Bioorganic & Medicinal Chemistry 19 (2011) 2751–2756

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Inhibitory effects of Mannich bases of heterocyclic chalcones on NO production by activated RAW 264.7 macrophages and superoxide anion generation and elastase release by activated human neutrophils

M. Vijaya Bhaskar Reddy [a], Tsong-Long Hwang [b,†], Yann-Lii Leu [b], Wen-Fei Chiou [c], Tian-Shung Wu [a,d,*]

[a] Department of Chemistry, National Cheng Kung University, Tainan 701, Taiwan, ROC
[b] Graduate Institute of Natural Products, College of Medicine, Chang Gung University, 259 Taoyan, Taiwan, ROC
[c] National Research Institute of Chinese Medicine, Taipei 112, Taiwan, ROC
[d] College of Pharmacy and Chinese Medicine Research and Development Center, China Medical University, Taichung, Taiwan, ROC

ARTICLE INFO

Article history:
Received 11 January 2011
Revised 17 February 2011
Accepted 18 February 2011
Available online 8 March 2011

Keywords:
Mannich bases of heterocyclic chalcone analogs
NO production inhibition
($O_2^{\cdot-}$) generation
Elastase release
$[Ca^{2+}]_i$ mobilization

ABSTRACT

Some chalcones exert potent anti-inflammatory activities. Mannich bases of heterocyclic chalcones inhibited nitric oxide (NO) production in lipopolysaccharide and interferon-γ stimulated RAW 264.7 macrophages. Also Formyl-Met-Leu-Phe and cytochalasin B induced superoxide anion generation ($O_2^{\cdot-}$) and elastase release in human neutrophils. Mannich bases of heterocyclic chalcone analogs exhibited potent inhibitory effects on NO production with IC_{50} values ranges between 10.5 and 0.018 μM, $O_2^{\cdot-}$ generation (IC_{50} 39.87–0.68 μM) and elastase release (IC_{50} 39.74–0.95 μM). Compound **29** (IC_{50} 0.055 μM) and **34** (IC_{50} 0.018 μM) were showed excellent inhibition on NO production. On the other hand, compounds **2** and **8** showed potent inhibition on $O_2^{\cdot-}$ generation and elastase release. Therefore, these four compounds may be new leads for development of anti-inflammatory activities. The structure–activity relationships are also discussed.

1. Introduction

Many laboratories and clinical studies evidenced that oxidative stress imposed by reactive oxygen species (ROS) plays a crucial role in the pathophysiology associated with atherosclerosis, neurodegradative diseases and carcinogenesis.[1] Oxidative stress developed when there is an imbalance between the generations of ROS and their removal, resulting in potential cell damages. ROS species include free radicals such as hydroxyl radicals, peroxy radicals and superoxide anion radicals; and other reactive species like hydrogen peroxide, singlet oxygen and nitric oxide (NO). Among these ROS species, nitric oxide (NO) is a biologically active and paramagnetic free radical, which it has a reactive short half-life.[2,3] NO is an important mediator involved in the regulation of many physiological and pathological processes including neurotransmission and smooth muscle relaxation. The free radical nature of NO and high reactivity of superoxide ($O_2^{\cdot-}$), with subsequent generation of $ONOO^-$, renders NO to a potent pro-oxidant molecule inducing potential oxidative damages toward cellular targets.[4,5] The formation of NO is catalyzed by the enzyme nitric oxide synthase (NOS) via the NADPH- and O_2-dependent oxidation of L-arginine.[6] Three distinct isoforms of NOS have been identified including constitutive endothelial NOS (eNOS), neuronal NOS (nNOS) and inducible NOS (iNOS). The endothelial and neuronal NOS are predominantly presented in the vascular endothelium and nervous system, respectively. The iNOS generate high levels of NO that modulates inflammations through multiple pathways and plays an important role in the regulation of immune reactions.[7,8] Overproduction of NO by iNOS implicates a number of diverse physiological and pathological processes, such as vasodilation, nonspecific host defense, ischemia, reperfusion injury, chronic or acute inflammation, rheumatoid arthritis and onset of colitis.[9] A variety of exogenous stimuli, including lipopolysaccharide (LPS), interferon gamma (INFγ), tumor necrosis factor (TNFα), other induced ultraviolet radiation and ozone,[10,11] stimulate the expression of iNOS.

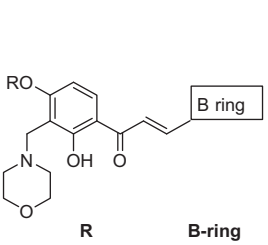
Human neutrophils are known to play important roles in the host defense against microorganisms. They are also critical in the pathogenesis of various diseases such as rheumatoid arthritis, ischemia, reperfusion injury, chronic obstructive pulmonary disease (COPD) and asthma.[12–16] In response to the diverse stimuli, activated neutrophils secrete series of cytotoxins, such as superoxide anion ($O_2^{\cdot-}$), granule proteases, and bioactive lipids. Despite this, currently there are only a few available agents that directly modulate neutrophils' pro-inflammatory responses in clinical practices. Therefore, the above mentioned inhibitory effects are

* Corresponding author. Tel.: +886 6 2747538; fax: +886 2 2740552.
E-mail address: tswu@mail.ncku.edu.tw (T.-S. Wu).
† This author has the same contribution as the first author.

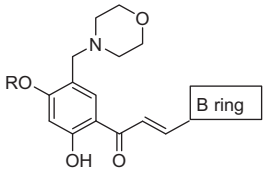
0968-0896/$ - see front matter Crown Copyright © 2011 Published by Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmc.2011.02.038

the important therapeutic targets for potential anti-inflammatory diseases.

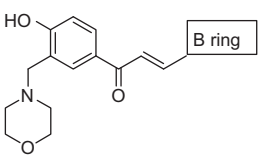
Mannich bases of chalcones and related compounds were displayed significant cytotoxicity towards murine P388 and L1210 leukemia cancer cell lines,[17] as well as a number of other tumor cell lines. Previously, we reported that the synthesis and biological evaluations of Mannich bases of heterocyclic chalcone analogs (**1**–**39**) (Scheme 1) are cytotoxic agents against four human cancer cell lines including PC-3 (prostate cancer), MCF-7 (human breast cancer), KB (nasopharyngeal carcinoma) and KB-VIN (vincristine-resistant KB subline).[18] To the best of our knowledge, there is no existing literatures focused on the inhibitory effects of NO production, $O_2^{\cdot-}$ generation and elastase release by Mannich bases of heterocyclic chalcones. In continuation of our interests



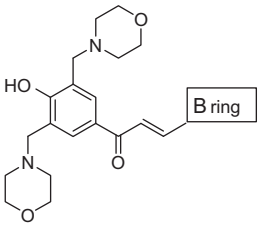
R	B-ring
1. H	2-pyridyl
2. Me	2-pyridyl
3. isopropyl	2-thiophene
4. prenyl	3-methyl-2-thiophene



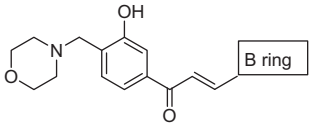
R	B-ring
5.ethyl	3-pyridyl
6. ethyl	2-furan
7. ethyl	5-methyl-2-furan
8. ethyl	2-pyridyl
9.ethyl	phenyl
10.ethyl	3-methyl-2-thiophene
11.methyl	3-pyridyl
12.methyl	phenyl
13.methyl	4-$CH_3OC_6H_4$
14.methyl	2-thiophene
15.methyl	2-furan
16.isopropyl	2-thiophene



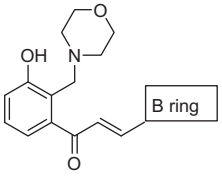
B-ring
17. 2-pyridyl
18. 3-pyridyl
19. 4-pyridyl
20. phenyl
21. 2-furan
22. 2-thiophene
23. 3-methyl-2-thiophene
24. 5-methyl-2-furan



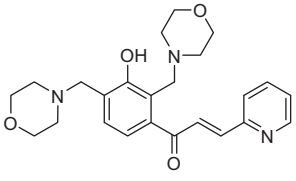
B-ring
25. 2-pyridyl
26. 3-methyl-2-thiophene



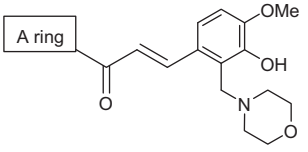
B-ring
27. 2-pyridyl
28. 3-pyridyl
29. 4-pyridyl



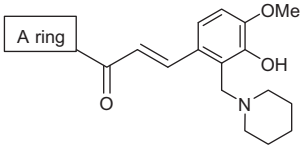
B-ring
30. 2-furan
31. 2-thiophene
32. 3-methyl-2-thiophene
33. phenyl



34.



A-ring
35. 3_6H_4-Me-C
36. 4-Me-C_6H_4



A-ring
37. 2-thiophene
38. 2-furan
39. 3-pyridyl

Scheme 1.

on Mannich bases of heterocyclic chalcone analogs, herein we describe the inhibition of NO production in LPS plus INF-γ activated RAW 264.7 macrophages in a preliminary in vitro test. Also cytotoxicity was evaluated by MTT (3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. Most of the compounds showed potent inhibition of NO production with IC_{50} values ranges between 10.5 and 0.018 μM comparing to the reference compound, AMG (amtolmetin guacyl) (IC_{50} 51.30 μM).

In addition, we examined the inhibitory effects on Formyl-Met-Leu-Phe (FMLP) and cytochalasin B (CB) stimulated $O_2^{\cdot-}$ generation and elastase release in human neutrophils. The IC_{50} values ranges between 0.68 and 39.87 μM and 0.95–39.74 μM, respectively. Among 39 compounds, **29** and **34** showed potent NO production inhibitions in LPS plus INF-γ activated RAW 264.7 macrophages with IC_{50} values of 0.055 and 0.018 μM, respectively. Compounds **2** and **8** showed potent inhibitory effects on FMLP/CB stimulated $O_2^{\cdot-}$ generation and elastase release in human neutrophils. In the further study, we evaluated compounds **2** and **21** for FMLP-activated increasing $[Ca^{2+}]_i$ mobilization in human neutrophils.

2. Results and discussions

To clarify the structure–activity relationships (SAR) of Mannich bases of heterocyclic chalcones **1**–**39**, the inhibition of NO production in LPS plus INF-γ activated RAW 264.7 macrophages, $O_2^{\cdot-}$ generation, and elastase release in FMLP/CB stimulated human neutrophils. Compounds **1** and **2** are 2-pyridyl group in ring-B; and hydroxyl and methoxyl groups at C-4′ position in ring-A, respectively. They showed equipotent inhibition of NO production in RAW 264.7 macrophages with an IC_{50} value of 1.7 μM. But **2** exhibited more potent inhibitory effects on FMLP/CB induced $O_2^{\cdot-}$ generation and elastase release with IC_{50} values of 0.68 and 0.95 μM than **1**($O_2^{\cdot-}$ IC_{50} 12.64 μM and elastase release IC_{50} of 21.17 μM). These results indicate that methoxy group at C-4′ position in which **2** is responsible for the enhancement of inhibitory effects on $O_2^{\cdot-}$ generation and elastase release in human neutrophils.

Analogs **3** and **16** are positional isomers, which have morpholine Mannich base group at C-3′ position and C-5′, respectively. Compound **3** (IC_{50} 2.21 μM) showed less inhibited NO production than **16** (IC_{50} 0.23 μM). Also turn **3** (IC_{50} 3.39 μM) showed more potent on $O_2^{\cdot-}$ generation than **16** (IC_{50} 19.39 μM). These results clearly indicate bulky Mannich base group at C-3′ position in **3** is weak in responding to inhibit NO production in RAW 264.7 cells. In addition, compound **4** (IC_{50} 0.93 μM) had prenyloxy group at C-4′ and 3-methyl-2-thiophene moiety as ring-B, showed more potent NO production inhibition than **3** (IC_{50} 2.21 μM).

Compounds **5–8** are 3-pyridyl, 2-furan, 5-methyl-2-furan and 2-pyridyl substitution pattern in ring-B, respectively, showed significant inhibition on NO production with IC_{50} values ranges between 10.50 and 16.50 μM. But with 3- and 2-pyridyl substitution in ring-B of compounds **5** and **8** exhibited more potent inhibition on $O_2^{\cdot-}$ generation in FMLP/CB induced human neutrophils with IC_{50} values of 2.42 and 0.89 μM, respectively. This indicated that pyridine ring was probably responsible for the enhancement of inhibition on $O_2^{\cdot-}$ generation and elastase release in human neutrophils.

The un-substituted phenyl group in B-ring, ethoxyl and methoxyl groups at C-4′ in **9** (IC_{50} 0.29 μM) and **12** (IC_{50} 0.20 μM), respectively, showed potent NO production inhibition in LPS plus IFN-γ induced RAW 264.7 macrophage cells.

Compound **14** (IC_{50} 3.61 μM) showed significantly inhibited NO production, when methoxyl group was replaced with isopropoxyl group at C-4′ position in ring-A, higher inhibition was observed in **16** (IC_{50} 0.23 μM). So isopropoxyl group at C-4′ position may be responsible for the enhancement of NO production inhibition in **16**. However, the reverse results were observed in the inhibition of $O_2^{\cdot-}$ generation for compounds **14** (IC_{50} 10.19 μM) and **16** (IC_{50} 19.39 μM).

Based on the in vitro preliminary results for compounds **5–16**, 3-pyridyl group as ring-B analogs (**5** and **11**), 2-pyridyl (**8**), phenyl (**9** and **12**) and 4-methoxyphenyl (**13**) showed more potent inhibitory effects in FMLP/CB induced $O_2^{\cdot-}$ generation than five membered heteroatomic ring-B substituted analogs (**6**, **7**, **10** and **14**–**16**).

Compounds **17**, **18** and **19** are 2, 3 and 4-pyridyl groups in ring-B, respectively. Among these, **18** (IC_{50} 0.82 μM) showed more potent NO production inhibition than **17** (IC_{50} 3.41 μM) and **19** (IC_{50} 4.14 μM). When the pyridyl moiety as ring-B was replaced with phenyl moiety, drastically decreased NO production inhibition was observed in compound **20** (IC_{50} 15.60 μM).

Compounds **21** (IC_{50} 0.16 μM) and **22** (IC_{50} 0.7 μM) are 2-furan and 2-thiophene groups as ring-B, respectively, were more potently inhibited NO production than methyl substituted analogs **23** (IC_{50} 1.39 μM) and **24** (IC_{50} 1.28 μM).

Analogs **27–29** had 2, 3 and 4-pyridyl moieties in ring-B, respectively, and hydroxyl and morpholine Mannich base groups at C-3′ and C-4′ positions, showed more potent inhibition on NO production in LPS plus INF-γ activated RAW 264.7 macrophages than the isomers **17–19**. The similar results were observed in FMLP/CB induced $O_2^{\cdot-}$ generation, elastase release, and inhibition in human neutrophils for compounds **27–29**. These results clearly indicate that C-4′ hydroxyl group has conjugation with carbonyl carbon in **17–19**, probably decreasing the inhibitory effects in both NO production in RAW 264.7 macrophage cell lines, $O_2^{\cdot-}$ generation and elastase release in human neutrophils.

Compounds **25** (IC_{50} 2.52 μM) and **26** (IC_{50} 0.44 μM) contained two Mannich base groups at C-3′ and C-5′ positions, showed more potent inhibitors of NO production than corresponding mono substituted Mannich base analogs **17** (IC_{50} 3.41 μM) and **23** (IC_{50} 1.39 μM). Analogs **30–33** significantly inhibited NO production with IC_{50} values ranges between 2.30 and 3.39 μM. Compound **34** (IC_{50} 0.018 μM) showed more potent inhibition of NO production than corresponding isomer **25** (IC_{50} 2.52 μM).

Compounds **21–26** and **30–32** have five membered heteroatomic substitution as ring-B, exhibited less inhibitory effects on both $O_2^{\cdot-}$ generation (IC_{50} 27.38–9.60 μM) and elastase release (IC_{50} 39.74 to −15.40 μM) in FMLP/CB induced human neutrophils.

Compound **34** with two Mannich base groups at C-2′ and C-4′ and hydroxyl group at C-3′ in ring-A, exhibited more potent inhibitory effects in FMLP/CB stimulated $O_2^{\cdot-}$ generation (IC_{50} 1.66 μM), elastase release (IC_{50} 1.66 μM) in human neutrophils, and inhibition of NO production (IC_{50} 0.018 μM) than **25**, which have Mannich base groups at C-3′ and C-5′ and hydroxyl at C-4′ position. The results indicate that hydroxyl group at C-4′ in ring-A was probably responsible for less inhibitory effects observed in all cases, due to hydroxyl group's conjugation with carbonyl carbon. The methyl substituted analogs **35** and **36** exhibited significant inhibitory effects on FMLP/CB stimulated $O_2^{\cdot-}$ generation and elastase release in human neutrophils (Tables 1–3).

3. Conclusions

In summary, a series of Mannich bases of heterocyclic chalcone analogs (**1**–**39**) were evaluated for their inhibition on NO production in activated RAW 264.7 macrophages, FMLP/CB induced $O_2^{\cdot-}$ generation, and elastase release in human neutrophils. Compounds **29** (IC_{50} 0.055 μM) and **34** (0.018 μM) exhibited potent inhibition on NO production in activated RAW 264.7 macrophages. The effects of compounds (**1–34**) on cell viability were determined by

Table 1
Nitric oxide production and cytotoxic effects of compounds **1**–**34** in LPS plus IFN-γ induced RAW 264.7 macrophages

Compound	NO inhibition IC_{50} (μM)	MTT assay IC_{50} (μM)
1	1.73	11.40
2	1.73	10.80
3	2.21	34.00
4	0.93	11.00
5	15.40	4.46
6	16.50	16.90
7	10.50	91.30
8	15.10	0.18
9	0.29	1.60
10	8.13	2060.00
11	11.80	2810.00
12	0.20	0.19
13	3.35	1860.00
14	3.61	148.00
15	6.06	1440.00
16	0.23	47.70
17	3.41	6.56
18	0.82	53.20
19	4.14	111.00
20	15.60	35.00
21	0.16	10.00
22	0.47	6830.00
23	1.39	ND[a]
24	1.28	0.006
25	2.52	190.00
26	0.44	56.90
27	0.72	78.00
28	0.23	ND
29	0.055	110.00
30	2.50	17.20
31	2.49	12.70
32	3.39	182.00
33	2.30	9.95
34	0.018	24.30
AMG	51.30	ND

[a] Not determined.

MTT assay in RAW 264.7 macrophages. No significant cytotoxicity was observed for compounds **29** (IC_{50} 110 μM) and **34** (24.30 μM) in RAW 264.7 macrophages. These data revealed that the inhibitory effect of compounds **29** and **34** on NO production at concentrations which were well below their cytotoxicity. However, some compounds (**5**, **6**, **12**, **17**, and **20**) had similar inhibitory potency for NO production and cytotoxicity. Therefore, we could not rule out the possibility that these compounds inhibited NO production due to their cytotoxicity in RAW 264.7 macrophages.

In addition, compounds **2** and **8** were potent inhibitors of FMLP/CB induced $O_2^{\cdot-}$ generation and elastase release in neutrophils. Further study showed that significant inhibition on FMLP-induced increase in $[Ca^{2+}]_i$ mobilization by compounds **2** was observed in human neutrophils. In contrast, **21** failed to change the $[Ca^{2+}]_i$ mobilization of human neutrophils caused by FMLP. The bacterial peptide FMLP is the first to be identified and is a highly potent leukocyte chemoattractant. FMLP activates neutrophils by binding to the G protein-coupled receptor (GPCR) on the membrane. Stimulation of GPCR induces the Ca^{2+} signal via activation of phospholipase C, which hydrolyzes phosphatidylinositol 4,5-bisphosphate into inositol trisphosphate and diacylglycerol, resulting in an increase in $[Ca^{2+}]_i$ and activation of PKC, respectively.[19] Many cellular functions of neutrophils, such as respiratory burst and degranulation, are regulated by the Ca^{2+} signals.[20] The magnitude and duration of $[Ca^{2+}]_i$ signal responses to GPCR are obviously important. Increases in $[Ca^{2+}]_i$ have profound effects on neutrophils, including the initiation of cytoskeletal changes, degranulation, and respiratory burst.[20,21] Our results suggest that the inhibition of human neutrophil $O_2^{\cdot-}$ generation and elastase release by compound **2** is through attenuation of calcium signaling pathway (Fig. 1).

Table 2
Effects of compounds on superoxide anion generation and elastase release by human neutrophils in response to FMLP/CB

Compound	Superoxide anion IC_{50} (μM)[a] or (Inh%)	Elastase IC_{50} (μM)[a] or (Inh%)
1	12.64 ± 0.36	21.17 ± 2.91
2	0.68 ± 0.05	0.95 ± 0.04
3	3.39 ± 0.22	NT[b]
4	(21.54 ± 3.41)**	NT[c]
5	2.42 ± 0.37	3.48 ± 0.29
6	(39.87 ± 4.67)**	NT[c]
7	19.95 ± 1.29	NT[c]
8	0.89 ± 0.07	1.24 ± 0.16
9	3.35 ± 0.24	NT[c]
10	19.95 ± 1.29	NT[c]
11	2.65 ± 0.17	4.52 ± 0.60
12	2.86 ± 0.06	NT[c]
13	5.77 ± 0.80	NT[c]
14	10.19 ± 0.92	NT[c]
15	15.81 ± 1.49	(17.92 ± 2.05)**
16	19.39 ± 3.82	NT[c]
17	15.17 ± 0.65	20.85 ± 2.54
18	23.52 ± 2.74	(20.38 ± 5.69)*
19	8.85 ± 0.47	13.65 ± 1.19
20	16.57 ± 1.44	(9.32 ± 2.41)*
21	(27.38 ± 4.00)**	(16.17 ± 6.00)
22	25.39 ± 2.24	(−19.29 ± 5.58)**
23	16.40 ± 3.43	18.20 ± 1.54
24	25.57 ± 1.81	(13.50 ± 5.52)
25	14.77 ± 0.68	18.89 ± 4.18
26	(33.78 ± 2,61)***	(−15.40 ± 4.79)*
27	2.63 ± 0.11	2.47 ± 0.67
28	5.96 ± 1.15	6.66 ± 0.82
29	1.60 ± 0.18	2.28 ± 0.42
30	18.19 ± 1.16	(39.74 ± 4.48)***
31	10.48 ± 1.06	24.31 ± 1.40
32	9.60 ± 1.77	18.61 ± 2.92
33	2.81 ± 0.43	5.19 ± 1.06
34	1.66 ± 0.43	1.44 ± 0.13
35	3.16 ± 0.03	2.95 ± 0.80
36	2.76 ± 0.16	6.97 ± 1.77
37	8.32 ± 0.82	NT[c]
38	4.18 ± 0.26	NT[c]
39	13.03 ± 1.73	20.08 ± 2.43
DPI[d]	1.02 ± 0.35	NT
PMSF[d]	NT	95.0 ± 25

Percentage of inhibition (Inh%) at 30 μM concentration. Results are presented as mean ± S.E.M. (n = 3–4). *P <0.05, **P <0.01, ***P <0.001 compared with the control value.

[a] Concentration necessary for 50% inhibition (IC_{50}).

[b] Compound **25** reacted with substrate.

[c] Compounds **4**, **6**, **7**, **9**, **10**, **12**–**14**, **16**, **37** and **38** had a strong absorbance at 405 nm.

[d] Diphenyleneiodonium (DPI, a NADPH oxidase inhibitor) and phenylmethylsulfonylfluoride (PMSF, a serine protease inhibitor) were used as the positive controls in the generation of superoxide anion and elastase release, respectively.

Table 3
Effects of compounds **2** and **21**, on the peak $[Ca^{2+}]_i$ and the time taken for this concentration to decline to half of its peak value ($t_{1/2}$) in FMLP-activated neutrophils

Drugs	Peak $[Ca^{2+}]_i$ values (nM)	Time taken to decline to half peak values ($t_{1/2}$) (s)
DMSO	280.75 ± 8.91	31.47 ± 3.90
2 (10 μM)	208.65 ± 15.35*	8.43 ± 1.42**
21 (10 μM)	276.51 ± 6.79	28.10 ± 2.11

Neutrophils labeled with fluo-3AM as described under Section 4 were stimulated with 0.1 μM FMLP in the presence of 1 mM Ca^{2+}, and fluorescence was monitored at 37 °C with stirring. All data are expressed as mean ± SEM. *P <0.05, **P <0.01, compared to the control value.

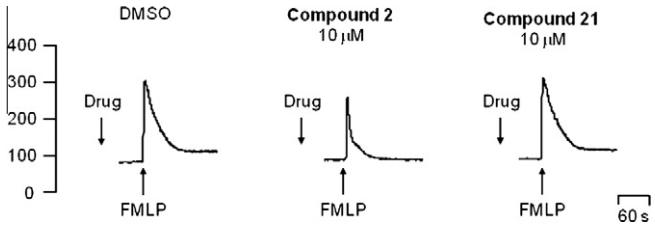


Figure 1. Typical traces of the effect of compounds **2** and **21** on mobilization in FMLP-activated human neutrophils. Human neutrophils were incubated with compounds **2** (10 μM) and **21** (10 μM) for 5 min before stimulation with FMLP. Mobilization of Ca^{2+} was determined in real time in a spectrofluorometer.

4. Materials and methods

4.1. Mannich bases of heterocyclic chalcone analogs

Thirty nine Mannich bases of heterocyclic derivatives (**1–39**) were synthesized as described previously,[18] and their chemical structures are shown in Scheme 1. The structures were confirmed by IR, ^{1}H, ^{13}C NMR, EIMS, HREIMS and elemental analysis.

4.2. Cell culture

The murine macrophages cell line, RAW 264.7 cells, was obtained from the American Type Culture Collection (ATCC, TIB 71, Rockville, MD). Cells were cultured in 75 cm^2 plastic flasks (Corning–Costar) with Dulbecco's modified Eagle's medium (DMEM), supplemented with antibiotics (100 U mL^{-1} penicillin A and 100 μg mL^{-1} of streptomycin), and 10% heat-inactivated fetal calf serum (FCS), and maintained in a 37 °C humidified incubator containing 5% CO_2 in air for 1 h. Non-adherent cells were removed by various pipetting after centrifugation, and adherent cells were cultured in 96-well plates with 200 μL of cultured medium reached until confluence (approximately 200,000 cells per well) followed by adding fresh medium containing LPS (1 μg mL^{-1}) and IFN-γ (50 U mL^{-1}).

4.3. Nitrite measurement

The RAW 264.7 cells were cultured in order to induce iNOS, fresh cultured medium containing LPS (1 μg mL^{-1}) and IFN-γ (50 U mL^{-1}) was added. Nitrite accumulation in the medium was measured at 24 h with the application of LPS (1 μg mL^{-1}) plus IFN-γ (50 U mL^{-1}). To assay the effect of drugs on nitrite production, compounds were added together with LPS/IFN-γ. Nitrite was measured by adding 100 μL of Griess reagent (1% sulfanilamide and 0.1% naphthylenediamine in 5% phosphoric acid) to 100 μL samples of medium. The optical density of 550 nm (OD_{550}) was measured with a microplate reader. Concentrations were calculated by comparison with OD_{550} of standard solutions of sodium nitrite prepared in the cultured medium.

4.4. Cell viability by MTT assay

Cell viability was determined by the mitochondria-dependent reduction of MTT [3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide] to formazan. Cells in 96-well plates were incubated at 37 °C with MTT (5 mg mL^{-1} for 4 h). Cultured medium was gently aspirated from each well, and then the MTT crystals were dissolved in acid-SDS (100 μL). The reduction of the MTT to formazan within the cells were quantitated by measurement of OD_{570} against OD_{630}.

4.5. Preparation of human neutrophils

Blood was taken from healthy human donors (20–32 years old) by veinpuncture, using a protocol approved by the institutional review board at Chang Gung Memorial Hospital. Neutrophils were isolated with a standard method of dextran sedimentation prior to centrifugation in a Ficoll Hypaque gradient and hypotonic lysis of erythrocytes.[22,23] Purified neutrophils that contained >98% viable cells, as determined by the trypan blue exclusion method, were re-suspended in a calcium (Ca^{2+})-free HBSS buffer at pH 7.4, and were maintained at 4 °C before use.

4.6. Measurement of superoxide anion ($O_2^{\cdot-}$) generation

The assay of $O_2^{\cdot-}$ generation was based on the SOD-inhibitable reduction of ferricytochrome *c*.[24] In brief, after supplementation with 0.5 mg/ml ferricytochrome *c* and 1 mM Ca^{2+}, neutrophils were equilibrated at 37 °C for 2 min and incubated with drugs for 5 min. Cells were activated with 100 nM FMLP for 10 min. When FMLP was used as a stimulant, cytochalasin B (CB, 1 μg/ml) was incubated for 3 min before activation by the peptide (FMLP/CB). Changes in absorbance with the reduction of ferricytochrome *c* at 550 nm were continuously monitored in a double-beam, six-cell positioned spectrophotometer with constant stirring (Hitachi U-3010, Tokyo, Japan).

4.7. Measurement of elastase release

Degranulation of azurophilic granules was determined by elastase release as described previously.[22] Experiments were performed using MeO-Suc-Ala-Ala-Pro-Val-*p*-nitroanilide as the elastase substrate. Briefly, after supplementation with MeO-Suc-Ala-Ala-Pro-Val-*p*-nitroanilide (100 μM), neutrophils (6×10^5 ml^{-1}) were equilibrated at 37 °C for 2 min and incubated with drugs for 5 min. Cells were activated by 100 nM FMLP and 0.5 μg ml^{-1} CB, and changes in absorbance at 405 nm were continuously monitored to assay elastase release. The results were expressed as the percentage of elastase release in the FMLP/CB-activated, drug-free control system.

4.8. Measurement of intracellular calcium concentration ($[Ca^{2+}]_i$)

Neutrophils were loaded with 2 μM fluo-3 AM at 37 °C for 45 min. After being washed, cells were re-suspended in Ca^{2+}-free HBSS to 3×10^6 cells/ml. The change in fluorescence was monitored using a Hitachi F-4500 spectrofluorometer (Tokyo, Japan) in a quartz cuvette with a thermostat (37 °C) and continuous stirring. The excitation wavelength was 488 nm, and the emission wavelength was 520 nm. FMLP (0.1 μM) was used to increase $[Ca^{2+}]_i$ in the presence or 1 mM Ca^{2+}. $[Ca^{2+}]_i$ was calibrated by the fluorescence intensity as follows: $[Ca^{2+}]_i = K_d \times [(F - F_{min})/(F_{max} - F)]$ *where, F* is the observed fluorescence intensity, F_{max} and F_{min} were obtained by the addition of 0.05% Triton X-100 and 20 mM EGTA, respectively; and K_d was taken to 400 nM.

Statistical analysis: Results are expressed as the mean ± S.E.M., and comparisons were made using Student's *t*-test. A probability of 0.05 or less was considered significant.

Acknowledgments

This work was supported by a grant from National Science Council, Taiwan, Republic of China, and Grant No.(OUA 95-3-2-021) from the National Cheng Kung University, Tainan, Taiwan R.O.C was awarded to T.S. Wu. This study is supported in part by Taiwan Department of Health Clinical Trail and Research Center of Excellence (DOH100-TD-B-111-004).

References and notes

1. Scandalios, J. G. *Braz. J. Med. Biol. Res.* **2005**, *38*, 995.
2. Palmer, R. M. J.; Ferrige, A. G.; Moncada, S. *Nature* **1987**, *327*, 524.
3. Beckman, J. S.; Beckman, T. W.; Chen, J.; Marshall, P. A.; Freeman, B. A. *Proc. Natl. Acad. Sci.* **1990**, *87*, 1620.
4. Luperchio, S.; Tamir, S.; Tannenbaum, S. R. *Free Radic. Biol. Med.* **1995**, *21*, 513.
5. Epe, B.; Ballmaier, D.; Roussyn, I.; Brivida, K.; Sies, H. *Nucleic Acids Res.* **1996**, *24*, 4105.
6. Nathan, C.; Xie, Q. W. *Cell* **1994**, *78*, 915.
7. Groves, J. T.; Wang, C. C. Y. *Curr. Opin. Chem. Biol.* **2000**, *4*, 687.
8. Aktan, F. *Life Sci.* **2004**, *75*, 639.
9. Kroncke, K. D.; Fehsel, K.; Kolb-Bachofen, V. *Clin. Exp. Immunol.* **1998**, *113*, 147.
10. Warren, J. B. *FASEB J.* **1994**, *8*, 247.
11. Pendino, K. J.; Laskin, J. D.; Shuler, R. L.; Punjabi, C. J.; Laskin, D. L. *J. Immunol.* **1993**, *151*, 7196.
12. Malech, H. L.; Gallin, J. I. *N. Engl. J. Med.* **1987**, *317*, 687.
13. Witko-Sarsat, V.; Rieu, P.; Descamps-Latscha, B.; Lesarve, P.; Halbwachs-Mecarelli, L. *Lab. Invest.* **2000**, *80*, 617.
14. Okajima, K.; Harada, N.; Uchiba, M. *J. Pharmacol. Exp. Ther.* **2002**, *301*, 1157.
15. Ennis, M. *Curr. Allergy Asthma Rep.* **2003**, *3*, 159.
16. Vinten-Johansen, J. *Cardiovasc. Res.* **2004**, *61*, 481.
17. Dimmock, J. R.; Kandepu, M. N.; Hetherington, M.; Quail, J. W.; Pugazhenthi, U.; Sudom, A. M.; Chamankhah, M.; Rose, P.; Pass, E.; Allan, T. M.; Halleran, S.; Szydlowski, J.; Mutus, B.; Tannous, M.; Manavathu, E. K.; Myers, T. G.; Clercq, E. D.; Balzarini, J. *J. Med. Chem.* **1998**, *41*, 1014.
18. Vijaya Bhaskar reddy, M.; Su, C. R.; Chiou, W. F.; Liu, Y. N.; Chen, R. Y. H.; Bastow, K. F.; Lee, K. H.; Wu, T. S. *Bioorg. Med. Chem.* **2008**, *16*, 7358.
19. Downey, G. P.; Fukushima, T.; Fialkow, L. *Curr. Opin. Hematol.* **1995**, *2*, 76.
20. Hwang, T. L.; Su, Y. C.; Chang, H. L.; Leu, Y. L.; Chung, P. J.; Kuo, L. M. *J. Lipid Res.* **2009**, *50*, 1395.
21. Tintinger, G.; Steel, H. C.; Anderson, R. *Clin. Exp. Immunol.* **2005**, *141*, 191.
22. Hwang, T. L.; Li, G. L.; Lan, Y. H.; Chia, Y. C.; Hsieh, P. W.; Wu, Y. H.; Wu, Y. C. *Free Radic. Biol. Med.* **2009**, *15*, 520.
23. Boyum, A.; Lovhaug, D.; Tresland, L.; Nordlie, E. M. *Scand. J. Immunol.* **1991**, *34*, 697.
24. Babior, B. M.; Kipnes, R. S.; Curnutte, J. T. *J. Clin. Invest.* **1973**, *52*, 741.