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Der Pharmacia Lettre, 2016, 8 (20):172-176
(http://scholarsresearchlibrary.com/archive.html)

Der Pharmacia Lettre
Scholars Research Library
ISSN 0975-5071
USA CODEN: DPLEB4

Inhibitory effects of Bichalcone derivatives on Superoxide anion generation ($O_2^{\bullet-}$) and elastase release by activated human neutrophils in response to FMLP/CB

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ABSTRACT

*Chalcones have a distinctive 1,3-diaryl propenone skeleton and exert numerous biological effects. Previously we synthesized (**1a-23**) bichalcones through the piperazine Mannich base linkage with different substitution on the both ring-B of the chalcone moieties. These compounds were subjected into the inhibitory effects on Formyl-Met-Leu-Phe (FMLP) and cytochalasin B (CB) stimulated $O_2^{\bullet-}$ generation and elastase release in human neutrophils. Among the tested compounds, **3-23** were exhibited potent elastase release inhibitory effects in activated human neutrophils with the IC_{50} values ranges from 4.98 μM to 50.87 μM*

Keywords: Mannich base linked bichalcones, superoxide generation ($O_2^{\bullet-}$) and elastase release.

INTRODUCTION

Chalcones possess a 1,3-diphenyl-2-propen-1-one basic skeleton in which two aromatic rings are connected by a three carbon *α, β*-unsaturated carbonyl system. They are intermediates in the biosynthesis of flavonoids, which are substances widespread in plants and with an array of biological activities; however, their structure differs considerably from the other members of the flavonoids family [1].Chalcones are reported with diverse biological activities including antihyperglycemic, [2] antibacterial,[3] antiplatelet,[4]antiulcerative,[5] antimalarial,[6] antiviral,[7] antileishmanial,[8] antioxidant,[9] antitubercular, [10] tyrosinase inhibiting,[11] anti-inflammatory,[12] and analgesic activities [13].

Neutrophils play a pivotal role in the defense of the human body against infections. However, overwhelming activation of neutrophils is known to elicit tissue damage. Human neutrophils are known to play important roles in the pathogenesis of various diseases, such as ischemic heart disease, acute myocardial infarction, sepsis, and atherogenesis [14–17]. In response to diverse stimuli, activated neutrophils secrete a series of cytotoxins, such as the superoxide anion ($O_2^{\bullet-}$), a precursor of other ROS, granule proteases, and bioactive lipids [18-19]. $O_2^{\bullet-}$ production is linked to the killing of invading microorganisms, but it can also directly or indirectly cause damage by destroying surrounding tissues. Neutrophil granules contain many antimicrobial and potentially cytotoxic substances. Neutrophil elastase is a major secreted product of stimulated neutrophils and a major contributor to the destruction

of tissue in chronic inflammatory disease [20]. Therefore, it is crucial to restrain respiratory burst and degranulation in physiological conditions while potentiating these functions in infected tissues and organs. Previously we synthesized [21] a series of bichalcone analogs through the piperazine Mannich base linkage with different substitutions on the ring-B of the chalcone moiety (**1a-23**) (Figure 1) were displayed potent activity against four human cancer cell lines including KB, A549, HCT-8 and DU145 and mechanism action of compound **23** in the HT-29 human colon adenocarcinoma cell line. In addition to this, compounds **3-23** were tested for the inhibition of nitric oxide (NO) production in murine microglial cells, **4** (IC_{50} 0.34 μM) and **11** (IC_{50} 0.5 μM) were exhibited more potent than a specific NOS inhibitor L-NAME (L-nitroarginine methyl ester) (IC_{50} 18.9 μM) [21]. Considering the pharmacological importance of bichalcone analogs (**1a-23)** through the piperazine Mannich base linkage with different substitution pattern in the ring-B, herein we describe the respiratory burst and degranulation caused by the inhibitory effects of superoxide anion generation ($O_2^{\bullet-}$) and elastase release in response to Fmlp/CB induced human neutrophils (Table 1). In this study we found that Mannich base linked bichalcones analogs were significantly inhibited in superoxide anion generation ($O_2^{\bullet-}$) and potent inhibition was observed in elastase release.

MATERIALS AND METHODS

Materials Bichalcone derivatives were synthesized (published results) [21] and dissolved in dimethyl sulfoxide (DMSO) to make stock solutions. Aprotinin, *N*-(2-((*p*-bromocinnamyl)amino)ethyl)-5-isoquinolinesulfonamide (H89), KT5720 (9*S*,10*S*,12*R*-2,3,9,10,11,12-hexahydro-10-hydroxy-9-methyl-1-oxo-9,12-epoxy-1*H*-diindolo(1,2,3-fg:3′,2′,1′-kl)pyrrolo(3,4-i)(1,6)benzodiazocine-10-carboxylic acid hexyl ester), leupeptin, phenylmethylsulfonyl fluoride (PMSF), 3-(1-(3-(amidinothio)propyl-1*H*-indol-3-yl))-3-(1-methyl-1*H*-indol-3-yl)maleimide (Ro318220), rolipram, and zaprinast were obtained from Calbiochem (La Jolla, CA, U.S.A.). Fluo-3 AM was purchased from Molecular Probes (Eugene, OR, U.S.A.). 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium monosodium salt (WST-1) was purchased from Dojindo Laboratories (Kumamoto, Japan). All other chemicals were obtained from Sigma (St Louis, MO, U.S.A.). When drugs were dissolved in DMSO, the final concentration of DMSO in cell experiments did not exceed 0.5% and did not affect the parameters measured.

Preparation of human neutrophils:
Blood was taken from healthy human donors (20~32 years old) by venipuncture, 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 resuspended in a calcium (Ca^{2+})-free HBSS buffer at pH 7.4, and were maintained at 4 °C before use.

Measurement of $O_2^{\bullet-}$ generation:
The assay of $O_2^{\bullet-}$ 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, 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 positioner spectrophotometer with constant stirring (Hitachi U-3010, Tokyo, Japan). Calculations were based on differences in the reactions with and without SOD (100 U/ml) divided by the extinction coefficient for the reduction of ferricytochrome *c* (ε = 21.1/mM/10 mm).

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 (5 x 10^5/ml) were equilibrated at 37 °C for 2 min and incubated with drugs for 5 min. Cells were activated by100 Nm fMLP and 0.5 μg/ml CB, and changes in absorbance at 405 nm were continuously monitored to assay elastase release. The results are expressed as the percent of the initial rate of elastase release in the fMLP/CB-activated, drug-free control system.

RESULTS AND DISCUSSION

We examined the inhibitory effects on Formyl-Met-Leu-Phe (FMLP) and cytochalasin B (CB) stimulated $O_2^{\bullet-}$ generation and elastase release in human neutrophils. Compounds **5** and **8** are 2-furan and 5-methyl-2-furan groups

in ring-B, respectively. Among these, **5** exhibited potent inhibitory effects on both superoxide generation ($O_2^{\bullet-}$) and elastase release in FMLP/CB induced human neutrophils. Compound **3** (IC_{50} 19.73 μM) was more potent than **20** (IC_{50} 35.36 μM), in elastase release inhibition, and they have only one structural difference. Both compounds possess a 2-pyridyl group as ring-B, but an additional methoxy group at C-3 position in **20** was responsible for decrease the elastase release inhibition. Compound **6** (IC_{50} 11.87 μM) was more potent than **7** (IC_{50} 35.67 μM) in elastase release inhibition, and the corresponding methoxy derivative **15** (IC_{50} 28.60 μM) exhibited less elastase inhibition observed in human neutrophils. These results indicate that methyl group on thiophene ring and methoxy group on benzene ring caused for the decrease elastase inhibition observed in compounds **7** and **15**. Compounds **10** (IC_{50} 3.46 μM), **11** (IC_{50} 6.20 μM) and **12** (IC_{50}3.52 μM) having phenyl, 4-methoxyphenyl and 3,4-methylenedioxy benzene groups in ring-B, respectively, were exhibited more potent inhibition observed in Fmlp/CB induced in superoxide anion generation ($O_2^{\bullet-}$). Compound **7** (IC_{50} 0.08 μM) is more potent than the corresponding methoxy derivative **16** (IC_{50} 2.92 μM) in superoxide anion generation, indicating that methoxy functionality was responsible for decrease the of superoxide anion generation inhibition in Fmlp/CB induced human neutrophils . Compounds **21** (IC_{50}19.35 μM) and **22** (IC_{50} 4.98 μM) are having pyrrole and N-methylpyrrole moieties in ring-B. These results clearly indicate methyl group play a major role for enhance the inhibition of elastase release in human neutrophils but compound **22** (IC_{50} 4.98 μM) was more potent elastase release inhibition observed than the corresponding bichalcone derivative **9** (IC_{50} 23.05 μM) in human neutrophils. Compound **23** (IC_{50} 6.67 μM) exhibited more potent inhibition observed in superoxide anion generation than **4** (IC_{50} 26.08 μM). Compound **19** having 2-chlorobenzene as ring-B of both chalcone moieties, which was potently inhibited Fmlp/CB induced superoxide anion generation in human neutrophils.

Based on the *in vitro* preliminary results observed for compounds**1a-23** in FMLP/CB stimulated elastase release in human neutrophils with IC_{50} values ranging from 4.98 μM to 50.87 μM compared to control PMSF (IC_{50} 95.0 μM). These data could be provided as the bichalcones linked with the Mannich base group for further design and development of anti-inflammatory agents.

Table 1 Effects of compounds (1a-23) 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 %)
1a	(15.33 ± 2.29)**	(-2.65 ± 4.43)
2a	(15.46 ± 2.76)**	(-4.90 ± 3.20)
3	(22.24 ± 3.46)**	(19.73 ± 6.01)*
4	(26.08 ± 3.78)**	(21.43 ± 3.11)**
5	(13.34 ± 3.16)*	(7.88 ± 2.45)*
6	(-1.29 ± 1.97)	(11.87 ± 3.26)*
7	(0.08 ± 0.79)	(35.67 ± 7.16)**
8	(26.68 ± 1.80)***	(14.47 ± 7.44)
9	(-0.73 ± 1.90)	(23.05 ± 4.07)**
10	(3.46 ± 3.09)	(18.74 ± 7.64)
11	(6.20 ± 2.56)	(36.95 ± 2.00)***
12	(3.52 ± 2.26)	(50.87 ± 7.22)**
13	(-2.31 ±1.40)	(23.08 ± 2.04)***
14	(10.20± 3.76)	(20.24 ± 2.24)***
15	(-1.40 ±2.69)	(28.60 ± 2.24)***
16	(2.92 ± 2.97)	(26.30 ± 1.70)***
17	(-0.06± 2.71)	(23.71 ± 3.21)**
18	(5.32 ± 0.94)**	(33.32 ± 2.27)***
19	(4.72 ± 1.77)	(31.55 ± 5.58)**
20	(27.27±6.27)*	(35.36 ± 7.54)**
21	(10.37 ± 6.39)	(19.35 ± 5.52)*
22	(-2.97± 6.58)	(4.98 ± 5.28)
23	(6.67 ± 1.18)	(27.95 ± 2.34)***
DPI[b]	1.02 ± 0.35	N
PMSF[b]	N	95.0 ± 25

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

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

[b]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.

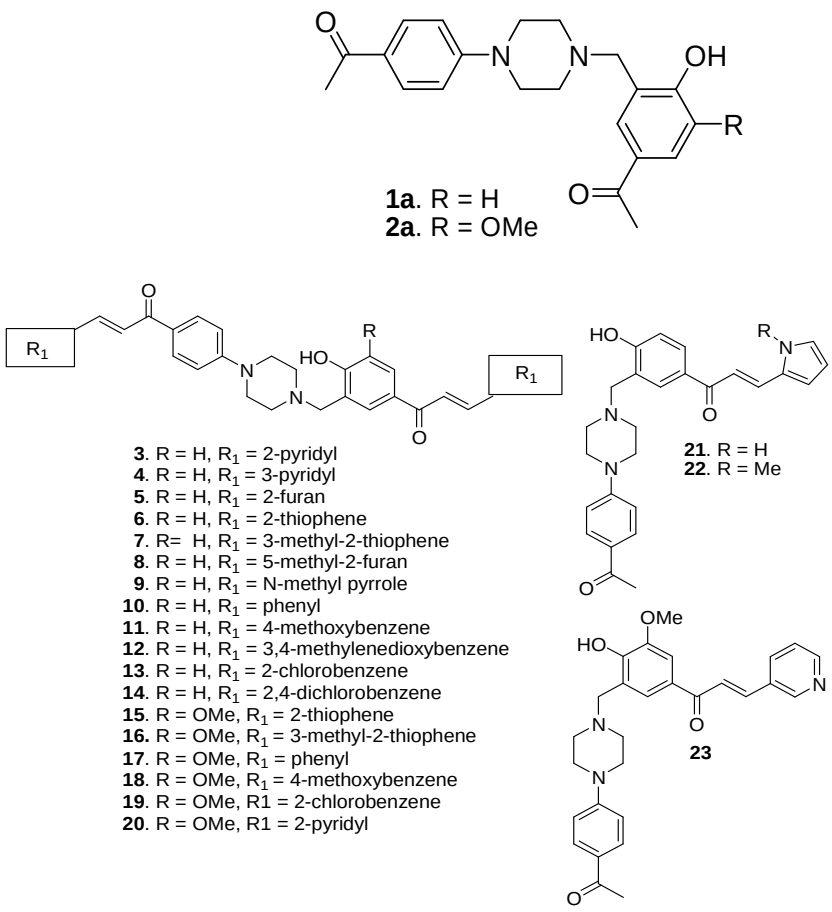

Figure 1: Bichalcone analogs 3-23

CONCLUSION

Present study described the inhibitory effects on Formyl-Met-Leu-Phe (FMLP) and cytochalasin B (CB) stimulated $O_2^{\bullet-}$ generation and elastase release in human neutrophils and the most active compounds with the IC_{50} values **5** (IC_{50} 7.88 μM), **6** (IC_{50} 11.87 μM), **21** (IC_{50} 19.35 μM) and **22** (IC_{50} 4.98 μM), respectively, against the inhibition of elastase release in activated human neutrophils.

Acknowledgments
This work was supported by a grant of 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 awarded to T. S. Wu. This study is supported in part by Taiwan Department of Health Clinical Trial and Research Center of Excellence (DOH100-TD-B-111-004).

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