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Allylmagnolol, a Novel Magnolol Derivative as Potent Antioxidant

Chi-Yuan Li,[a] Yvonne Wang[b,] and Ming-Kuan Hu[b,*]

[a]*Department of Anesthesiology, Tri-Service General Hospital, National Defense Medical Center, 325, Chenggung Road, Section 2, Taipei 114, Taiwan*

[b]*School of Pharmacy, National Defense Medical Center, 161, Minchuan East Road, Section 6, Taipei 114, Taiwan*

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Abstract—We reported the discovery of potent antioxidants based on magnolol, a naturally occurring biphenolic obtained from the bark of *Magnolia officinalis*. The allylmagnolols **3a**,**b** were synthesized via *O*-alkylation of the biphenols followed by Claisen rearrangement. In-vitro using enhanced chemiluminescence (CL) and flow cytometric assays in whole cells revealed that both **3a** and **3b** displayed promising free radical scavenging effects in PMA- and LPS-stimulated models as compared with magnolol. Further DNA labeling analysis for cytotoxicity indicated that these analogues show no cytotoxic effects for the scavenging of the oxygen-derived free radicals under PMA-stimulated concentrations. The results from 3,3′-bisallylmagnolol (**3b**) suggested that the naturally occurring constituent was suitable to be a lead compound for the development of potential antioxidants for certain diseases.

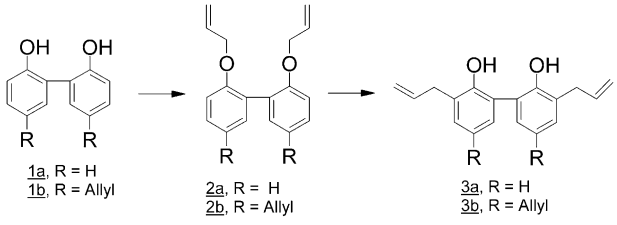
Introduction

Recent contributions on the drug development have revealed that there has been a blooming of interest in the discovery of natural constituents or their semi-synthetic derivatives as a source of novel pharmacological agents.[1] Importantly, most of these natural products have been used in traditional medicine or alternative therapies.[2] Magnolol (**1b**, Scheme 1), a naturally biphenolic constituent of *Magnolia officinalis*, has been found to exhibit strong scavenging effects against hydroxyl radicals under certain disease-orientated models. For instance, it can attenuate peroxidative damage, improve survival of rats with sepsis, and protect cortical neuronal cells from chemical hypoxia in rats.[3,4] Meanwhile, *M. officinalis*, known in Chinese folk medicine as *houpo*, has long been utilized for treating stomach disorders, cardiovascular and allergic diseases such as thrombosis, bronchial asthma.[5–7] On the basis of those reported investigations and traditional effects of the herb, it was strongly conceivable that magnolol can be a suitable lead compound for the development of free radical scavengers as novel antioxidants. Magnolol possesses an unusual biphenolic structure with two *para*-allyl groups.[8] Its potent anti-oxidative activities were presumably attributed to the hydroxyl group at the biphenolic moiety. In the course of structural and biological investigations for magnolol and search for potent antioxidants, we here report to synthesize its allyl analogues and evaluated their radical scavenging activities.

Results and Discussion

Chemistry

The synthesis of allylmagnolols **3a**,**b** were accomplished with phenolic *O*-allylation followed by Claisen rearrangement[9] as illustrated in Scheme 1. Therefore, 2,2′-biphenol or magnolol was treated with allyl bromide in the presence of potassium carbonate to afford 2,2′-bis(allyloxy)-biphenyls **2a**,**b**. The following Claisen rearrangement of



Scheme 1.

*Corresponding author. Tel.: +886-2-8792-3100x18996; fax: +886-2-8792-3169; e-mail: hmk@ndmctsgh.edu.tw

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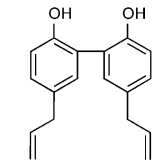
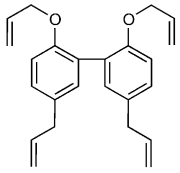
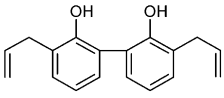
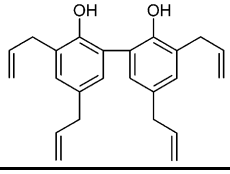
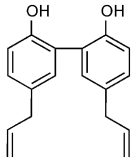
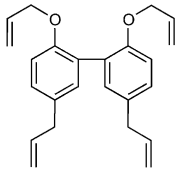
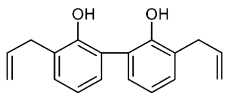
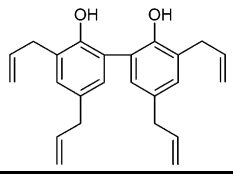
Table 1. Scavenging effects of magnolol analogues on PMA-stimulated oxygen metabolites

Compd	Compd concn (μM)	10^{-2}	10^{-1}	1	10
		% of positive control			
	Probucol	97±13	106±9	102±15	133±20
1b		84±10	112±15	91±18	122±12
2b		77±7	101±13	91±17	111±10
3a		26±6[a]	26±9[a]	46±11[a]	50±14[a]
3b		56±13	62±14	21±9[a]	47±11[a]

Values are the mean±SD, $n=4$.
[a] $p<0.001$ compared with control.

Table 2. Scavenging effects of magnolol analogues on LPS-stimulated oxygen metabolites

Compd	Compd concn (μM)% of positive control	10^{-2}	10^{-1}	1	10
		% of positive control			
	Probucol	107±12	124±15	82±8	98±9
1b		81±9	81±7	68±5[a]	74±8
2b		93±8	82±6	66±7[a]	87±5
3a		112±10	99±8	68±7[a]	65±10[a]
3b		93±9	90±6	66±7[a]	95±5

Values are the mean±SD, $n=4$.
[a] $p<0.01$ compared with control.

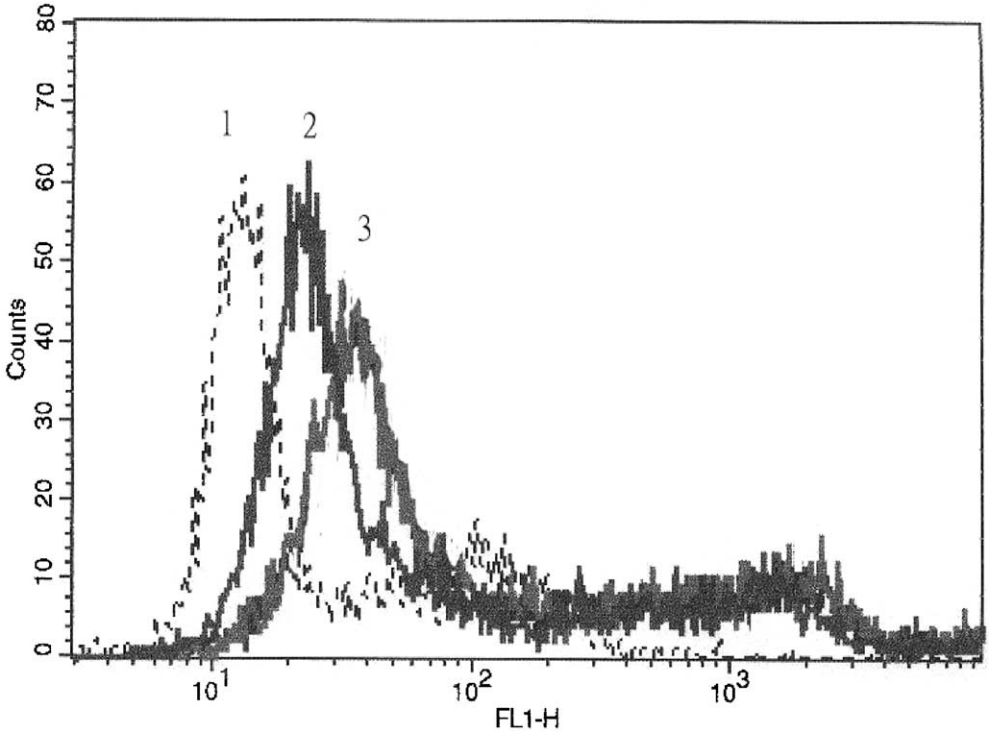


Figure 1. Flow cytometric analysis of a representative experiment. PMA-stimulated neutrophils, with (2) or without (3) magnolol analogues pretreatment, and control level (1) were quantitated by FACSort. FL1-H means fluorescent intensity; *y*-axis indicates cell counts.

Table 3. Scavenging effects of compounds on the PMA-induced production of H_2O_2 at 20 min after addition of PMA

Compd concn (μM)	% Inhibition of DCF		
	Magnolol (**1**)	**3a**	**3b**
10^0	51.1±8.5[a]	>100	>100
10^{-1}	32.3±3.1[a]	>100	>100
10^{-2}	8.4±2.3	>100	29.3±4.2[a]
10^{-3}	—[b]	82.7±9.3	9.0±2.6[a]
10^{-4}	—	30.0±4.6[a]	—

Values shown are means±SD, $n=4$.
[a]$p<0.01$ compared with control.
[b]No inhibitory effects.

2a was performed in heated phenol to give the desire 3,3′-bisallylbiphenol (**3a**) as a pale purple oil in 58% yield, but the product readily turned deep purple in 2 h at room temperature; presumably, it was sensitive to the atmosphere. Similarly, 3,3′-bisallylmagnolol (**3b**) was obtained as a pale yellow oil in 67% yield from **2b** and it is chemically stable under ambient conditions for months.

Anti-oxidant activities

The measurement of anti-oxidizing activities of the biphenolic analogues was conducted with means of PMA/lucigenin-dependent enhanced chemiluminescence of human whole blood.[10] This was utilized to evaluate the scavenging effects of test compound on the reactive oxygen metabolites such as hydrogen peroxide and superoxide radicals mainly derived from neutrophils. 3,3′-Bisallylmagnolol (**3b**) displayed the strongest anti-oxidant activity among the test chemicals. It was 8-fold more potent radical scavenging effects (20% of control) than magnolol (90% of control) at 1 μM level. Meanwhile, the 3,3′-bisallylbiphenyl (**3a**) showed similar scavenging potency even at lower concentration of 0.01 mM, but chemically it is less stable and readily turned deep purple in a short time at ambient conditions.Impressively, this antioxidant effect was overwhelmingly stronger than that of probucol, a classic antioxidant (Table 1). As indicated in this study, allyl ether **2b** showed the least scavenging effects among these tested compounds. It is well accepted that the oxygen-derived free radical scavenging of phenolic compound is mainly due to the existing hydroxyl group and its hydrogen-donating ability.[11] Thus, masking hydroxyl group of compound **2b** resulted in decreased potency against free radicals. On the other hand, the electron-deficient allyl group of magnolol was presumably to show synergetic effects on its biological activity. Therefore, the *ortho*-allyl epimer **3a** and derivative **3b** were prepared and they were more potent among the tested compounds under PMA-induced models. These results suggested that the electron-deficient allyl group at the *ortho*-position of magnolol improves its radical affinity of phenolic moiety more promising than that at the *para*-position.

Accordingly, the inhibition of lipopolysaccharide (LPS)-induced macrophage activation of the whole blood was executed to evaluate anti-oxidizing actions of magnolol analogues against the nitric oxide-related free radicals.[12] As described in Table 2, these analogues of magnolol were a little more potent than probucol in this model, but they were shown no improvements on scavenging effects as compared with magnolol at the concentrations in the range of 0.1–1.0 μM levels. These results indicated

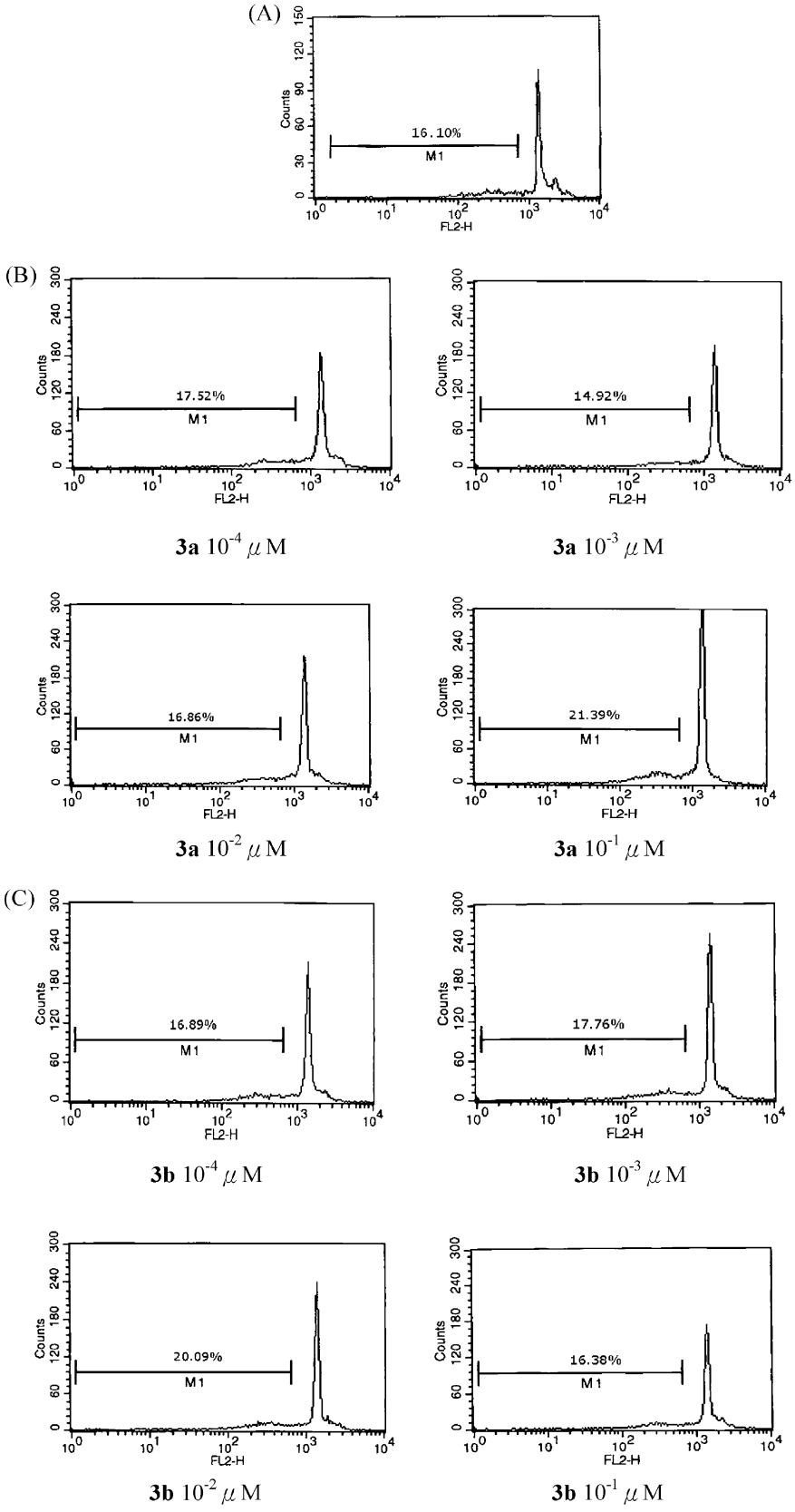


Figure 2. DNA fluorescence histograms of PI-stained cells for various concentrations of magnolol analogues **3a** (Panel B) and **3b** (Panel C) after 24 h incubation at 37 °C. Panel A represented the control set. FL2-H represented fluorescent intensity and M1 showed the percent of Sub-G1 phase.

that these phenolic-containing compounds were not active against nitric oxide-related reactive species induced by endotoxin, which nevertheless have been known to be sensitive to vitamin E or certain selenocontaining compounds.[13]

We further used a flow cytometric method that enabled us to measure intracellular reactive oxygen species (e.g., $O_2^{-\cdot}$ and H_2O_2) changes in PMA-stimulated neutrophils with or without magnolol analogues pretreatment.[10,14] Figure 1 illustrates the fluorescence intensity measured in a representative experiment and Table 3 summarizes the results of four experiments of PMA-stimulated accumulation of intracellular H_2O_2 as measured by DCF fluorescence. The results indicate that both **3a** and **3b** showed more potent than magnolol on inhibition of PMA-induced H_2O_2 production. At higher concentrations (**3a** $> 10^{-2}$ μM; **3b** $> 10^{-1}$ μM), they not only effectively inhibit the generation of PMA-induced H_2O_2 but also simultaneously scavenge the intracellular H_2O_2 by neutrophils.

As the results of above studies, both electrodeficient *ortho*-allyl groups of compounds **3a** and **3b** seemed to display a synergistic effct on antioxidative activity especially at PMA-stimulated model. The electrodeficient properties of the allyl group was assumed to improve radical scavenging effects via interaction with the phenolic oxygen. Chemically, **3b** was suprisingly unstable while it gradually turned to slight purple color within 2 h as it was prepared. promising candidate with strong antioxidative activity.

In order to distinguish if the potent scavenging effects of these magnolol analogues on the PMA-stimulated reactive oxygen species was related to cytotoxic effects, a flow-cytometric analysis of centrifuged cell pellets stained with propidium iodide (PI) in hypotonic fluorochrome solution was used to evaluate on DNA labeling.[15] Figure 2 illustrates the DNA fluorescence flow cytometric profiles after 24 h incubation in medium alone or in varieties of concentrations of magnolol analogues. From 10^{-4} to 10^{-1} μM levels, **3a** and **3b** indicate the similar DNA fragmentation profiles at sub-G1 phase to the control set. These results indicated that these tested compounds show no detrimental cytotoxity during the scavenging of oxygen-derived free radicals under PMA-stimulated concentrations.

Conclusion

Recent investigations proved that magnolol showed to prevent ischaemia/reperfusion injury by inhibiting PMA-activated neutrophil adhesion.[10] This might be accounted for its blocking the accumulation and production of reactive oxygen-derived species in whole cells. As a whole, This study found out that both 3,3′-bisallylbiphenyl (**3a**) and 3,3′-bisallylmagnolol (**3b**) showed prominently improved anti oxidative activities without cytotoxic effects under cell-based assay. Chemically **3b** is a stable magnolol derivative compared with **3a** and therefore should be chosen for further evaluation on certain free radical damage due to its promising results from our studies.

Experimental

Chemistry

All reagents were commercial materials and were used directly unless otherwise noted. DMF was dehydrated over 4 Å molecular sieve. NMR spectra were recorded on a Varian Gemini at 300 MHz for ^{1}H and at 75 MHz for ^{13}C. Elemental analyses were determined using a Perkin Elmer 240 EA analyzer. Chromatography refers to flash chromatography on silica gel (silica gel 60, 230–400 mesh ASTM, E. Merck). Melting points were recorded on a Thomas Hoover capillary melting point apparatus in open capillary tubes and are uncorrected.

Magnolol (5,5′-bisallylbiphenyl-2,2′-diol, 1b).[16] The dried stem bark *M. officinalis* (1.2 kg) was soaked with in anhydrous ethanol at room temperature for 3 days. Concentration of the solvent gave a dark brown syrup alcoholic extract (350 gm), which was taken to silica gel chromatography (*n*-hexane/EtOAc = 20:1→15:1 as eluents). The desired magnolol was obtained (5.2 g) as a white solid: mp 99–101° (lit.[8] 100–102 °C); ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.13–6.92 (m, 6H, Ar–H), 6.05–5.93 (m, 2H, 2 CH=), 5.14–5.07 (m, 4H, 2 $=CH_2$), 3.37 (d, $J = 6.6$ Hz, 4H, 2 Ar-CH_2); UV λ_{max} (EtOH) nm (ε): 290 (5370); FABMS (NBA as matrix): *m*/*z* $[M+H]^+$ 266.1.

2,2′-Bis(allyloxy)biphenyl (2a). To a solution of 2,2′-biphenyl (3.72 g, 20 mmol) and allyl bromide (3.4 mL, 40 mmol) in actone (50 mL) was treated with potassium carbonate (5.52 g, 40 mmol) in an ice bath. The resulting mixture was heated under reflux for 6 h and then concentrated to give, after silica gel chromatography (*n*-hexane/EtOAc = 15:1), a pale yellow oil (3.76 g, 71%): UV λ_{max} (95% EtOH): 288 (log ε 2.56); ^{1}H NMR (300 MHz, $CDCl_3$) 7.33–6.94 (m, 8H, Ar–H), 5.98–5.88 (m, 2H, 2 CH=), 5.26–5.12 (m, 4H, 2 $=CH_2$), 4.52–4.50 (m, 4H, 2 O–CH_2); FABMS *m*/*z* $[M]^+$ 266.1; HR-FABMS: exact mass calcd for $C_{18}H_{18}O_2$, 266.1307, found 266.1316.

5,5′-Diallyl-2,2′-bis(allyloxy)-biphenyl (2b). To a solution of magnolol (2.66 g, 10 mmol) and allyl bromide (1.7 mL, 40 mmol) in acetone (30 mL) was treated with potassium carbonate (2.75 g, 20 mmol) in an ice bath. The resulting mixture was heated under reflux for 6 h and then concentrated to give, after silica gel chromatography (*n*-hexane/EtOAc = 15:1), a pale yellow oil (2.97g, 86%): ^{1}H NMR (300 MHz, $CDCl_3$) 7.20–6.89 (m, 6H, Ar-H), 6.09–5.89 (m, 4H, 4 CH=), 5.30–5.08 (m, 8H, 4 $=CH_2$), 4.49 (m, 4H, 2 O–CH_2), 3.38 (d, $J = 6.6$ Hz, 4H, 2 Ar–CH_2); FABMS *m*/*z* $[M]^+$346.1; HR-FABMS: exact mass calcd for $C_{24}H_{26}O_2$, 346.1934, found 346.1935.

3,3′-Bisallylbiphenyl-2,2′-diol (3a). A mixture of biphenyl ether **2a** (0.71 g, 2.67 mmol) in phenol (2 g) was

heated under reflux for 10 h and then diluted with ethyl acetate and water. The organic layer was dried over sodium sulfate, filtered, and evaporated in vacuum to give, after silica gel chromatography (*n*-hexane/EtOAc = 10:1), a yellow oil (0.47 g, 67%): ^{1}H NMR (300 MHz, $CDCl_3$) 7.23–6.80 (m, 6H, Ar–H), 6.18–6.02 (m, 2H, 2 CH=), 5.56 (s, 2H, 2 OH), 5.22–5.15 (m, 4H, 2 $=CH_2$), 3.52 (d, J = 6.5 Hz, 4H, 2 Ar–CH_2), FABMS: m/z [M]+ 266.1, HR-FABMS: exact mass calcd for $C_{18}H_{18}O_2$, 266.1307, found 266.1302.

3,3′-Bisallylmagnolol (3b). A mixture of biphenyl ether **3** (0.6 g, 1.73 mmol) in phenol (1.5 g) was heated under reflux for 6 h and diluted with ethyl acetate and water. The organic layer was dried over sodium sulfate, filtered, and evaporated in vacuum to give, after silica gel chromatography (*n*-hexane/EtOAc = 10:1), a pale brown oil (0.35 g, 58%): ^{1}H NMR (300 MHz, $CDCl_3$) 7.04 (s, 2H, Ar–H), 6.97 (s, 2H, Ar–H), 6.12–5.92 (m, 4H, 2 CH=), 5.43 (s, 2H, 2 OH), 5.21–5.07 (m, 8H, 4 $=CH_2$), 3.48 (d, J = 6.5 Hz, 4H, 2 Ar–CH_2), 3.35 (d, J = 6.7 Hz, 4H, 2 Ar–CH_2), FABMS: m/z [M]+ 346.1, HR-FABMS: exact mass calcd for $C_{24}H_{26}O_2$, 346.1934, found 346.1936.

Evaluation of anti-oxidative activities

Measurement of chemiluminescence. The measurement of antioxidant activities was using enhanced chemiluminescence (CL).

A. To a sample containing 180 μL of heparinized whole bloods in a 96-well plate was added the tested compound with gradient concentration followed by PMA (2 μg/mL) or LPS (10 μg/mL) and lucigenin (1 μg/mL). After it stood for 15 min at dark conditions, the final volume of each well was set to 200 μL with the addition of phosphate buffer saline (PBS). Chemiluminescence was monitored in a luminometer (Packard LumiCount) and the peak height was recorded in mV. Two negative models, which were devoid of either lucigenin or PMA, and a positive model that contained both lucigenin and PMA were conducted as background for control.

B. According to the above experiment, LPS (10 mg/mL) was used to replace PMA and chemiluminescence was monitored in a luminometer (Packard LumiCount). The peak height was recorded in mV. Two negative models, which were devoid of either lucigenin or LPS, and a positive model that contained both lucigenin and LPS were conducted as background for control.

Measurement of hydrogen peroxide production

Preparation of cells for assay. Put 3 mL of heparinized whole blood in a 50-mL conical tube, and add 47 mL of RBC lysing buffer (150 mM NH_4Cl, 10 mM $NaHCO_3$, 1 mM EDTA). Rotate the tubes for 10 min at room temperature on a bench rocker, and then centrifuge at 400*g* or 10 min. Wash the cells with PBS and adjust the concentration to 2.0×100 cell/mL. Take 1 mL of the resulting supernatant into the tube and centrifuge at 150 *g* for 10 min. Decant the supernatant and the resulting precipitate pellet was used for assay.

Analysis of hydrogen peroxide production. The cell suspension, preincubated for 15 min with DCFA-DA (20 μM, final concentration), was treated with tested compounds with gradient concentrations. The resulting suspension was added with PBS to give a final volume of 400 mL and stood for 10 min. After the addition of PMA as a trigger, the resulting suspension was incubated for 20 min and detected by a flow cytometer (FACScan, Becton Dickinson). Another cell suspensions with and without PMA were prepared as a positive control and a referred background, respectively.

DNA labeling analysis

To 3 mL of heparinized whole blood in a 50-mL conical tube was added 47 mL of RBC lysing buffer. The resulting mixture centrifuged for 10 min (400*g*). The supernatant was removed and the centrifuged cell was washed with PBS and incubated in RPMI 1640 medium (10% FBS, 1% P/S).

To the 24-well costar was transferred the cell suspension (1×10^6 cell/mL) followed by tested compounds and incubated at 37 °C for 24 h. The cell suspension was centrifuged (150*g*) for 10 min and the resulting precipitate was treated with 1 mL of propidium iodide solution (PI, 50 μg/mL in 0.1 μg/mL sodium citrate and Triton X-100, Sigma). The mixed cells were incubated for 24 h in the dark at 37° and measured using a flow cytometer (FACScan, Becton Dickinson), relative to a negative control.

Statistical analysis

The data are presented as the mean ± SD for the number of experiments indicated in the legends. Statistical analysis was evaluated using Student's t-test, and $P < 0.01$ was regarded as significantly different.

Acknowledgements

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