet-}$ radicals resulting in the decrease of signal areas of PBN/OH and DMPO/OOH, respectively. Neuropharmacology of **18** was investigated in mice in comparison with chroman amide **12P**, a potent neuroprotective-radical scavenger. **18** at 100 mg/kg, i.p. was the promising compound as it showed significant suppression (18.16%) on the hypermotility induced by methamphetamine (MAP), but did not reduce locomotor activity in normal condition as **12P** did. The suppression demonstrated the enhancement of brain delivery and the antagonism against aberrant dopamine release. In water maze test, **18** (100 mg/kg, i.p.) as well as **12P** (200 mg/kg) and tacrine (3 mg/kg), significantly reduced the learning and memory impairment induced by scopolamine (0.5 mg/kg), **18** was more potent than **12P**. These results support the enhanced brain delivery of **18** as well as provide additional evidence for the role of radical scavenger in the modulation of brain neurotransmitters in the aberrant condition.

Keywords: Hydroxylnicotinyl amide, Lipid peroxidation, Radical scavenger, Locomotor, Memory, Water maze.

INTRODUCTION

Free radicals in brain could represent one of the main causes of the degenerative process associated with aging and neurodegenerative diseases such as Alzheimer's dementia and Parkinson's disease.[1,2] It has been demonstrated that a general reduction of the antioxidant protection mechanisms occurs during aging.[3] In enzymatic system, an age-dependent decrease in superoxide dismutase activity was reported and in nonenzymatic system, levels of antioxidants i.e. ascorbate,[4,5] glutathione[6,7] and α-tocopherol[8-9] have been shown to decrease in brain. The reduction or prevention of free radical generation was attempted as one of the potential therapeutic approach for Alzheimer's disease and other pathological dysfunction in memory.[10-13] Several studies reported the effect of antioxidants against scopolamine-induced amnesia or in the aged mice. The neuroprotective effect of ascorbic acid has been shown to relate to nootropic drugs and other antiamnesic drug.[14] Idebenone, a synthetic compound acting as free radical scavenger, has shown efficacy in degenerative and vascular dementia.[15-17] BN80933, a dual inhibitor of neuronal nitric oxide synthase and lipid peroxidation,

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represents a class of potentially useful therapeutic agents for the treatment of stroke or trauma and possibly neurodegenerative disorders that involve both nitric oxide (NO) and reactive oxygen species (ROS).[18] Other antioxidants namely lazaroids (21-aminosteroids),[19] nitric oxide blockers,[20-21] pyrrolopyrimidines,[22] LY341122[23] and derivatives of antioxidative vitamins are under investigation for neuroprotection and antidementia.

In former studies, series of chroman amide and nicotinyl amide derivatives were synthesized as radical scavenging agents for cerebroprotection. Chroman amide **12**, the most potent compound, inhibited *ex vivo* lipid peroxidation and showed *in vivo* neuro protective efficacy in traumatic head injury and anoxia.[24] Chroman amide **12P** which the O-acetyl group was served to protect the active hydroxyl group for the delivery to the target organ showed significant suppression on the hypermotility induced by methamphetamine and reduced the learning and memory impairment induced by scopolamine.[25] As nicotinamide and piperazine containing compounds were found to be efficient in vascular and neurodegenerative dementia, it is of interest to further structure modification of compound in nicotinyl amide series.[26-31] The low antilipid peroxidation activity *in vitro* of hydroxy nicotinyl amides **5-8** in previous study is possibly due to the low log *P* and high reactivity of active hydroxy functional group. The connection between nicotinyl amide and piperazine in the structure of new **18** was aimed to improve neuroprotective efficacy. Radical-scavenging abilities of new **18** were evaluated by EPR and antilipid peroxidation action. The *in vivo* test on improvement of scopolamine-induced amnesia using water maze was measured to demonstrate the role of radical scavenger in neuroprotection. In addition to oxygen radicals, dopamine is released after brain injury and ischemia finally leading to membrane damage and cell death. The *in vivo*

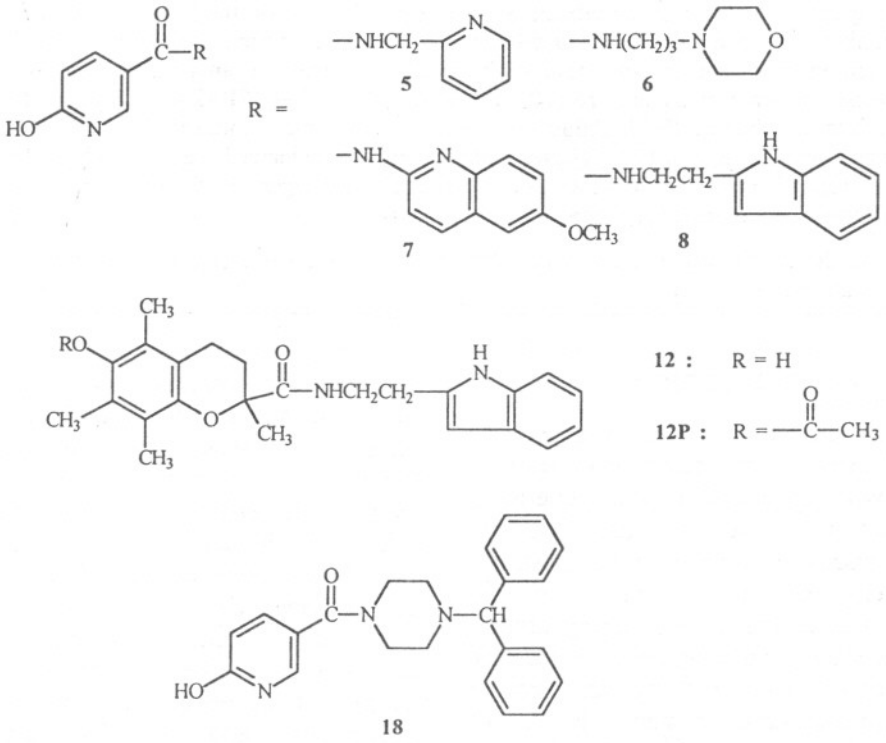

Figure 1. Structures of hydroxynicotinyl amides **5-8**, chroman amides **12**, **12P** and new **18**.

test on suppression of the methamphetamine (MAP)-induced hyper motility by **18** was therefore investigated.

MATERIALS AND METHODS

Synthesis

Melting points of the compounds were determined on a Buchi capillary melting point apparatus and uncorrected. Infrared (IR) spectra were run on FTIR Nicolet 500 as a potassium bromide pellet. Proton nuclear magnetic resonance (^{1}HNMR) spectra were obtained with a JEOL JNM-A-500 (500 MHz). Chemical shifts were reported in ppm related to the internal standard, tetramethylsilane. Thin-layer chromatography was carried out on Merck Kieselgel 60 F_{254} plates and the purified compound showed a single spot. Analytical results from an elemental analyzer (Perkin Elmer PE 400) obtained were within ± 0.4% of the theoretical values. All starting materials were commercially available.

6-Hydroxy-3-pyridinecarbonyl-2′-(Diphenylmethyl) piperazine **(18)**

Carbonyldiimidazole (0.324 g, 2 mmol) was added in small portion to a solution of 6-hydroxynicotinic acid (0.278 g, 2 mmol) in molecular sieve-dried pyridine (10 ml) and the mixture was stirred for 2 hours at room temperature. Then, 1-diphenylmethyl piperazine (0.504 g, 2 mmol) in molecular sieve-dried pyridine (2 ml) was added dropwise to the stirring mixture. The reaction mixture was allowed to stir for 24 h and then evaporated to dryness under reduced pressure. The resulting residue was purified by passing through a silica gel column (eluent: chloroform/methanol, 12:1). Recrystallization from ethanol/water gave compound **18** 0.595 g (79.82% yield), mp 232-233°C. IR (KBr) (cm^{-1}): 3447(O-H), 3061-3020(aromatic C-H), 2968-2860 (aliphatic C-H), 2805-2785(N^+-H), 1669(C=O ester), 1628-1599(aromatic C=C, cyclic C=N, N-H), 1452-1432(N-CO), 1288-1246(aromatic C-N), 1118-1002(C-O), 770-710(aromatic C-H, monosubstitution). ^{1}H NMR ($CDCl_3$): δ 2.44(m, 4H, piperazine-3′ and 5′), 3.69(m, 4H, piperazine-2′ and 6′), 4.29(s, 1H, N-C<u>H</u>-ph_2), 6.56(d, J 9.38 Hz,1H, H3), 7.21(m, 2H, *p*-phenyl-H), 7.30(m, 4H, *m*- phenyl-H), 7.43(d, J 7.19 Hz, 4H, *o*-phenyl-H), 7.56(dd, J 2.37 and 9.39 Hz, 1H, H4), 7.62(d, J 2.00 Hz, 1H, H6). TLC: stationary phase-silica gel 60 F_{254}, mobile phase-chloroform/methanol (8:1); R_f of 1-diphenyl methylpiperazine = 0.25, R_f of compound **18** = 0.63, while 6-hydroxynicotinic acid stayed at the baseline. Elemental analysis for $C_{23}H_{23}N_3$ - Calculated: C, 73.99; H, 6.17; N, 11.26. Found: C, 73.68; H, 6.12; N,= 11.25.

Evaluation of radical scavenging ability

Effect on lipid peroxidation (in vitro)

Mice brains were obtained from freshly slaughtered mice. The brains were homogenized in ice-cold phosphate buffer 40 mM pH 7.4 (1:19 w/v) and kept in ice bath. Lipid peroxidation was assessed by the formation of thiobarbituric acid-reactive substances (TBARS). The fluorescence of TBARS was exited at 515 nm and measured at 553 nm (emission wavelength). Test compounds were prepared in DMSO and were diluted serially. IC_{50} values of **18** was determined.

Effect on lipid peroxidation (ex vivo)

Compounds **18** (100 mg/kg and 150mg/kg) or vehicle (DMSO) was administered subcutaneously to each group of 5 male Swiss albino mice (27-33 g). The brain was excised 1 h after administration of test compounds or vehicle and frozen in dry ice. The frozen brains were homogenized in ice-cold potassium phosphate buffer (1:19 w/v). The mixture was centrifuged after addition of perchloric acid (35% v/v). Thiobarbituric acid (1% v/v in 50% glacial acetic acid) was added to the supernatant and the mixture was heated to 100°C for 15 min. The fluorescence of TBARS was measured.

Electron paramagnetic resonance study(EPR)

The reaction mixtures (1 ml) were prepared and transferred immediately to a quartz EPR flat cell, which was in turn inserted into the cavity of the JOEL (JES-RE2X) EPR spectrometer with the following parameters: magnetic field strength 335.10 mT, microwave power 20 mW, modulation

amplitude 1x1000 mT, and modulation width 0.5x0.1 mT. The evaluation of $HO^{\bullet}$ trapping ability was examined using α-phenyl-*tert*-butylnitrone (PBN) as spin trap. $CuSO_4/H_2O_2$ reaction was used to generate $HO^{\bullet}$. Test compound **18** (final concentration, 5 mM) and PBN (final concentration, 50 mM) were added to a test tube containing the freshly solution mixture of $CuSO_4$ (final concentration, 0.1 mM) and H_2O_2 (final concentration, 10 mM) and a final volume was adjusted to 1.0 ml by phosphate buffer pH 7.4. For the evaluation of $O_2^{\bullet-}$ trapping ability was measured using 5,5-dimethyl-1-pyrroline-1-oxide (DMPO) as spin trap. Test compound (final concentration, 2.5 mM) and DMPO (final concentration, 50 mM) was added to the test tube containing the freshly solution mixture of xanthine oxidase (0.04 units/ml), xanthine (final concentration, 0.4 mM) and deferoxamine (final concentration, 1 mM) and buffer was added to a final volume. As test compound dissolving in ethanol and ethanol itself is a $HO^{\bullet}$ trap, thus control containing an equal volume of ethanol was used. The spectra were recorded at 5 min after mixing the solution on an EPR spectrometer.

Evaluation of neuropharmacological effects

Determination of locomotor activity[25]

Male Swiss albino mice, weighing 25-35 g were used. Fifteen or twenty animals were housed in a cage with free access to water in a temperature and humidity controlled room and was subjected to a 12:12 h light-dark cycle. Mice were divided into 4 groups of 10 animals. In the first group, test compounds (**12P**, **18**) was administered i.p. at the dose of 50-100 mg/kg. One hour after treatment with test compounds, MAP dissolved in normal saline solution (NSS) at the dose of 1 mg/kg was administered i.p. In the second group, mice were treated as group 1 except that NSS was injected instead of MAP. The other two groups are negative control and positive control. The drug vehicle (15% tween 80) and then the MAP vehicle (NSS) were administered in negative control group. The drug vehicle (15% tween 80) and then MAP (1 mg/kg) were administered as positive control group. Three minutes after administration of MAP or NSS in drug treated group and positive control or MAP's vehicle (normal saline) in negative control group, locomotor activity of mice in the 12"× 12" chamber was observed for 5 minutes. The area in the chamber was divided into 16 squares, the area of each square was about the size of the mice. The locomotor activity was recorded in term of the number of squares per minute that the mice occupied in 5 minutes. Significant increase in locomotor activity induced by MAP of the positive control group was observed. The locomotor activity of mice in drug treated groups were determined to evaluate the effect of test compounds on the locomotor activity (group 2) as well as on the MAP induced hypermotility (group 1). Two tail student's *t*-test was used for statistical analysis.

Determination of the improvement of the cognitive deficits induced by scopolamine[25]

The effect of **12P** and **18** on the spatial cognitive deficits induced by scopolamine in mice in water maze test was determined. Fifteen or twenty male ICR mice (SLC, Japan), weighing 30-40 g, were housed in a cage with free access to water in a temperature and humidity controlled room and were subjected to a 12:12 h light-dark cycle (light on 7.00-19.00). In a circular pool, 70 cm-diameter and 28 cm-depth, a dark platform (6 × 10 × 15 cm) was placed 1 cm below the water level in the middle of fixed quadrant. The temperature of the water was adjusted to 25±1°C. The training period continued for 5 days to reach the steady state of escape latency. Mice were placed in a pool of water and allowed to swim freely for 90 sec, 4 trials/day. The time that mice escaped and reached the platform was recorded. On day 6, the platform was taken off from the pool and the mice were allowed to swim 2 times from the different quadrants for 90 sec. The swimming time in the quadrant (Q1) where the platform was formerly located was recorded. On day 7-8, platform was placed in the same position again. Mice were allowed to swim to reach platform 2 times for 90 sec. On day 9, test compounds or vehicle (15% tween 80) were administered i.p. One hour after injection of test compounds, scopolamine hydrobromide (0.5 mg/kg, i.p.) was administered to induce amnesia. Thirty minutes after

scopolamine injection, the mice were placed in the water pool which platform was taken out, they were allowed to swim 2 times from the different quadrants for 90 sec as the same condition as day 6. The swimming time at Q1 was recorded and compared with the swimming time of the same animal on day 6. Tacrine were tested as a reference compound of cholinesterase inhibitor. Pairwise multiple comparison was tested followed by Student-Newman-Keuls method. Differences at $p<0.05$ were considered as statistically significant.

RESULTS AND DISCUSSION

Hydroxynicotinyl amides **5-8** were reported previously to have lower inhibitory action against lipid peroxidation than the chroman amides which possibly due to low lipophilicity.[24] The new **18**, 6-hydroxy-3-pyridinecarbonyl-2′-(diphenylmethyl) piperazine, with increased lipophilicity (log*P* of 3.62 vs. -0.13-2.50 of **5-8**) was synthesized and evaluated. The ability of **18** to scavenge radical was evaluated through the determination of lipid peroxidation inhibitory activity both *in vitro* and *ex vivo* using thiobarbituric method. Electron paramagnetic resonance (EPR) study was performed to verify the scavenging abilities against $HO^{\bullet}$ and $O_2^{\bullet-}$ radicals.

In addition to oxygen radicals, dopamine is released after brain injury and ischemia. There are several possible mechanisms through which dopamine could exacerbate the cell damage produced by cerebral ischemia and trauma. The oxidation of dopamine by molecular oxygen results in the formation of superoxide anion.[32] The reaction between dopamine and hydroxyl radicals generates the neurotoxin, 6-hydroxydopamine.[33] Experimental evidence suggests that decrease in brain dopamine level protects brain tissues from ischemic damage.[34] Oxygen radicals and dopamine are components of an interactive cascade leading to membrane damage and cell death. Consequently, compounds capable of regulating these factors might be able to limit post-traumatic tissue damage and enhance neurological recovery. Antagonism of the metamphetamine (MAP)-induced hypermotility resulting from dopamine release in the CNS[35] by **18** and **12P** were therefore investigated. In addition, it is interested to assess the new synthetic **18** for the ability to improve learning and memory impairment as antioxidants such as ascorbic acid alone or in combination with oxiracetam were found to improve cognitive deficits on radial maze[36-37] as well as water maze test.[38-39]

These two *in vivo* tests on suppression of MAP-induced locomotor hypermotility and improvement of scopolamine-induced amnesia using water maze aim to demonstrate the enhanced brain delivery as well as the radical scavenger role in the neuroprotection of new hydroxynicotinyl amide **18**.

Synthesis

The new hydroxynicotinyl **18** amide was synthesized by carbonyldiiimidazole coupling method. A coupling agent, N,N′-carbonyldiimidazole, reacted readily with carboxylic acid in a 1:1 molar ratio to form acylimidarole. Acylimidazole is very reactive carboxylic acid derivative, therefore, nucleophilic groups such as amine are easily attracted to the carbonyl to form an amide. The desired compound was separated from the reaction mixture by column chromatography and further purified by recrystallization. Percentage yields was high, 79.82 %. The infrared absorption spectrum of **18** showed OH stretching at 3447 cm^{-1} and N^{+}-H stretching at 2785-2805 cm^{-1} which was due to the formation of quaternary ammonium from tautomerization of hydroxyl proton at position of pyridine. The characteristic stretching of amide bond were carbonyl stretching at 1669 cm^{-1} together with the vibration of N-CO function at 1432-1452 cm^{-1}. The ^{1}NMR spectra of **18**, the characteristic proton peak of hydroxynicotinyl portion contained one singlet of pyridine OH at 13.18 ppm. The typical peaks of pyridine protons in position 3, 4 and 6 were shown at 6.56, 7.56 and 7.62 ppm, respectively. The proton peaks of piperazine side chain of **18**, were two multiplets, at 2.44, for protons and 3.69 ppm, respectively. The signal peak of one proton in methine group adjacent of piperazine ring was exhibited as singlet peak at 4.29 ppm.

Evaluation of radical scavenging ability

The new **18** and **12P** and trolox were tested for the inhibitory effect against lipid peroxidation both *in vitro* and *ex vivo* using thiobarbituric acid method. The ability of **18** to scavenge $HO^{\bullet}$ and $O_2^{\bullet-}$ radicals was determined by ESR-spin trapping method. The partition coefficients as lop *P* values were calculated by software Molgen 4.0.

Effect on lipid peroxidation

Homogenate of mice whole brain was used as the source of lipid in the test system because of its high polyunsaturated fatty acid content and the liability to lipid oxidation. The resultant lipid peroxidation was measured from the pink product of the reaction between malondialdehyde and thiobarbituric acid. The inhibition of lipid peroxidation activity represented in term of concentration inhibiting 50% of lipid peroxidation, IC_{50}. The linear regression plots between percent inhibition values and the concentration of all test compounds showed r^2 value ranging from 0.92-0.99. The IC_{50} and log *P* were displayed in Table 1. The lipophilicity of new **18** was in between chroman amide **12P** and trolox but the lipid peroxidation inhibitory action was less potent than trolox. These were possibly due to some other factors besides lipophilicity that governed the inhibitory action against of lipid peroxidation. The reason may be the tautomerization of hydroxyl group on pyridine nucleus, thus the efficiency of the active hydroxyl group to react with biological radicals was less than the hydroxyl on the chroman nucleus. The new **18** was continued for *ex vivo* evaluation of antilipid peroxidation in mice. One hour after injection of **18** (100 and 150 mg/kg, s.c.), the mice were killed and brains were excised for the evaluation. The *ex vivo* antilipid peroxidation results were shown in Table 1. The *ex vivo* antilipid peroxidation of **18** was 12.10% inhibition at 150 mg/kg in spite of low activity *in vitro* (IC_{50} = 1.25 mM). These indicated that **18** with increased lipophilicity effectively reached the brain, the target site. Moreover, other factors apart from hydroxyl group such as the nicotinamide and piperazine portion could play additional role contributing to the *ex vivo* action.

Table 1. Lipophilicity and effect on lipid peroxidation

Cpds	Lipid peroxidation			Log *P*
	in vitro IC_{50} (μM) (n = 3)	*ex vivo* (n = 5)		
		dose (mg/kg)	% inhibition	
18	1.25×10^3	100	3.46	3.62
		150	12.10*	
12P	0.58	100	20.27**	4.30
Trolox	56.35	-	-	3.19

*$p < 0.01$, **$p < 0.001$ vs. control

EPR-spin trapping study

As $HO^{\bullet}$ and $O_2^{\bullet-}$ radicals are the predominant radicals contributing to cellular damage in biological system, the ability of **18** to trap $HO^{\bullet}$ and $O_2^{\bullet-}$ radicals was investigated by EPR-spin trapping method. The EPR spectra of $HO^{\bullet}$ generated by $H_2O_2/CuSO_4$ system in the presence of PBN or mixture of PBN and **18** were showed in Figure 2.

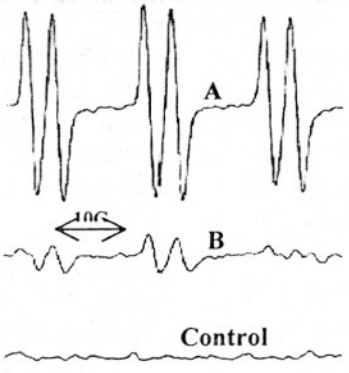

Figure 2. EPR spectrum A obtained from the addition of PBN (final concentration of 50 mM) to $H_2O_2/CuSO_4$ system. EPR spectrum B was collected under the same conditions as A except that the mixture of PBN and **18** (final concentration of 50 mM and 5 mM, respectively) was added.

The formation of the spin adduct PBN/$^{\bullet}$OH was evidenced by the appearance of the characteristic 2:2:2 EPR hyperfine splitting pattern. Compound **18** was found to reduce the signal of PBN/OH adduct as showed in Figure 2B that verified the capability of **18** to scavenge $HO^{\bullet}$. For the evaluation of $O_2^{\bullet-}$ trapping ability, the formation of spin adduct of DMPO/OOH from the reaction of xanthine oxidase and xanthine in the presence of

diethylenetriamine pentaacetic acid and DMPO was evidenced by the appearance of the characteristic of 1:2:2:1 EPR hyperfine splitting pattern (Figure 3).

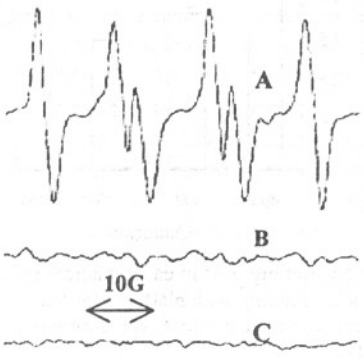

Figure 3. EPR spectrum A obtained from the addition of DMPO (final concentration of 50 mM) to xanthine oxidase, xanthine and deferoxamine. EPR spectra B and C were collected under the same conditions as A except that the reaction mixtures also contained compound 18 (final concentration, 25 mM) (B) or SOD (30 units/ml) (C), respectively.

The formation of the superoxide radicals and consequently its spin adduct (DMPO/OOH) was confirmed by the absence of signals in the presence of superoxide dismutase (SOD). It appeared that **18** suppressed the $O_2^{\bullet-}$ generation resulting in the decrease of DMPO/$^{\bullet}$OOH adduct. The effectiveness in the suppression of $HO^{\bullet}$ and $O_2^{\bullet-}$ radicals strongly suggested that **18** was an effective radical scavenger. In comparison with chroman amide **12**, which EPR signals of chromanoxyl radical appeared after scavenging radicals while **18** did not. New **18** would be a promising candidate since it did not posses the pro-oxidant property.

Neuropharmacological determination

Effect on locomotor activity and MAP-induced hypermotility

Compound **18** and chroman amide **12P** were evaluated for the effect on the locomotor activity as well as the effect on the suppression of methamphetamine (MAP), a dopamine releaser, induced hypermotility in mice. Dopamine can exacerbate the cell damage produced by cerebral ischemia and trauma, the oxidation of dopamine by molecular oxygen results in the formation of superoxide anion leading to the generation of 6-hydroxydopamine, the neurotoxin. The test compound (100 mg/kg) in 15% tween 80 was injected i.p. 1 h before MAP injection (1 mg/kg in saline) and locomotor activity was measured before and after MAP injection. The result (Table 2) showed that **18** as well as **12P** exhibited significant effect against MAP-induced hypermotility. New **18** (100 mg/kg) suppressed MAP-induced hypermotility in the lesser extent than **12P**, 18.16% of **18** versus 54.36% of **12P**, but **18** did not reduce the locomotor activity in normal condition like **12P**. These results indicated that new **18** were a potential compound since it suppressed the MAP-induced hypermotility without reducing the locomotor activity in normal condition in mice.

Determination of the improvement of cognitive deficits induced by scopolamine

New **18** was investigated in comparison with **12P** for the action to improve learning and memory impairment induced by scopolamine, anticholinergic drug, on water maze test in mice. The test compounds were injected i.p. one hour, prior to injection of scopolamine 0.5

Table 2. Effect on MAP-induced hypermotility

Treatment (n = 5-10)	Normal condition[a]		MAP-induced[a]	
	No.sq./min Mean(SEM)	% Locomotor activity	No.sq./min Mean(SEM)	% Suppression of hypermotility
vehicle	20.66(1.86)	100	33.26(2.12)$^{\#\#}$	0.00
12P	6.60(1.73)$^{\#\#}$	31.95	15.18(1.47)**	54.36
18	21.48(2.31)	103.97	27.22(2.34)*	18.16

[a]The test compound in 15% tween 80 (100 mg/kg) was injected i.p. 1 h before MAP injection (1 mg/kg in saline) and locomotor activity was measured after MAP or MAP vehicle injection. $^{\#}p<0.05$, $^{\#\#}p<0.001$ vs. control and $^{*}p<0.05$,$^{**}p<0.001$ vs. MAP

mg/kg i.p. After training the mice for 5 days, the mice remembered the platform's position and the escape latency of water maze test reduced to steady state (Table 3).

Table 3. Steady state on day 5 of the training period

Day	Escape latency (sec) Mean ± SEM (n = 26)
1	39.94 ± 3.94
2	21.90 ± 2.03
3	14.17 ± 1.63
4	10.51 ± 1.38
5	8.24 ± 1.03

On the day 6, the platform was taken from the pool, the trained mice escaped and swam in the quadrant where the platform was formerly located. It was in contrast on day 1, the untrained mice escaped and searched randomly for the platform by swimming in all quadrants. Without platform on day 6, the training mice spent more time in the quadrant 1 that formerly located the platform ($p < 0.05$, Figure 4). Dose of scopolamine to induce learning and memory impairment was initially determined at 0.5 mg/kg, then the mice spent more time to reach platform ($p < 0.05$). Without platform, after being induced amnesia by scopolamine, the mice spent less time at quadrant1, in contrast with the trained mice that spent more time in quadrant 1.

Compounds 18 and 12P were tested for the effect to improve the cognitive deficits caused

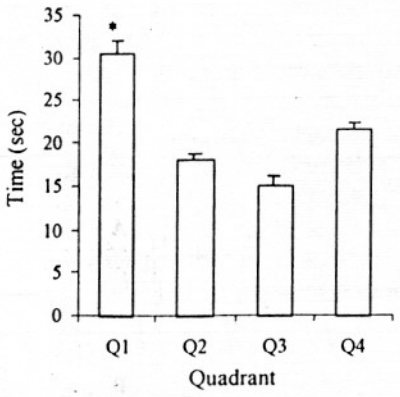

Figure 4. Swimming time in each quadrant on day 6 (no platform) after training with platform for 5 days, Q1 was the quadrant where the platform was formerly located; n = 26, $*p < 0.05$.

by scopolamine. The impairment of cognitive deficits would result in decrease of swimming time at quadrant 1 and the improvement of cognitive deficits would increase the swimming time in quadrant 1. The result of this assay were illustrated in Figure 5. Tacrine, anticholinesterese drug, was used as a positive reference compound and displayed significant improvement in cognitive deficits induced by scopolamine at the dose of 3 mg/kg. New 18 exerted significant improvement of the cognitive deficits induced by scopolamine in the dose of 100 mg/kg better than 12P that showed significant improvement in the higher dose of 200 mg/kg.

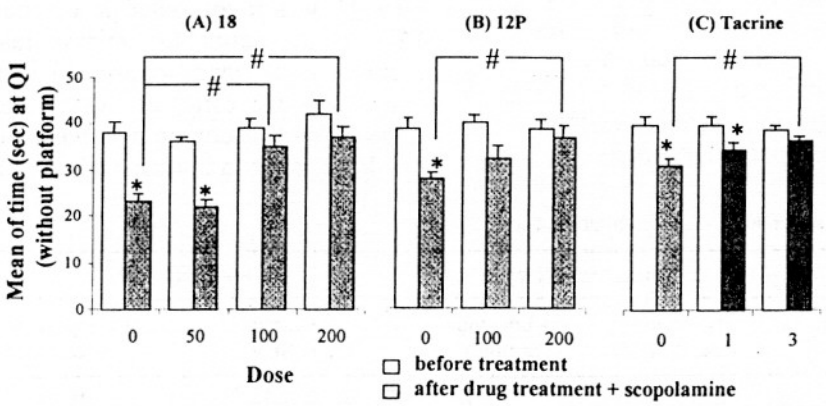

Figure 5. Effect of compounds 18 (A), 12P (B) and, tacrine (C) on scopolamine (0.5 mg/kg) induced cognitive deficits on day 6. Each column represents the mean ± SEM of swimming time that mice spent at the quadrant of platform formerly located (Q1) within 90 sec from 2 different starting quadrants (n=8). $\#p < 0.05$ vs. drugs and scopolamine treatment, $*p < 0.05$ vs. control (before drug and scopolamine treatment) (Student-Newman- Keuls method).

In conclusion, **18** gave substantial *ex vivo* inhibitory action against lipid peroxidation in mice brain regardless of low *in vitro* inhibition. **18** had the effect on suppression of hypermotility induced by MAP, although the suppression was less than chroman **12P**, hydroxynicotinyl amide **18** had no effect on locomotor activity in normal condition as **12P** did. In addition, **18** was found to improve learning and memory deficits caused by scopolamine at the lower dose than **12P**. Other factors beside the active hydroxyl group may contribute to the activity of **18** as the hydroxyl group on pyridine ring was less reactive than the hydroxyl group on chroman nucleus due to tautomerization. The good *in vivo* activity of new **18** was possibly attributed to the nicotinamide and piperazine portion. In addition, **18** was not a pro-oxidant since it scavenged the radicals and reduced the signals of spin trapping adduct without giving an EPR signal. Further pharmacological investigation is required to reveal the mechanism regarding the effect of **18** on learning and cognition.

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REFERENCES

1. Carrol EC, Barry H, Edward TB. Oxygen radicals and human disease. *Annal Inter Med* 1987; 107: 526-45.
2. Halliwell B, Gutteridge JMC. Cross CE. Free radicals, antioxidants, and human disease Where are we now? *J Lab Clin Med* 1992; 119(6): 598-620.
3. Hajimohammadreza I, Brammer M. Brain membrane fluidity and lipid peroxidation in Alzheimer's disease. *Neurosci Lett* 1990; 112: 333-7.
4. Adlard BPF, De Souza SW, Moon S. The effect of age retardation and asphyxia on ascorbic acid concentrations in developing brain. *J Neurochem* 1973; 21: 877-881.
5. Knight JA. The process and theories of aging. *Ann Clin Lab Sci* 1995; 25:1-12.
6. Benzi G. Pastoris O. Mazzatico F. Villa RF. Age related effect induced by oxidative stress on the cerebral glutathione system. *Neurochem Res* 1989;14: 473-81.
7. Hazelton GA, Lang CA. Glutathione contents of tissues in the aging mouse. *Biochem J* 1980; 188: 25-30.
8. Meydani M, Macanley JB, Blumberg JB. Influence of dietary vitamin E, selenium and age on regional distribution of α-tocopherol in the rat brain. *Lipids* 1986; 21: 786-91.
9. Williams LR. Oxidative stress, age, related neurodegeneration and the potential for neurotrophic treatment. *Cerebrovasc Brain Metab Rev* 1995;7: 55-73.
10. Behl C. Alzheimer's disease and oxidative stress: implications for novel therapeutic approaches. *Prog Neurobiol* 1999; 57: 301-23.
11. Grundman M, Corey Blool J, Thal LJ. Perspertives in clinical Alzeimer's disease research and the development of antidementia drugs. *J Neural Transm Suppl* 1998; 53: 255-75.
12. Hall ED. Cerebral ischemia, free radicals and antioxidant protection. *Biochem Soc Trans* 1993; 21: 334-9.
13. Parntti L, Senin U, Mecocci P. Cognitive enhancement therapy for Alzheimer's disease. *Drugs* 1997;33: 752-68.
14. Angelis LD, Furlan C. The effects of ascorbic acid and oxiracetam on scopolamine-induced amnesia in a habituation test in aged mice. *Neurobiol Learn Memo* 1995; 64: 119-24.
15. Zs-Nagy I, Floyd RA. EPR spin trapping studies on the $HO^{\bullet}$ free radical reaction of Idebenone. *Arch Gerontol Geriatr* 1990; 11: 215-31.
16. Bergamasco B, Villardita C, Coppi R. Idebenone in the treatment of multi-infarct dementia: a randomised double-blind placebo controlled multicentre trial *Arch Gerontol Geriatr* 1992; 15: 2371-8.
17. Nappi G, Bono G, Merlo P. Long-term Idebenone treatment of vascular and degenerative brain disorders of the elderly. *Arch Gerontol Geriatr* 1992; 15: 261-9.
18. Chabrier PE, Michel A, Spinnewyn B, Auvin S, Cornet S, Demerle-Pallardy C, Guilmard-Favre C, Marin JG, Pignol B. BN80933, a dual inhibitor of neuronal nitric oxide synthase and lipid peroxidation: a promising neuroprotective strategy. *Proc Natl Acad Sci* 1999; 96: 10824-9.
19. Schultz JB, Henshaw DR, Siwek D. Involvement of free radicals in excitotoxicity *in vivo*. *J Neurochem* 1995;64: 2239-7.
20. Matsumoto K, Yobimoto K, Thi Thu Huong N, Abdel-Fattah M, Hien TV, Watanabe H. Psychological stress-induced enhancement of

brain lipid peroxidation via nitric oxide systems and its modulation by anxiolytic and anxiogenic drugs. *Brain Res* 1999; 839: 74-84.

21. Schultz JB, Matthews RT, Jenkins BG. Blockade of neuronal nitric oxide synthase protects against excitotoxicity *in vivo*. *J Neurosci* 1995; 15: 8419-29.
22. Sethy BH, Wu H, Oostveen JV. Neuroprotective effects of the pyrrolopyrimidine U-104067F in 3-acetylpyridine treated rats. *Exp Neurol* 1996; 140: 79-83.
23. Huh PW, Belayev L, Zhao W, Clemens JA, Panetta JA, Busto R, Ginsberg MD. Neuroprotection by LY341122; A novel inhibitor of lipid peroxidation against focal ischemic brain damage in rats. *Eur J Pharmacol* 2000; 389: 79-88.
24. Vajragupta O, Toasaksiri S, Boonyarat C, Wongkrajang Y, Peungvicha P, Watanabe H, Boonchoong P. Chroman amide and nicotinyl amide derivatives: Inhibition of lipid peroxidation and protection against head trauma. *Free Rad Res* 2000; 32: 145-55.
25. Vajragupta O, Monthakantirat O, Wongkrajang Y, Watanabe H, Peungvicha P. Chroman amide 12P: Inhibition of lipid peroxidation and protection against learning and memory impairment. *Life Sci* 2000; 67:1725-34.
26. Kamat JP, Devasagayam TP. Nicotinamide (vitamin B3) as an effective antioxidant against oxidative damage in rat brain mitochondria. *Redox Rep* 1999; 4(4): 179-84.
27. Issam A, Ayoub E, Jian L, Christopher S, Ogilvy M, Flint B, Maynard IM. Nicotinamide reduces infarction up to two hours after the onset of permanent focal cerebral ischemia in Wistar rats. *Neurosci Lett* 1999; 259: 21-4.
28. Malcon C. Kaddurah-Daouk K, Beal MF. Neuroprotective effects of creatine administration against NMDA and malonate toxicity. *Brain Res* 2000; 860(1-2): 195-8.
29. Crowley LL, Payne CM, Bernstein H, Bernstein C, Roe D. The NAD+ precursors nicotinic acid and nicotinamide protect cells against apoptosis induced by a multiple stress inducer deoxycholate. *Celll Death Di* Issam *ffer* 2000; 7(3): 314-26.
30. Akimoto T. Quantitative analysis of the kinetic constant of reaction of N,N'-propylenedinicotinamide with the hydroxyl radical using dimethyl sulfoxide and deduction of its structure in chloroform. *Chem Pharm Bull (Tokyo)* 2000; 48(4): 467-76.
31. Maurice T, Privat A. SA4503, a novel cognitive enhancer with σ_1 receptor agonist properties facilitates NMDA receptor-dependent learning in mice. *Eur J Pharmacol* 1997; 328: 9-18.
32. Cadet JL, Sheng P, Ali S, Rothman R, Carlson E, Epstein C. Attenuation of methamphetamine-induced neurotoxicity in copper/zinc superoxide dismutase transgenic mice. *J Neurochem* 1994; 62: 380-3.
33. Schmidt CJ, Ritter JK, Sonsalla PK, Hanson GR, Gibb JW. Role of Dopamine in the neurotoxic effects of methamphetamine. *J Pharmacol Exp Ther* 1985; 233: 539-44.
34. Clemens JA, Phebus LA. Dopamine depletion protects striatal neurons from ischemia-induced cell death. *Life Sci* 1988; 42: 707-13.
35. Yamamoto BK, Zhu W. The effects of methamphetamine on the production of free radicals and oxidative stress. *J Pharmacol Exp Ther* 1998; 187: 107-14.
36. Socci DJ, Crandall BM, Arendash GW. Chronic antioxidant tratment improves the cognitive performance of aged rats. *Brain Res* 1995; 693: 88-94.
37. Morel P, Fauconneau B, Page G, Mirbeau T, Huguet F. Inhibitory effects of ascorbic acid on dopamine uptake by rat striatal synaptosomes: relationship in lipid peroxidation and oxidation of protein sulfhydryl groups. *Neurosci Res* 1998; 32: 171-9.
38. Hodges H. Maze procedures: the radial-arm and water maze. *Cognitive Brain Res* 1996; 3:167-81.
39. Gallagter M, Burwell R, Burchinal M. Severity of spatial learning impairment in aging: development of a learning index for performance in the Morris water maze. *Behav Neurosci* 1993; 4: 618-26.

ฤทธิ์ปกป้องสมองของสารประกอบไฮดรอกซีนิโคทินิลเอไมด์ 18 ต่อภาวะการเกิด Lipid peroxidation และภาวะความจำบกพร่อง

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การวิจัยนี้เป็นการพัฒนาสารต้านอนุมูลอิสระไฮดรอกซีนิโคทินิลเอไมด์ 18 เพื่อใช้เป็นสารปกป้องสมองถึงแม้ว่าสาร 18 จะออกฤทธิ์ยับยั้งการเกิด lipid peroxidation ในหลอดทดลองต่ำ แต่พบว่าสารนี้สามารถออกฤทธิ์ต่อสมองของหนูถีบจักรเมื่อทดลองแบบนอกร่าง ผลดังกล่าวชี้ให้เห็นว่าสาร 18 สามารถส่งผ่านเข้าสู่สมองซึ่งเป็นอวัยวะเป้าหมายได้อย่างมีประสิทธิผล เมื่อศึกษาด้วยวิธี spin trapping ด้วยเครื่องมือ Electron paramagnetic resonance (EPR) พบว่าสาร 18 สามารถจับอนุมูล HO• โดยลดขนาดสัญญาณของ PBN/OH และ PBN/OOH ได้อย่างมีนัยสำคัญ จากการเปรียบเทียบฤทธิ์ทางเภสัชวิทยาของสารประกอบไฮดรอกซีนิโคทินิลเอไมด์ 18 ต่อระบบประสาทกับสารประกอบโครแมนเอไมด์ 12P ซึ่งมีฤทธิ์แรงในการต้านอนุมูลอิสระในสมอง โดยฉีดสาร 18 เข้าช่องท้องของหนูถีบจักรขนาด 100 มก./กก. พบว่า มีฤทธิ์ลดการเคลื่อนที่ที่มากเกินปกติอันเกิดจากการกระตุ้นด้วย methamphetamine (MAP) อย่างมีนัยสำคัญ โดยสามารถลดได้ 18.16% แต่ไม่ลดการเคลื่อนที่ในหนูภาวะปกติ จากผลดังกล่าวแสดงให้เห็นถึงความสามารถของสาร 18 ในการเพิ่มการนำส่งยา และต้านฤทธิ์ dopamine ที่หลั่งมากเกินปกติ นอกจากนี้ในการทดลอง water maze โดยการฉีดสาร 18 ในขนาด 100 มก./กก. และสารประกอบ 12P ขนาด 200 มก./กก. เข้าบุเยื่อช่องท้องของหนูถีบจักร พบว่าสารต้านอนุมูลอิสระทั้งสองสามารถลดภาวะบกพร่องของการเรียนรู้และความจำอันเนื่องจากการได้รับ scopolamine ได้อย่างมีนัยสำคัญเช่นเดียวกับ tarcrine ในขนาด 3 มก./กก. ทั้งนี้สาร 18 มีฤทธิ์แรงกว่าสารประกอบ 12P ผลโดยสรุปแสดงให้เห็นว่าสาร 18 สามารถเพิ่มการนำส่งยาไปยังสมอง และยังสนับสนุนบทบาทของสารต้านอนุมูลอิสระ ในการปรับสารสื่อประสาทในสมองเมื่อเกิดภาวะผิดปกติ