JPP Journal of Pharmacy and Pharmacology

JPP 2005, 57: 1191–1197

Received January 29, 2005
Accepted April 25, 2005
DOI 10.1211/jpp.57.9.0014
ISSN 0022-3573

# Effects of 4-piperidinomethyl-2-isopropyl-5-methylphenol on oxidative stress and calcium current

Mei-Han Huang, Li-Fang Liao, Shih-Hsien Kuo, Chi-Lan Chen and Ai-Yu Shen

## Abstract

4-Piperidinomethyl-2-isopropyl-5-methylphenol (THPI) was synthesized by reaction of thymol with piperidine and formaldehyde. The biological effect of THPI on superoxide anion scavenging activity, antiplatelet activity and calcium current inhibition were investigated. THPI (50 $\mu$M) was shown to be a scavenger of superoxide radicals in human neutrophils stimulated with *N*-formyl-Met-Leu-Phe (66% inhibition). Since superoxide anions are essential for platelet aggregation and L-type $Ca^{2+}$-channel activity, we further found that THPI inhibited platelet aggregation induced by arachidonic acid (IC50 $46.80 \pm 6.88$ $\mu$M). The effect of THPI on $Ca^{2+}$ current in NG108-15 cells was investigated using the whole-cell voltage-clamp technique. THPI inhibited voltage-dependent L-type $Ca^{2+}$ current ($I_{Ca,L}$). The IC50 value of THPI-induced inhibition of $I_{Ca,L}$ was $3.60 \pm 0.81$ $\mu$M. THPI caused no change in the overall shape of the current–voltage relationship of $I_{Ca,L}$. This indicates that THPI is an inhibitor of $I_{Ca,L}$ in NG108-15 cells. Therefore, the channel-blocking properties of THPI may contribute to the underlying mechanism by which it affects neuronal or neuroendocrine function. Furthermore, no significant cytotoxic effects of THPI (0.3–50 $\mu$M) were observed in NG108-15 cells. The results indicate that THPI is a potential reactive oxygen species scavenger and may prevent platelet aggregation or inhibit L-type $Ca^{2+}$-channel activity, possibly by scavenging reactive oxygen species.

Department of Medical Technology, Fooyin University, Ta-Liao, Kaohsiung County, Taiwan

Mei-Han Huang

Department of Applied Chemistry, Fooyin University, Ta-Liao, Kaohsiung County, Taiwan

Li-Fang Liao

Basic Medical Science Education Center, Fooyin University, Ta-Liao, Kaohsiung County, Taiwan

Shih-Hsien Kuo, Chi-Lan Chen, Ai-Yu Shen

**Correspondence:** Ai-Yu Shen, Basic Medical Science Education Center, Fooyin University, Ta-Liao Kaohsiung Hsien, Taiwan. E-mail: mt002@mail.fy.edu.tw

**Acknowledgements and funding:** The authors thank MrYing-Zhi Lu for neutrophil preparation. This work was supported by the National Science Council (NSC 93-2320-B-242-010), Taiwan.

## Introduction

Previous studies have shown that the introduction of Mannich bases to phenolic compounds is often accompanied by increased bioactivity, including antimicrobial, cardiovascular and anticonvulsant activity (Shen et al 1995, 1999; Gul et al 2001, 2002). In addition, a general increase in the total levels of glutathione in Jurkat cells, with simultaneous inhibition of interleukin-2 production, in the presence of Mannich bases has been demonstrated (Geng et al 2002). Many phenolic compounds, such as thymol, are known for their antioxidative potential, which has been shown to be beneficial in the prevention of oxidative stress related disorders (Tepe et al 2004). In our preliminary trials, 4-piperidinomethyl-2-isopropyl-5-methylphenol (THPI), a Mannich base of thymol, was found to possess free radical scavenging activity. Reactive oxygen species contribute to a variety of diseases, including coronary arteriosclerosis, immunodeficiencies and multiple organ failure (Ames et al 1993; Partrick et al 1996). Chemical scavengers that can eliminate or decompose pathogenic free radicals would therefore be expected to work as therapeutic agents in these diseases. Moreover, reactive oxygen superoxide is also shown to be critical in the pathogenesis of blood coagulation (Hubbard et al 2003; Olas et al 2003). Platelet aggregation is the main mediator involved in haemostasis and thrombosis formation. Agents that inhibit the aggregation of platelets have been used in the prevention and treatment of thrombic disease (Coller 1992). To determine if THPI affects platelet function, the biochemical effects of THPI on superoxide formation and platelet aggregation were investigated.

A recent study demonstrated that oxidative stress induced by free radicals selectively regulates the activity of L-type $Ca^{2+}$ channels in cultured rat dentate granule cells (Akaishi et al 2004). The enhancement of $Ca^{2+}$ current was inhibited by glutathione, an antioxidant, and nifedipine, an L-type $Ca^{2+}$-channel blocker (Sevanian et al 2000). Thymol could suppress membrane currents through ion channels, such as voltage-dependent $K^+$, $Na^+$ or $Ca^{2+}$ currents in muscle cells or neurons (Haeseler et al

2002; Szentandrassy et al 2003). NG108-15 neuroblastoma and glioma hybrid cells have been widely used in electrophysiology and pharmacological research (Meves et al 1999). This cell line is known to express $Ca^{2+}$ currents (Seabrook et al 1994). Therefore, we also examined the effect of THPI on L-type $Ca^{2+}$ current ($I_{Ca,L}$) and cytotoxicity in NG108-15 cells.

## Materials and Methods

### Drugs and solutions

Piperidine was obtained from the Tokyo Chemical Industry Co. (Tokyo, Japan). Thymol, arachidonic acid, bovine serum albumin, sodium citrate, dimethylsulfoxide (DMSO), Tris-HCl, cytochrome C, and xanthine oxidase were obtained from Sigma Chemical Corporation (St Louis, MO, USA). All other chemicals were of analytical grade. To record inwardly rectifying $K^+$ current, the high $K^+$, $Ca^{2+}$-free solution contained the following (mM): KCl 130, NaCl 10, $MgCl_2$ 3, glucose 5.5 and HEPES-KOH buffer 10 (pH 7.4). To record membrane potentials, the patch pipette was filled with the following solution (mM): KCl 140, $MgCl_2$ 1, $Na_2ATP$ 3, $Na_2GTP$ 0.1, EGTA 0.1 and HEPES-KOH buffer 5 (pH 7.2). To record $Ca^{2+}$ current, $K^+$ ions inside the pipette solution were replaced with equimolar $Cs^+$ ions, and the pH was adjusted to 7.2 with CsOH. Stock solution of THPI was prepared in DMSO and diluted with Tyrode solution to give a final concentration in DMSO of 0.1%.

### Preparation of THPI

Melting points were determined on a Yanagimoto MP-3 micromelting apparatus and are uncorrected. IR spectra were obtained on a Shimadzu IR-408 spectrophotometer. $^1$H NMR spectra were recorded on a Varian Gemini T-500 spectrometer at the National Sun Yat-Sen University (Kaohsiung, Taiwan) and are expressed in parts per million ($\delta$), with tetramethylsilane used as an internal standard. Mass spectra recorded for the purposes of structure confirmation were obtained on a Jeol JMS-HX 110 mass spectrometer at the National Sun Yat-Sen University. Elemental analysis was performed on a CHN-O-Rapid Heraeus elemental analyser at the National Cheng-Kung University (Tainan, Taiwan); analyses were within $\pm$ 0.4% of the theoretical values. Thin-layer chromatography was carried out on precoated silica gel $F_{254}$ chromatographic plates (Kieselgel 60 F-254, 0.2 mm; Merck, Darmstadt, Germany) and eluted with diethyl ether/methanol (8:2).

THPI was prepared according to the Mannich reaction (Cummings & Shelton 1960). Formaldehyde (37%, 0.025 mol) was added to a stirred solution of piperidine (0.025 mol) with thymol (0.025 mol) dissolved in 50 mL methanol. The mixture was gradually heated and refluxed for 12 h. The solvent was then removed, the oily residue was dissolved in ether overnight and the product was recrystallized from ether.

THPI ($C_{16}H_{25}NO$; Figure 1): 63% yield; m.p. 105–107°C; IR (nuzol) $cm^{-1}$: 3453, 2959, 1609, 1082.

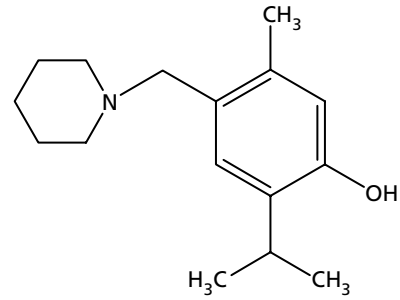


**Figure 1** Chemical structure of 4-piperidinomethyl-2-isopropyl-5-methylphenol.

$^1$H NMR (500 MHz, DMSO-$d_6$) $\delta$: 6.86 (s, 1H), 6.53 (s, 1H), 3.22 (s, 2H), 3.11 (m, 1H), 2.50 (br, OH), 2.27 (br, 4H), 2.16 (s, 3H), 1.44 (br, 4H), 1.37 (br, 2H), 1.11 (d, J = 6.4 Hz, 6H). MSm/z (%): 247(3.07), 162(16.5), 86(56.7), 84(100). Anal. calcd. for $C_{16}H_{25}NO$ (247): C, 77.73; H, 10.12; N, 5.67. Found: C, 77.64; H, 10.19; N, 5.65.

### Superoxide anion scavenging activity in a cell-free system

Free radical scavenging activity was determined by the cytochrome C test. Superoxide anion was assayed spectrophotometrically by a cytochrome C reduction method described by McCord & Fridovich (1969) and calculated from the increase in absorption at 550 nm, the maximum absorption of reduced cytochrome C.

### Superoxide anion scavenging activity in neutrophils

*Preparation of neutrophils*

Experimental protocols were approved by our Institutional Review Board in accordance with international guidelines and informed consent was obtained from all subjects who participated in the study. Venous blood samples were collected from healthy volunteers, 20–30 years of age, into syringes containing heparin (20 units $mL^{-1}$). Neutrophils were isolated by the Ficoll gradient centrifugation method, followed by hypotonic lysis of contaminating erythrocytes (Tomita et al 1984). Briefly, each blood sample was mixed with an equal volume of 3% dextran solution to allow sedimentation of erythrocytes. The upper leukocyte-rich layer was then collected. To remove residual erythrocytes, the pellet was re-suspended in 20 mL cold 0.2% NaCl for 30 s, followed by addition of 20 mL cold 1.6% NaCl to restore tonicity. The remaining neutrophils were then pelleted, washed twice with ice-cold phosphate buffer solution and resuspended in an adequate volume of ice-cold Hank's buffered saline until further manipulation. The preparation contained more than 95% neutrophils, estimated by counting 200 cells under a microscope after Giemsa staining.

*Measurement of superoxide anion*

Purified human neutrophils ($1 \times 10^6$ cells $mL^{-1}$) were pre-incubated at 37°C in 1-mL cuvettes with or without THPI (50 $\mu$M). After addition of cytochrome C, the reaction was initiated with *N*-formyl-Met-Leu-Phe (fMLP; 1 $\mu$M). After 30 min, the reaction was terminated and superoxide anion generation was measured by the superoxide dismutase-inhibited reduction of ferri-cytochrome C in a dual-beam spectrophotometer at 550 nm as previously described (Roberts et al 1990). Extracellular superoxide anion release was monitored by kinetic assays. The kinetics of neutrophil superoxide anion release varied with different activating stimulation and exhibited non-linear kinetics with respect to incubation time.

### Platelet aggregation activity

Blood was obtained from healthy volunteers, 20–30 years of age, and withdrawn into a siliconized glass syringe containing sodium citrate (3.8 g $dL^{-1}$: 1 vol per 9 vols of blood). Platelet-rich plasma was prepared by centrifugation for 10 min at 90 *g* at room temperature. A washed human platelet suspension was prepared from platelet-rich plasma according to procedures described by Ko et al (1994). Platelets were counted using the Hemalaser 2 (Sebia, Molineaux, France) and adjusted to a concentration of $3 \times 10^8$ platelets $mL^{-1}$. Platelet pellets were finally suspended in Tyrode's solution containing bovine serum albumin (0.35%). Briefly, platelets were pre-incubated with THPI for 3 min, followed by the addition of arachidonic acid (100 $\mu$M). Aggregation was measured at 37°C by the turbidimetric method (O'Brien 1962) using an aggregometer (Chromo-Log Co., Havertown, PA, USA). The percent aggregation was calculated as described by Teng et al (1988).

### Electrophysiological measurements

The clonal strain NG108-15 cell line, originally formed by Sendai virus-induce fusion of the mouse neuroblastoma clone N18TG-2 and the rat glioma clone C6 BV-1, was obtained from the European Collection of Cell Cultures (Salisbury, Wiltshire, UK). NG108-15 cells were dissociated and a sample of the cell suspension was placed in a recording chamber affixed to the stage of an inverted phase-contrast microscope (Diaphot-200; Nikon, Tokyo, Japan). Cells were bathed at room temperature (20–25°C) in normal Tyrode's solution containing 1.8 mM $CaCl_2$. Patch pipettes that had a resistance of 3–5 MΩ in bathing solution were prepared from borosilicate glass capillary tubes (Kimax-51; Vineland, NJ, USA) using a vertical two-stage electrode puller (PB-7; Narishige, Tokyo, Japan) and the tips were fire-polished with a microforge (MF-83; Narishige). Ionic currents passing through the whole cell (whole-cell configuration) were measured with the aid of an RK-400 patch amplifier (Biologic, Claix, France) (Hamill et al 1981). The voltage pulses were digitally generated at a rate of 0.1 Hz by means of a programmable stimulator (SMP-311; Biologic). All potentials were corrected for the liquid junction potential that would develop at the tip of the pipette when the composition of the pipette solution was different from that of the bath solution. THPI was added to the bath to obtain the final concentrations indicated.

### Data recording and analyses

The signals consisting of voltage and current tracings were monitored with a digital storage oscilloscope (model 1602; Gould, Valley View, OH, USA) and simultaneously recorded on a digital audio tape recorder (model 1204; Biologic). To calculate the percentage inhibition of THPI, each cell was depolarized from −50 mV to 0 mV, and the current amplitude of $I_{Ca,L}$ after adding THPI was compared with the control value. The concentration-dependent effect of THPI to inhibit $I_{Ca,L}$ was fitted to a Hill function with a non-linear least-square fitting algorithm (Wu et al 2001). That is, percentage inhibition $= E_{max}/\{1 + (IC50^n/[C]^n)$, where [C] is the concentration of the compound, n and IC50 are the Hill coefficient and the concentration of compounds that induced half-maximal inhibition, respectively, and $E_{max}$ is the compound-induced maximal inhibition of $I_{Ca,L}$.

### Cytotoxicity testing

NG108-15 cells ($5 \times 10^4$ $mL^{-1}$) were cultured at 37°C in a 96-well microplate containing 100 U $mL^{-1}$ penicillin G potassium and 100 $\mu$g $mL^{-1}$ streptomycin in a humidified atmosphere containing 5% $CO_2$. The cells were then treated with THPI in graded concentrations and incubated for 24 h. A colourimetric method (Alley et al 1988) for measuring cell number was used for quantifying cell densities in microtitre plates. The resulting optical density of each well was measured at 450 and 650 nm using an enzyme-linked immunosorbent assay reader (Dynatech Laboratories, Chantilly, VA, USA).

### Statistical analysis

All values are presented as mean ± s.e.m. Data were analysed by one-way analysis of variance followed by post-hoc Dunnett's test for pair-wise comparison. Statistical significance was defined as $P < 0.05$.

## Results

### Superoxide anion scavenging activity of THPI

The antioxidant activity of THPI (10–100 $\mu$M) was determined by the cytochrome C test in a cell-free system (Figure 2). In addition, fMLP was used to determine the superoxide anion scavenging activity of THPI in human neutrophils (Figure 3). fMLP (1 $\mu$M) treatment caused an elevation of superoxide anion release in neutrophils. THPI (50 $\mu$M) inhibited superoxide anion release by 66%. The results indicate that THPI may be a potent superoxide scavenger in human neutrophils.

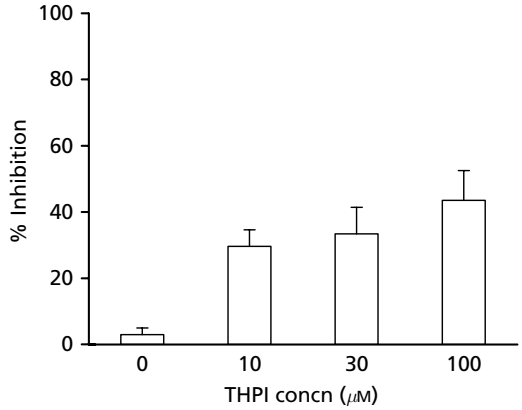


**Figure 2** Scavenging effects of 4-piperidinomethyl-2-isopropyl-5-methylphenol (THPI) in the cytochrome C test. Percentage inhibition is presented as mean ± s.e.m., n = 3–4.

**Table 1** Effects of 4-piperidinomethyl-2-isopropyl-5-methylphenol (THPI) on platelet aggregation induced by arachidonic acid

| Compound | Concentration ($\mu$M) | Aggregation (%) |
|---|---|---|
| Control | 0 | 89.9 ± 0.9 |
| THPI | 12.5 | 87.2 ± 1.6*** |
| | 25 | 83.8 ± 1.1*** |
| | 50 | 66.8 ± 1.2*** |
| | 100 | 0 ± 0*** |
| Indometacin | 30 | 41.17 + 3.1** |

Washed human platelets were pre-incubated with 0.2% dimethylsulfoxide (control), 30 $\mu$M indometacin or various concentrations of THPI at 37°C for 3 min before the addition of 100 $\mu$M arachidonic acid. Percentage aggregation is presented as mean ± s.e.m., n = 4–5. $^{**}P < 0.01$, $^{***}P < 0.001$, significantly different compared with control.

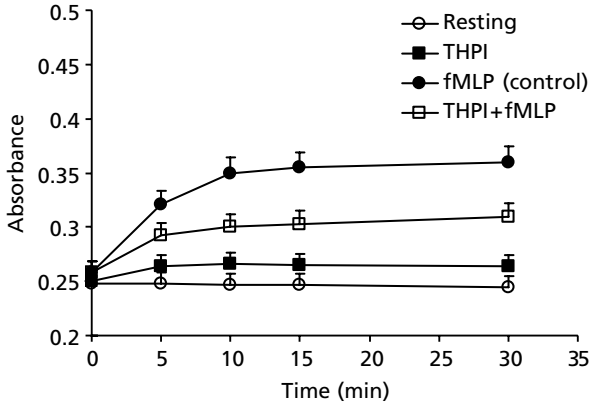


**Figure 3** Inhibitory effects of 4-piperidinomethyl-2-isopropyl-5-methylphenol (THPI) on superoxide anion release from human neutrophils stimulated with *N*-formyl-Met-Leu-Phe (fMLP). Neutrophils were pre-incubated with Hank's buffered saline (resting) or compounds (50 $\mu$M) for 10 min at 37 °C before the addition of fMLP (1 $\mu$M) for superoxide anion release. Superoxide anion was monitored at different times as indicated. Values are presented as mean ± s.e.m., n = 4–5.

### Effects of THPI on platelet aggregation

THPI inhibited arachidonic acid-induced platelet aggregation in a concentration-dependent manner. The IC50 of THPI for arachidonic acid (100 $\mu$M)-induced platelet aggregation was 46.80 ± 6.88 $\mu$M (Table 1).

### Inhibitory effect of THPI on voltage-dependent L-type $Ca^{2+}$ current ($I_{Ca,L}$) in NG108-15 cells

To determine if there was any effect of THPI on $I_{Ca,L}$ in NG108-15 cells, the cells were bathed in normal Tyrode's solution containing 1.8 mM $CaCl_2$ and depolarized from −50 to 0 mV to evoke $I_{Ca,L}$. As shown in Figure 4, THPI had a depressant effect on the amplitude of $I_{Ca,L}$, with no

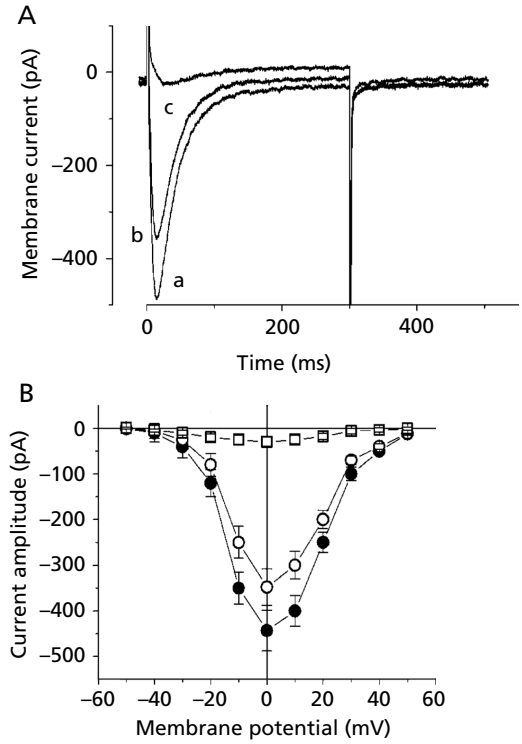


**Figure 4** Inhibitory effect of 4-piperidinomethyl-2-isopropyl-5-methylphenol (THPI) on voltage-dependent L-type $Ca^{2+}$ current ($I_{Ca,L}$) in NG108-15 cells. Cells were bathed in normal Tyrode's solution containing 1.8 mM $CaCl_2$ and the recording pipettes were filled with a $Cs^+$-containing solution. In order to evoke $I_{Ca,L}$, each cell was depolarized from −50 to 0 mV for a duration of 300 ms. A. Original current traces obtained for the control (a), and in the presence of 1 $\mu$M (b) and 10 $\mu$M (c) THPI. B. The averaged current–voltage relationships of $I_{Ca,L}$ in the control (●), and during exposure to 1 $\mu$M (○) and 10 $\mu$M (□) THPI. Each point represents the mean ± s.e.m., n = 5–8.

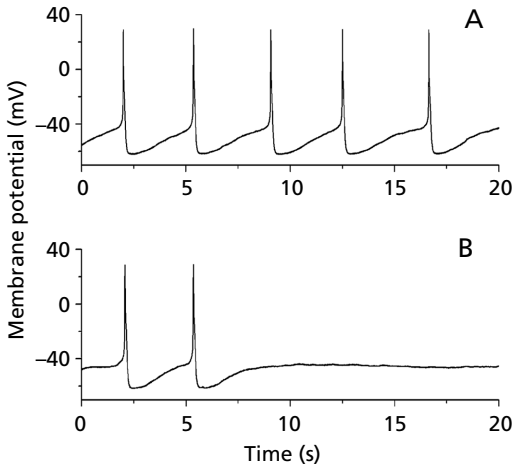


**Figure 5** Inhibitory effect of 4-piperidinomethyl-2-isopropyl-5-methylphenol (THPI) on the firing of action potentials in NG108-15 cells. Cells were bathed in normal Tyrode's solution containing 1.8 mM $CaCl_2$ and the recording pipettes were filled with a $K^+$-containing solution. Original potential traces shown in A and B were obtained in the absence and presence of 1 $\mu$M THPI, respectively.

change in the current–voltage relationships of $I_{Ca,L}$. For example, when cells were depolarized from −50 to 0 mV, THPI, at concentrations of 1 and 10 $\mu$M, significantly decreased $I_{Ca,L}$ to $348 \pm 39$ and $31 \pm 12$ pA (n = 6), respectively, from a control value of $443 \pm 45$ pA (n = 7).

### Inhibitory effect of THPI on spontaneous action potentials in NG108-15 cells

The effect of THPI on the repetitive firing of action potentials in NG108-15 cells was also investigated. As shown in Figure 5, THPI (10 $\mu$M) caused a reduction in the firing frequency of action potentials in these cells. The firing frequency was significantly reduced from $0.15 \pm 0.03$ to $0.01 \pm 0.02$ Hz (n = 4). The THPI-mediated inhibition of the spontaneous discharge can conceivably be explained by its blockade of $I_{Ca,L}$.

### Cytotoxic effects of THPI in NG108-15 cells

After 24 h incubation with various concentrations of THPI (0.3–50 $\mu$M), no significant cytotoxic effects were observed in cultured NG108-15 cells.

## Discussion

Reaction of thymol with formaldehyde and secondary amine in a molar ratio of 1:1:1 in methanol produced THPI. The structure of THPI (Figure 1) was confirmed by IR, $^1$H-NMR, and electron ionization mass spectral data. The IR spectra of THPI displayed the characteristic O-H stretching vibration at 3374–3489 $cm^{-1}$. The $C_4$-H group was absent in the $^1$H-NMR spectrum. The singlet assigned to the $C_3$-H group was at 6.53 ppm and the singlet assigned to the $C_6$-H group was at 6.86 ppm. The proton at the 4 position was replaced by the piperidinomethyl group. The purity of THPI was also determined by elemental analyses. Analyses found by C, H and N elements were within ±0.1% of the theoretical values.

This study aimed to determine if THPI possesses antioxidant properties. A fine balance between reactive oxygen species and endogenous antioxidants, including superoxide dismutase, glutathione peroxidase and catalase, is believed to exist. Any disturbance of this balance in favour of reactive oxygen species causes an increase in oxidative stress and initiates subcellular damage. Chemical scavengers that can eliminate or decompose pathogenic free radicals would be expected to work as therapeutic agents in such conditions. This study showed that superoxide anion generated from the hypoxanthine–xanthine oxidase reaction system was suppressed by THPI (Figure 2). This indicates that THPI possesses antioxidant activity capable of scavenging superoxide radicals in-vitro.

The present study also showed that THPI could function as an inhibitor of oxidation induced by fMLP in human neutrophils. Neutrophils are major components of non-specific cell-mediated immune responses and play a vital role in host defence and inflammation (Smith 1994). However, excessive neutrophil function was implicated in the pathogenesis of capillary leak syndromes, resulting in decreased perfusion and end-organ damage (Windsor et al 1993). One mechanism through which excessive neutrophils might cause early end-organ injury is an indiscriminate adherence to capillary endothelium followed by an inappropriately stimulated elaboration and extracellular release of reactive oxygen metabolites (Weiss 1989). Superoxide anion is the proximal reactive oxygen metabolite and is generated by specialized NADPH oxidase activity (Chanock et al 1994; Partrick et al 1996). The human fMLP receptor is a G-protein-linked chemotactic receptor that activates the NADPH oxidase (Boulay et al 1990). We found that fMLP-induced neutrophil superoxide anion release is inhibited by THPI.

Since a role for NADPH oxidase in arachidonic acid-mediated platelet superoxide anion production was reported (Pignatelli et al 1998), we wanted to find out if THPI interferes with platelet function via arachidonic acid. In human washed platelets, THPI showed inhibition of aggregation induced by arachidonic acid in a concentration-dependent manner. Agonist-induced platelet activation results in a number of rapid biochemical changes; one of the earliest events is a rapid elevation in the intracellular calcium level (Majerus et al 1985). It is generally accepted that an elevation of cytoplasm-free calcium mediates the cytoskeleton rearrangement during platelet shape change, the secretion of granule contents and triggering of platelet aggregation (Detwiler et al 1978). The results suggest that the antiplatelet activity of THPI may be related to antioxidant effects and the inhibition of calcium

mobilization. It also highlights the benefit of THPI in protecting against cardiovascular disease when hyperactivity of platelets is observed.

It has been reported that calcium-channel antagonists can prevent oxidant-induced damage to endothelial cells, which is caused by the formation of reactive oxygen species and the increase in intracellular free calcium (Sevanian et al 2000). It is noteworthy that THPI was effective in suppressing $I_{Ca,L}$ in NG108-15 cells in a concentration- and voltage-dependent manner. In addition, THPI could reduce the firing of action potentials. These results suggest that THPI can interact directly with L-type $Ca^{2+}$ channels; the inhibition of L-type $Ca^{2+}$ channels may be one of the underlying ionic mechanisms of THPI-induced change in the functional activity of neurons or neuroendocrine cells. Oxidative injury appeared to be linked with oxidation of sulfhydryl groups on ion channels in the cellular membrane (Kourie 1998). Our results lead us to propose that the inhibitory effect of THPI on $I_{Ca,L}$ may be related to the decreased production of reactive oxygen species.

### Conclusion

THPI inhibits platelet aggregation induced by arachidonic acid. This may be partly due to the ability of THPI to scavenge superoxide anions, which is a mediator of platelet aggregation (Iuliano et al 1997; Pignatelli et al 1998). Moreover, the inhibitory effect of THPI on $I_{Ca,L}$ may be associated with the decreased generation of reactive oxygen species, with no significant cytotoxicity in NG108-15 cells. There is much evidence indicating the use of antioxidants to retard the progression of arteriosclerosis and to ameliorate systemic disorders (Bankson et al 1993; Knight 1995). The present results suggest that THPI may have potential in the prevention of various disorders involving free radicals.

## References


Akaishi, T., Nakazawa, K., Sato, K., Saito, H., Ohno, Y., Ito, Y. (2004) Hydrogen peroxide modulates whole cell $Ca^{2+}$ currents through L-type channels in cultured rat dentate granule cells. *Neurosci. Lett.* **356**: 25–28

Alley, M. C., Scudiero, D. A., Monk, A., Hursey, M. Y., Czerwinski, M. J., Fine, D. L., Abbott, B. J., Mayo, J. G., Shoemaker, R. H., Boyd, M. R. (1988) Feasibility of drug screening with panels of human tumor cell lines using a microculture tetrazolium assay. *Cancer Res.* **48**: 589–601

Ames, B. N., Shigenaga, M. K., Hagen, T. M. (1993) Oxidants, antioxidants, and the degenerative diseases of aging. *Proc. Natl Acad. Sci. USA* **90**: 7915–7922

Bankson, D. D., Kestin, M., Rifai, N. (1993) Role of free radicals in cancer and atherosclerosis. *Clin. Lab. Med.* **13**: 463–480

Boulay, F., Tardif, M., Brouchon, L., Vignais, P. (1990) The human N-formylpeptide receptor. Characterization of two cDNA isolates and evidence for a new subfamily of G-protein-coupled receptors. *Biochemistry* **29**: 11 123–11 133

Chanock, S. J., el Benna, J., Smith, R. M., Babior, B. M. (1994) The respiratory burst oxidase. *J. Biol. Chem.* **269**: 24 519–24 522

Coller, B. S. (1992) Antiplatelet agents in the prevention and therapy of thrombosis. *Annu. Rev. Med.* **43**: 171–180

Cummings, T. F., Shelton, J. R. (1960) Mannich reaction mechanisms. *J. Org. Chem.* **25**: 419–423

Detwiler, T. C., Charo, I. F., Feinman, R. D. (1978) Evidence that calcium regulates platelet function. *Thromb. Haemost.* **40**: 207–211

Geng, B., Fleming, P. R., Umlauf, S., Lin, A., Pallai, P. V. (2002) The synthesis and selective IL-2 inhibitory activity of bis piperazine-phenol Mannich adducts. *Bioorg. Med. Chem. Lett.* **12**: 775–778

Gul, H. I., Ojanen, T., Vepsalainen, J., Gul, M., Erciyas, E., Hanninen, O. (2001) Antifungal activity of some mono, bis and quaternary Mannich bases derived from acetophenone. *Arzneimittelforschung* **51**: 72–75

Gul, H. I., Calis, U., Vepsalainen, J. (2002) Synthesis and evaluation of anticonvulsant activities of some bis Mannich bases and corresponding piperidinols. *Arzneimittelforschung* **52**: 863–869

Haeseler, G., Maue, D., Grosskreutz, J., Bufler, J., Nentwig, B., Piepenbrock, S., Dengler, R., Leuwer, M. (2002) Voltage-dependent block of neuronal and skeletal muscle sodium channels by thymol and menthol. *Eur. J. Anaesth.* **19**: 571–579

Hamill, O. P., Marty, A., Neher, E., Sakmann, B., Sigworth, F. J. (1981) Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflugers Arch. Eur. J. Physiol.* **391**: 85–100

Hubbard, G. P., Wolffram, S., Lovegrove, J. A., Gibbins, J. M. (2003) The role of polyphenolic compounds in the diet as inhibitors of platelet function. *Proc. Nutr. Soc.* **62**: 469–478

Iuliano, L., Colavita, A. R., Leo, R. (1997) Oxygen free radicals and platelet aggregation. *Free Radic. Biol. Med.* **22**: 999–1006

Knight, J. A. (1995) Diseases related to oxygen-derived free radicals. *Ann. Clin. Lab. Sci.* **25**: 111–121

Ko, F. N., Wu, C. C., Kuo, S. C., Lee, F. Y., Teng, C. M. (1994) YC-1, a novel activator of platelet guanylate cyclase. *Blood* **84**: 4226–4233

Kourie, J. I. (1998) Interaction of reactive oxygen species with ion transport mechanisms. *Am. J. Physiol.* **275**: 1–24

Majerus, P. W., Wilson, D. B., Connolly, T. M., Bross, T. E., Neufeld, E. J. (1985) Pathways of phosphoinositide metabolism in human platelets. *Adv. Prostaglandin Thromboxane Leukot. Res.* **15**: 109–112

McCord, J. M., Fridovich, I. (1969) Superoxide dismutase. An enzymic function for erythrocuprein (hemocuprein). *J. Biol. Chem.* **244**: 6049–6055

Meves, H., Schwarz, J. R., Wulfsen, I. (1999) Separation of M-like current and ERG current in NG108-15 cells. *Br. J. Pharmacol.* **127**: 1213–1223

O'Brien, J. R. (1962) Platelet aggregation II. Some results from a new method of study. *J. Clin. Pathol.* **15**: 452–455

Olas, B., Wachowicz, B., Stochmal, A., Oleszek, W. (2003) Inhibition of oxidative stress in blood platelets by different phenolics from *Yucca schidigera* Roezl. bark. *Nutrition* **19**: 633–640

Partrick, D. A., Moore, F. A., Moore, E. E., Barnett, C. C., Jr, Silliman, C. C. (1996) Neutrophil priming and activation in the pathogenesis of postinjury multiple organ failure. *New Horiz.* **4**: 194–210

Pignatelli, P., Pulcinelli, F. M., Lenti, L., Gazzaniga, P. P., Violi, F. (1998) Hydrogen peroxide is involved in collagen-induced platelet activation. *Blood* **91**: 484–490

Roberts, P. J., Devereux, S., Pilkington, G. R., Linch, D. C. (1990) Fc gamma RII-mediated superoxide production by phagocytes is augmented by GM-CSF without a change in Fc gamma RII expression. *J. Leukoc. Biol.* **48**: 247–257

Seabrook, G. R., McAllister, G., Knowles, M. R., Myers, J., Sinclair, H., Patel, S., Freedman, S. B., Kemp, J. A. (1994) Depression of high-threshold calcium currents by activation of human D2 (short) dopamine receptors expressed in differentiated NG108-15 cells. *Br. J. Pharmacol.* **111**: 1061–1066

Sevanian, A., Shen, L., Ursini, F. (2000) Inhibition of LDL oxidation and oxidized LDL-induced cytotoxicity by dihydropyridine calcium antagonists. *Pharm. Res.* **17**: 999–1006

Shen, A. Y., Hwang, M. H., Roffler, S., Chen, C. F. (1995) Cytotoxicity and antimicrobial activity of some naphthol derivatives. *Arch. Pharm.* **328**: 197–201

Shen, A. Y., Tsai, C. T., Chen, C. L. (1999) Synthesis and cardiovascular evaluation of N-substituted aminonaphthols. *Eur. J. Med. Chem.* **34**: 877–882

Smith, J. A. (1994) Neutrophils, host defense, and inflammation: a double-edged sword. *J. Leukoc. Biol.* **56**: 672–686

Szentandrassy, N., Szentesi, P., Magyar, J., Nanasi, P. P., Csernoch, L. (2003) Effect of thymol on kinetic properties of Ca and K currents in rat skeletal muscle. *BMC Pharmacol.* **3**: 9

Teng, C. M., Lee, L. G., Lee, C. Y. (1988) Ferlan I. Platelet aggregation induced by equinatoxin. *Thromb. Res.* **52**: 401–411

Tepe, B., Daferera, D., Sokmen, M., Polissiou, M., Sokmen, A. (2004) In vitro antimicrobial and antioxidant activities of the essential oils and various extracts of *Thymus eigii* M. Zohary et P.H. Davis. *J. Agric. Food Chem.* **52**: 1132–1137

Tomita, T., Momoi, K., Kanegasaki, S. (1984) Staphylococcal delta toxin-induced generation of chemiluminescence by human polymorphonuclear leukocytes. *Toxicon* **22**: 957– 965

Weiss, S. J. (1989) Tissue destruction by neutrophils. *N Engl. J. Med.* **320**: 365–376

Windsor, A. C., Mullen, P. G., Fowler, A. A., Sugerman, H. J. (1993) Role of the neutrophil in adult respiratory distress syndrome. *Br. J. Surg.* **80**: 10–17

Wu, S. N., Lo, Y. K., Chen, H., Li, H. F., Chiang, H. T. (2001) Rutaecarpine-induced block of delayed rectifier $K^+$ current in NG108-15 neuronal cells. *Neuropharmacology* **41**: 834–843