European Journal of Medicinal Chemistry 45 (2010) 3111–3115

Contents lists available at ScienceDirect

European Journal of Medicinal Chemistry

journal homepage: http://www.elsevier.com/locate/ejmech

Original article

Synthesis and evaluation of benzoxazinone derivatives on activity of human neutrophil elastase and on hemorrhagic shock-induced lung injury in rats

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ARTICLE INFO

Article history:
Received 17 November 2009
Received in revised form
25 March 2010
Accepted 29 March 2010
Available online 3 April 2010

Keywords:
Human neutrophil elastase
Benzoxazinones
Superoxide anion
Hemorrhagic shock
Anti-inflammatory agents

ABSTRACT

A new series of benzoxazinone analogs were designed, synthesized, and assayed to determine their effects on superoxide anion generation and neutrophil elastase (NE) release in formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP)-activated human neutrophils. Of these, compounds **6**–**10** showed a potent dual inhibitory effect on NE release and superoxide anion generation. In contrast, compounds **11**–**15** exhibited highly selective and potent inhibitory activities on NE release. These results indicate that the inhibitory activity on NE release in FMLP-activated human neutrophils depended on the position of chloro-substituent in the A ring. On the other hand, **13** significantly attenuated the increase in myeloperoxidase (MPO) activity and edema in the lung of rats after trauma-hemorrhagic shock. Therefore, these compounds could be developed as new NE inhibitors.

1. Introduction

Neutrophils play a pivotal role in the defense of the human body against infections. In addition, neutrophil infiltration is a common pathological feature in acute inflammatory disorders [1]. In response to diverse stimuli, activated neutrophils secrete a series of cytotoxins, such as superoxide anion ($O_2^{\cdot-}$), a precursor of other reactive oxygen species, granule proteases, and bioactive lipids [2]. Of these, neutrophil elastase (NE) a 30-kDa glycoprotein chymotrypsin-like serine proteinase is stored in the azurophil granules of neutrophils in its active form and is released following neutrophil exposure to inflammatory stimuli [3]. Although a vigorous response by neutrophils to infection and injury is necessary for host defense, overly aggressive or inappropriate neutrophil responses can result in deleterious inflammatory conditions and tissue destruction [1]. High concentrations of reactive oxygen species (ROS) and NE produced by activated neutrophils in the sputum of patients has been implicated in the pathogenesis of many acute and chronic pulmonary diseases including asthma, chronic obstructive pulmonary disease, cystic fibrosis, and acute respiratory distress syndrome [3,4,5]. Of these, NE is a major secreted product of stimulated neutrophils and a major contributor to the destruction of tissue in chronic inflammatory disease [6,7]. For example, NE exists in high concentrations in the airway secretions of patients with chronic inflammatory airway diseases and induces overproduction of MUC5AC mucin, a major component of airway mucus [8]. In addition, NE is present within atherosclerotic plaques where it contributes to matrix degradation and weakening of the vessel wall associated with complications such as aneurysm formation and plaque rupture [9]. Furthermore, high NE activity is found in synovial fluid from patients with non-infectious knee joint synovitis and rheumatoid arthritis [10].

NE has a broad substrate specificity to break down extracellular matrices, cytokines, clotting factors, adhesion molecules, and components of the complement cascade [11,12,13]. Potential substrates of NE include extracellular matrices, cytokines, clotting factors, adhesion molecules, and components of the complement cascade [11,12]. As a result of this broad substrate specificity and the ability of this enzyme to produce tissue injury, NE has been involved in the pathogenesis of various inflammatory conditions and indeed it is often used as both a predictor and an indicator of inflammatory disease severity [12]. Since NE is important in the pathogenesis of inflammatory diseases, NE may represent a potential target in the development of new anti-inflammatory agents. However, there are currently only a few agents available that

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doi:10.1016/j.ejmech.2010.03.046

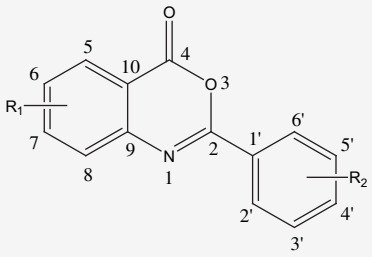
directly modulate NE responses in clinical practice. Therefore, research and development of a new generation of anti-inflammatory agents are needed and are in progress.

Substituted benzoxazin-4-ones have been well characterized as heterocyclic acylating agents of serine proteases [14]. These compounds inhibit serine proteases according to a mechanism which involves an acyl enzyme intermediate [14]. Many benzoxazin-4-ones have been synthesized and assayed for their effects on NE activities [15,16,17,18,19,20]. Furthermore, in a previous study, we synthesized and evaluated the effects of a series of 2,8-substituted benzoxazinones on the inhibition of NE release and $O_2^{\bullet-}$ generation in formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP)-activated human neutrophils [3]. Of these compounds, most of the 2-phenyl-8-chloro-benzoxazinones (**1**–**5**) showed a dual inhibitory effect on NE release and $O_2^{\bullet-}$ generation. In the present study, in order to examine the structural determinants required for the anti-inflammatory bioactivity of benzoxazinones and to develop more potent and selective inhibitors of NE, a new series of chloro-substituted 2-phenylbenzoxazinones were synthesized and pharmacologically characterized.

2. Chemistry

In a previous SAR study, the compounds with a substitution on the 6-position were found to be unfavorable [14]. Therefore, in the present investigation, a series of 5-/ or 7-substituted 2-phenylbenzoxazinone derivates were prepared by reacting 2-amino-4-chlorobenzoic acid and 2-amino-6-chlorobenzoic acid with the corresponding substituted benzoyl chlorides as described in a previous pathway [3,21]. All compounds (**6**–**15**, Table 1) were pure and were characterized by spectroscopic data, as shown in the Section 5.

Table 1
Anti-inflammatory activities of synthetics in FMLP-activated human neutrophils.



Compounds	Structures		IC_{50} (nM)[a]	
	R_1	R_2	$O2^{\bullet-}$ generation	NE release
DPI[b]			969.7 ± 394.6	NT
PMSF[b]			NT[c]	109518.5 ± 25039.7
Elastatinal[b]			NT	56450.0 ± 9130.0
1	8–Cl	2′–F	gt;25000[d]	6030.0 ± 650.0[d]
2	8–Cl	2′–Cl	8660.0 ± 1440.0[d]	1890.0 ± 1100.0[d]
3	8–Cl	2′–Br	6560.0 ± 3090.0[d]	1690.0 ± 300.0[d]
4	8–Cl	2′–CH_3	17490.0 ± 2690.0[d]	gt;25000[d]
5	8–Cl	2′–OCH_3	10030.0 ± 590.0[d]	1910.0 ± 140.0[d]
6	7–Cl	2′–F	1381.8 ± 129.6	1141.0 ± 144.4
7	7–Cl	2′–Cl	2680.5 ± 90.8	836.8 ± 68.4
8	7–Cl	2′–Br	2002.1 ± 158.5	536.3 ± 91.8
9	7–Cl	2′–CH_3	ND[e]	ND[e]
10	7–Cl	2′–OCH_3	1206.1 ± 127.1	227.5 ± 47.4
11	5–Cl	2′–F	23393.8 ± 1232.9	77.1 ± 15.3
12	5–Cl	2′–Cl	gt;25000	47.1 ± 9.6
13	5–Cl	2′–Br	gt;25000	80.8 ± 10.3
14	5–Cl	2′–CH_3	gt;25000	47.6 ± 4.3
15	5–Cl	2′–OCH_3	6475.5 ± 1408.0	64.4 ± 12.9

[a] The IC_{50} values are presented as mean ± S.E.M. ($n = 3$).
[b] Diphenyleneiodonium (DPI) [30], phenylmethylsulfonyl fluoride (PMSF) [31], and elastatinal [32] were used as positive control in anti-inflammatory assay.
[c] None test.
[d] The biological data of compounds **1**–**5** came from the literature directly [11].
[e] No data due to insolubility.

Table 2
Inhibitory effects of compounds **10**–**15** on activity of human NE in a cell-free conditioned medium.

Compounds	IC_{50} (nM)[a]
PMSF[b]	95219.3 ± 19225.8
10	612.4 ± 179.8
11	52.7 ± 4.5
12	30.7 ± 4.0
13	24.8 ± 1.9
14	57.1 ± 9.2
15	40.0 ± 4.6

[a] The IC_{50} values are presented as mean ± S.E.M. ($n = 3$).
[b] Phenylmethylsulfonyl fluoride (PMSF) was used as a positive control in this assay.

3. Results and discussion

In the present study, ten chloro-substituted 2-phenylbenzoxazinones were synthesized and their inhibitory effects on NE release and $O_2^{\bullet-}$ generation induced by FMLP were compared. All the synthetics showed a potent inhibitory effect on NE release (Table 1). Compounds **6**–**10** showed a potent dual inhibitory effect on NE release and $O_2^{\bullet-}$ generation. These effects were decreased or disappeared when the chlorine was substituted at C-5 and C-8. Conversely, compounds **11**–**15**, with a chloro-substitution at C-5 exhibited highly selective inhibitory effects on NE release with IC_{50} values of 77.1, 47.1, 80.8, 47.6, and 64.4 nM, respectively. Our results indicate that compounds **11**–**15** showed more activity in the inhibition of NE release than the corresponding chloro-substitution at C-7 (**6**–**10**) and C-8 (**1**–**5**) by about 100- to 2000-fold, suggesting that the inhibitory effect on NE release in FMLP-activated human neutrophils depended on the position of the chloro-substituent in the A ring. Although 2-(2′-substituted-phenyl) benzoxazin-4-ones have been well known acylating agents of serine proteases. In addition, the substitutions at C-2′ were affect acylating rate [14]. However, our results indicate the substitutions at C-2′ of 5-chloro compounds did not enhance activity significantly. We propose 5-chloro compounds may be as covalent binders of serine proteases via a Michael addition-elimination.

In a cell-free conditioned medium (Table 2) [22], compounds **11**–**15** also showed potent inhibitory effects on NE activity with IC_{50} values of 52.7, 30.7, 24.8, 57.1, and 40.0 nM, respectively. Therefore, we next evaluated these analogs for their enzymatic activity on elastase from human leukocytes [23]. The results showed that these compounds directly inhibited elastase activity with IC_{50} values below 50 nM (Table 3).

It is well known that neutrophils are activated following hemorrhagic shock [24], and the subsequent accumulation of

Table 3
Inhibitory effects of compounds **10**–**15** on enzymatic activity of elastase from human leukocytes.

Compounds	IC_{50} (nM)[a]
PMSF[b]	52161.7 ± 1073.4
10	221.5 ± 59.2
11	30.8 ± 5.8
12	28.7 ± 6.2
13	29.5 ± 6.7
14	48.8 ± 14.2
15	15.4 ± 1.9

[a] Elastase (EC 3.4.21.37) was incubated with compounds for 2 min. Elastase activity was measured using ELISA reader at 405 nm, as described under Section 5. All data are presented as mean ± S.E.M. ($n = 3$–4).
[b] Phenylmethylsulfonyl fluoride (PMSF) was used as a positive control in this assay.

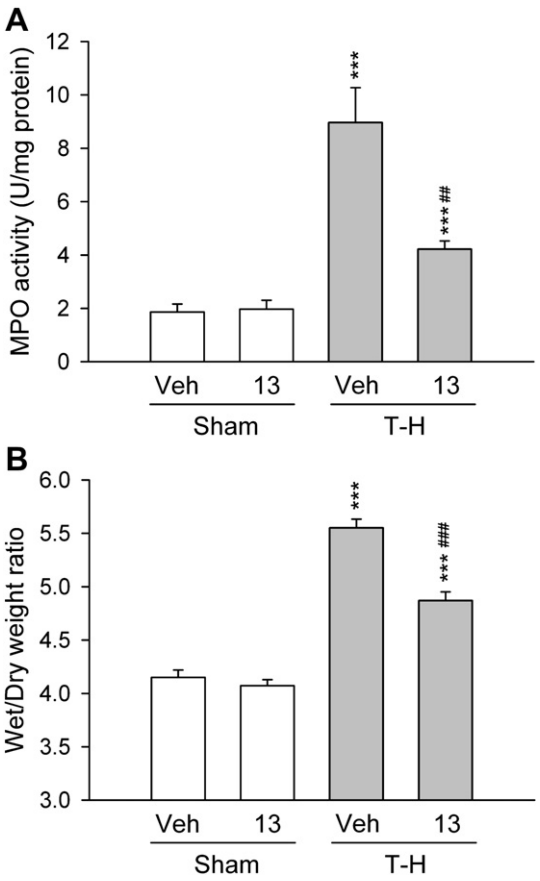


Fig. 1. Effect of compound **13** on lung injury in rats after a sham operation (Sham) or trauma-hemorrhage and resuscitation (T-H). Sprague-Dawley rats were treated with compound **13** (1 mg/kg of body weight, intravenously), or an equal volume of the vehicle (~0.2 mL, 10% DMSO). After 24 h, the MPO activity (A) and wet/dry weight ratio (B) in the lung tissue were assayed, as described in Section 5.1. Data are shown as mean ± SEM of 6 rats in each group. Statistical analysis was performed using Student's *t*-test or one-way analysis of variance (ANOVA) followed by Tukey's multiple-comparison test. A value of $p < 0.05$ was considered statistically significant. $^{***}p < 0.001$ compared to Sham; $^{\#\#}p < 0.01$; $^{\#\#\#}p < 0.001$ compared to T-H + Veh.

neutrophils in the lung is associated with lung injury [25, 26]. Therefore, regulation of the activity of blood neutrophils is important in controlling lung damage caused by hemorrhagic shock [27]. Herein, the protective effect of **13** in the lung injury following trauma-hemorrhagic shock were examined. The results showed no significant differences in the MPO activity and wet/dry weight ratio between vehicle and compound **13** treated groups in sham-operated animals. Administration of compound **13** (1 mg/kg of body weight) significantly attenuated the increase in the MPO activity and wet/dry weight ratio after trauma-hemorrhagic shock; however, these injury parameters remained higher than in sham-operated animals (Fig. 1). These data indicated that **13** attenuated hemorrhagic shock-induced lung injury in rats.

4. Conclusion

In this study, the synthesis and pharmacologic evaluation of a new series of chloro-substituted 2-phenylbenzoxazinones were described. The bioassay results of these compounds showed dramatic relationships between the structure of these compounds and their anti-inflammatory effects. The data indicated that the inhibition of human NE activity depended on the position of the chloro-substituent in the A ring. Therefore, these compounds are new NE inhibitors and could be developed as novel lead anti-inflammatory agents. Furthermore, these agents might also serve as a novel adjunct for improving depressed lung function following adverse circulatory conditions.

5. Experimental

5.1. Materials and methods

Unless stated otherwise, the chemicals were acquired from commercial sources and used without further purification. 2-Amino-4-chlorobenzoic acid, 6-amino-4-chlorobenzoic acid, 2-fluorobenzoyl chloride, 2-chlorobenzoyl chloride, 2-bromobenzoyl chloride, 2-methylbenzoyl chloride, and 2-methoxybenzoyl chloride were purchased from ACROS. Compound purity was checked by thin layer chromatography (TLC) on precoated silica gel plates (Merck, Kieselgel 60 F_{254}, layer thickness 0.25 mm). NMR spectra were performed in 400 and 100 MHz for ^1H and ^{13}C, respectively. ^1H NMR: $CDCl_3$ as solvent, $\delta = 7.265$ ppm and ^{13}C NMR: $CDCl_3$ as solvent, $\delta = 77.0$ ppm. Low resolution ESI-MS spectra were obtained on an API 3000™ (Applied Biosystems) in positive or negative mode (solvent: CH_3OH).

5.1.1. General procedure for synthesis of chloro-substituted 2-phenylbenzoxazinones (**6**–**15**)

5-/or 7-Chloro-substituted 2-phenylbenzoxazinones, compounds **6**–**15** (Table 1), were prepared by reaction of 2-amino-4-chlorobenzoic acid or 6-amino-4-chlorobenzoic acid, with corresponding substituted benzoyl chlorides in pyridine solution as described [3,21]. The reaction mixture was stirred at room temperature for 24 h. The pyridine was evaporated at reduced pressure, and further subjected to purification by silica column chromatography. All products were fully characterized using spectral data.

*5.1.1.1. 7-Chloro-2-(2-fluorophenyl)-4H-benzo[d][1,3]oxazin-4-one (**6**).* Yield: 85%, ^1H NMR (400 MHz, $CDCl_3$): $\delta = 8.16$ (1H, d, $J = 8.0$ Hz), 8.11 (1H, dt, $J = 2.0$, 8.0 Hz), 7.70 (1H, d, $J = 2.0$ Hz), 7.56 (1H, m), 7.50 (1H, dd, $J = 2.0$, 8.0 Hz), 7.29 (1H, br.t, $J = 8.0$ Hz), 7.22 (1H, dd, $J = 8.0$, 8.0 Hz). ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 161.5$ (s, $J_{C-F} = 260.0$ Hz), 158.3 (s), 147.7 (s), 143.0 (s), 134.4 (d, $J_{C-F} = 8.4$ Hz), 131.2 (d), 131.2 (s), 129.8 (d), 129.2 (d), 127.2 (d), 124.3 (d), 124.3 (s), 117.3 (d, $J_{C-F} = 21.2$ Hz), 115.3 (s). ESI-MS *m/z* 298 (100) $[M + Na]^+$, 300 (31).

*5.1.1.2. 7-Chloro-2-(2-chlorophenyl)-4H-benzo[d][1,3]oxazin-4-one (**7**).* Yield: 75%, ^1H NMR (400 MHz, $CDCl_3$): $\delta = 8.18$ (1H, d, $J = 8.0$ Hz), 7.90 (1H, dd, $J = 2.0$, 8.0 Hz), 7.72 (1H, d, $J = 2.0$ Hz), 7.53 (2H, m), 7.48 (1H, dd, $J = 2.0$, 8.0 Hz), 7.40 (1H, dt, $J = 2.0$, 8.0 Hz). ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 158.4$ (s), 157.7 (s), 147.5 (s), 143.1 (s), 133.6 (s), 132.6 (d), 131.5 (d), 131.2 (d), 129.8 (d), 129.8 (s), 129.5 (d), 127.2 (d), 126.9 (d), 115.3 (s). ESI-MS *m/z* 314 (100) $[M + Na]^+$, 316 (61).

*5.1.1.3. 7-Chloro-2-(2-bromophenyl)-4H-benzo[d][1,3]oxazin-4-one (**8**).* Yield: 67%, ^1H NMR (400 MHz, $CDCl_3$): $\delta = 8.20$ (1H, dd, $J = 3.0$, 8.0 Hz), 7.86 (1H, br.d, $J = 8.0$ Hz), 7.73 (1H, dd, $J = 2.0$, 8.0 Hz), 7.73 (1H, br.s), 7.54 (1H, br.d, $J = 8.0$ Hz), 7.46 (1H, t, $J = 8.0$ Hz), 7.39 (1H, t, $J = 8.0$ Hz). ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 158.4$ (s), 158.2 (s), 147.3 (s), 143.1 (s), 134.4 (d), 132.6 (d), 131.6 (s), 131.5 (d), 129.9 (d), 129.5 (d), 127.5 (d), 127.2 (d), 121.8 (s), 115.3 (s). ESI-MS *m/z* 360 (100) $[M + Na]^+$, 358 (72).

*5.1.1.4. 7-Chloro-2-(2-methylphenyl)-4H-benzo[d][1,3]oxazin-4-one (**9**).* Yield: 62%, ^1H NMR (400 MHz, $CDCl_3$): $\delta = 8.16$ (1H, dd, $J = 0.4$, 8.4 Hz), 8.04 (1H, br.dd, $J = 2.0$, 8.0 Hz), 7.68 (1H, dd, $J = 0.4$, 2.0 Hz), 7.48 (1H, dd, $J = 2.0$, 8.4 Hz), 7.44 (1H, dt, $J = 2.0$, 8.0 Hz), 7.33 (2H, br.t, $J = 8.0$ Hz), 2.72 (3H, s). ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 159.4$

(s), 158.9 (s), 147.9 (s), 142.8 (s), 139.5 (s), 132.0 (d), 131.9 (s), 130.2 (d), 129.7 (d), 129.2 (s), 128.9 (d), 127.0 (d), 127.2 (d), 126.1 (d), 115.1 (s), 22.3 (q). ESI-MS *m*/*z* 294 (100) $[M+Na]^+$, 292 (32).

*5.1.1.5. 7-Chloro-2-(2-methoxyphenyl)-4H-benzo[d][1,3]oxazin-4-one (**10**).* Yield: 58%, ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 8.14$ (1H, d, $J = 8.4$ Hz), 7.85 (1H, dd, $J = 2.0$, 8.4 Hz), 7.68 (1H, d, $J = 2.0$ Hz), 7.50 (1H, dt, $J = 2.0$, 8.4 Hz), 7.46 (1H, dd, $J = 2.0$, 8.4 Hz), 7.05 (1H, t, $J = 8.4$ Hz), 7.03 (1H, t, $J = 8.4$ Hz), 3.92 (3H, s). ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 159.0$ (s), 158.8 (s), 158.7 (s), 148.0 (s), 142.6 (s), 133.5 (d), 131.4 (d), 129.6 (d), 128.8 (d), 126.9 (d), 120.5 (d), 119.9 (s), 115.2 (s), 112.1 (d), 56.0 (q). ESI-MS *m*/*z* 310 (100) $[M + Na]^+$, 312 (30).

*5.1.1.6. 5-Chloro-2-(2-fluorophenyl)-4H-benzo[d][1,3]oxazin-4-one (**11**).* Yield: 64%, ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 8.10$ (1H, dt, $J = 2.0$, 8.0 Hz), 7.69 (1H, t, $J = 8.0$ Hz), 7.60 (1H, dd, $J = 0.4$, 8.0 Hz), 7.55 (2H, m), 7.28 (1H, t, $J = 8.4$ Hz), 7.21 (1H, dd, $J = 8.4$, 8.0 Hz). ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 161.4$ (s, $J_{C-F} = 259.3$ Hz), 155.6 (s), 148.9 (s), 135.9 (s), 134.3 (d, $J_{C-F} = 9.1$ Hz), 131.7 (s), 131.1 (d, 2C), 126.4 (d), 126.0 (s), 124.3 (d, $J_{C-F} = 3.7$ Hz), 121.2 (s), 117.3 (d, $J_{C-F} = 21.2$ Hz), 114.7 (s). ESI-MS *m*/*z* 298 (100) $[M + Na]^+$, 300 (34).

*5.1.1.7. 5-Chloro-2-(2-chlorophenyl)-4H-benzo[d][1,3]oxazin-4-one (**12**).* Yield: 51%, ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 7.90$ (1H, dd, $J = 1.6$, 8.0 Hz), 7.71 (1H, t, $J = 8.0$ Hz), 7.61 (1H, dd, $J = 1.6$, 8.0 Hz), 7.57 (1H, dd, $J = 1.6$, 8.0 Hz), 7.51 (1H, dd, $J = 1.6$, 8.0 Hz), 7.47 (1H, dt, $J = 1.6$, 8.0 Hz), 7.40 (1H, dt, $J = 1.6$, 8.0 Hz). ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 157.2$ (s), 155.8 (s), 148.7 (s), 136.0 (d), 133.5 (s), 132.5 (d), 131.4 (d), 131.3 (d), 131.3 (s), 131.2 (d), 129.7 (s), 126.9 (d), 126.4 (d), 114.7 (s). ESI-MS *m*/*z* 314 (100) $[M + Na]^+$, 316 (64).

*5.1.1.8. 5-Chloro-2-(2-bromophenyl)-4H-benzo[d][1,3]oxazin-4-one (**13**).* Yield: 63%, ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 7.85$ (1H, dd, $J = 1.6$, 8.0 Hz), 7.72 (1H, dd, $J = 1.6$, 8.0 Hz), 7.72 (1H, t, $J = 8.0$ Hz), 7.62 (1H, dd, $J = 1.6$, 8.0 Hz), 7.58 (1H, dd, $J = 1.6$, 8.0 Hz), 7.45 (1H, dt, $J = 1.6$, 8.0 Hz), 7.38 (1H, dt, $J = 1.6$, 8.0 Hz). ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 157.8$ (s), 155.8 (s), 148.6 (s), 136.0 (d), 134.4 (d), 132.6 (d), 131.7 (s), 131.4 (d), 131.4 (d), 131.3 (s), 127.5 (s), 126.4 (d), 121.8 (s), 114.7 (s). ESI-MS *m*/*z* 360 (100) $[M + Na]^+$, 358 (69).

*5.1.1.9. 5-Chloro-2-(2-methylphenyl)-4H-benzo[d][1,3]oxazin-4-one (**14**).* Yield: 60%, ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 8.03$ (1H, br.d, $J = 8.0$ Hz), 7.68 (1H, t, $J = 8.0$ Hz), 7.58 (1H, dd, $J = 1.2$, 8.0 Hz), 7.52 (1H, dd, $J = 1.2$, 8.0 Hz), 7.43 (1H, dt, $J = 1.2$, 8.0 Hz), 7.32 (2H, br.t, $J = 8.0$ Hz), 2.72 (3H, s). ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 158.9$ (s), 156.2 (s), 149.1 (s), 139.4 (s), 135.8 (d), 132.0 (d), 131.9 (d), 131.9 (s), 130.7 (d), 130.2 (d), 129.1 (s), 126.2 (d), 126.1 (d), 114.5 (s) 22.2 (q). ESI-MS *m*/*z* 294 (100) $[M + Na]^+$, 292 (31).

*5.1.1.10. 5-Chloro-2-(2-methoxyphenyl)-4H-benzo[d][1,3]oxazin-4-one (**15**).* Yield: 49%, ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 7.85$ (1H, dd, $J = 1.6$, 8.0 Hz), 7.67 (1H, t, $J = 8.0$ Hz), 7.59 (1H, br.dd, $J = 1.6$, 8.0 Hz), 7.51 (1H, dd, $J = 1.6$, 8.0 Hz), 7.50 (1H, dt, $J = 1.6$, 8.0 Hz), 7.06 (1H, br.t, $J = 8.0$ Hz), 7.03 (1H, br.t, $J = 8.0$ Hz), 3.92 (3H, s). ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 158.7$ (s), 156.4 (s), 149.3 (s), 135.7 (d), 133.5 (d), 132.6 (s), 131.3 (d), 130.7 (d), 126.2 (d), 125.1 (s), 121.3 (s), 120.5 (d), 114.6 (s), 112.1 (d), 56.0 (q). ESI-MS *m*/*z* 310 (100) $[M + Na]^+$, 312 (32).

5.2. Biological assays

5.2.1. 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 the hypotonic lysis of erythrocytes. Purified neutrophils that contained gt; 98% viable cells, as determined by the trypan blue exclusion method, were resuspended in Ca^{2+}-free HBSS buffer at pH 7.4 and maintained at 4 °C before use.

5.2.2. Measurement of $O_2^{\cdot-}$ generation

The $O_2^{\cdot-}$ generation assay was based on the superoxide dismutase (SOD)-inhibitable reduction of ferricytochrome *c*. In brief, after supplementation with 0.5 mg/ml ferricytochrome *c* and 1 mM Ca^{2+}, neutrophils (6×10^5/ml) were equilibrated at 37 °C for 2 min and incubated with compounds for 5 min. Cells were activated with 100 nM FMLP for 10 min. When FMLP was used as a stimulant, 1 μg/ml cytochalasin B (CB) was incubated for 3 min before activation. Changes in absorbance with the reduction of ferricytochrome *c* at 550 nm were continually monitored in a double-beam, six-cell positioner spectrophotometer with constant stirring (Hitachi U-3010, Tokyo, Japan) [22,27].

5.2.3. Measurement of NE release

Degranulation of azurophilic granules was determined by NE release. Experiments were performed using MeO-Suc-Ala-Ala-Pro-Val-p-nitroanilide as the NE substrate. Briefly, following supplementation with MeO-Suc-Ala-Ala-Pro-Val-p-nitroanilide (100 μM), neutrophils (6×10^5/ml) were equilibrated at 37 °C for 2 min and incubated with the compounds for 5 min. Cells were activated by FMLP (100 nM) in the presence of CB (0.5 μg/ml), and changes in absorbance at 405 nm were continuously monitored to determine NE release. The results are expressed as a percentage of NE release in the FMLP/CB-activated, drug-free control system.

To determine whether the compounds exhibited an inhibitory effect on NE activity, a direct NE activity assay was performed in a cell-free conditioned medium. Neutrophils (6×10^5/ml) were incubated for 20 min in the presence of FMLP (100 nM)/CB (2.5 μg/ml) at 37 °C. Cells were then centrifuged at 1000 *g* for 5 min at 4 °C in order to collect NE from the supernatant. The NE supernatant was equilibrated at 37 °C for 2 min and incubated with the compounds for 5 min. Following incubation, the NE substrate, MeO-Suc-Ala-Ala-Pro-Val-p-nitroanilide (100 μM), was added to the reaction mixtures. Changes in absorbance at 405 nm were continuously monitored for 10 min to assay NE activity [23,27].

5.2.4. Enzyme activity assay

Enzyme activity assay of NE was conducts in pH 7.4 HBSS buffer containing 0.03 U/ml human NE (EC 3.4.21.37, Sigma, St. Louis, MO, USA). The assay buffer was incubated with compounds for 2 min at 25 °C before the addition of NE substrate MeO-Suc-Ala-Ala-Pro-Val-p-nitroanilide (200 μM). Assays were performed in 96-well microtiter plates. The reaction mix was allowed to proceed for 30 min. Activity of human NE was measured using ELISA reader at 405 nm.

5.2.5. Trauma-hemorrhagic shock procedure

A rat model of trauma-hemorrhagic shock was used in this study [27,28]. Thirty-two male Sprague-Dawley rats (275–325 g) obtained from the National Science Council, Taipei, Taiwan were divided into 4 groups of 8 animals each, according to a table of random numbers. They were housed in an air-conditioned room under a reversed light–dark cycle and allowed at least 1 week to adapt to the environment. Before initiation of the experiment, they were starved overnight but were allowed water *ad libitum*. The rats were anesthetized by isoflurane (Attane, Minrad, Bethlehem, PA) inhalation prior to performing a 5-cm midline laparotomy in the abdomen. The abdomen was closed in layers, and catheters were

placed in both femoral arteries and the right femoral vein (polyethylene [PE-50] tubing; Becton Dickinson, Sparks, MD). The wounds were bathed with 1% lidocaine (Elkins-Sinn, Cherry Hill, NJ) throughout the surgical procedure to reduce postoperative pain. Rats were then allowed to awaken and were bled, and the mean blood pressure was maintained at 40 mmHg. This level of hypotension persisted until the animals could not maintain a mean blood pressure of 40 mmHg unless additional fluid in the form of Ringer's lactate was administered. This time was defined as the maximum bleed-out, and the amount of withdrawn blood was noted. Following this, the rats were maintained at a mean blood pressure of 40 mmHg until 40% of the maximum bleed-out volume was returned in the form of Ringer's lactate. The animals were then resuscitated with four times the volume of the shed blood over 60 min with Ringer's lactate. The time required for maximum bleed-out was ~45 min, the volume of the maximum bleed-out was ~60% of the calculated circulating blood volume [29], and the total hemorrhage time was ~90 min. Thirty minutes before the end of the resuscitation period, the rats received compound **13** (1 mg/kg of body weight, intravenously), or an equal volume of the vehicle (~0.2 ml, 10% DMSO, Sigma). The catheters were then removed, the vessels ligated, and the skin incisions closed with sutures. Sham-operated animals underwent the surgical procedure, which included a laparotomy in addition to ligation of the femoral artery and vein, but neither hemorrhage nor resuscitation was carried out. Vehicle or compound **13** was also administered to sham-operated rats after the catheters were put in place. The animals were then returned to their cages and allowed food and water *ad libitum*. The animals were sacrificed 24 h after the end of resuscitation. The current study was approved by the Institutional Animal Care and Use Committee of Chang Gung Memorial Hospital. All animal experiments were performed according to the guidelines of the Animal Welfare Act and the Guide for Care and Use of Laboratory Animals from the National Institutes of Health, Taiwan.

5.2.6. Preparation of lung tissue

Twenty-four hours after completing fluid resuscitation or the sham operation, the animals were anesthetized with isoflurane and sacrificed. The chest was opened and the left side of the lung was obtained after clamping the hilum. Excess blood was blotted dry, and the left upper lobe of the lung was measured for tissue water content. Other tissue sections were stored at −80 °C until analyzed [27].

5.2.7. Water content assay

In a separate cohort, the left upper lobe of the lung was weighed, and dried for 24 h at 80 °C. The water content of lung tissue was calculated as a wet/dry weight ratio [27].

5.2.8. Measurement of myeloperoxidase (MPO) activity

MPO activity in whole-lung homogenates was determined as described previously [27]. Briefly, equal weights (100 mg wet weight) of lung from various groups were suspended in 1 ml buffer (0.5% hexadecyltrimethylammonium bromide in a 50 mM phosphate buffer; pH 6.0) and sonicated at 30 cycles, twice, for 30 s on ice. Homogenates were cleared by centrifuging at 2000 *g* and 4 °C, and the supernatants were stored at −80 °C. Protein contents of samples were determined using the Bio-Rad (Hercules, CA) assay kit. Samples were incubated with the substrate, *o*-dianisidine hydrochloride. This reaction was carried out in a 96-well plate by adding 290 μl of 50 mM phosphate buffer, 3 μl substrate solution (containing 20 g/l *o*-dianisidine hydrochloride), and 3 μl H_2O_2 (20 mM). The sample (10 μl) was added to each well to start the reaction. Standard MPO (Sigma, St. Louis, MO) was used in parallel to determine the MPO activity in the sample. The reaction was stopped by adding 3 μl sodium azide (30%). The light absorbance at 460 nm was read. MPO activity was determined using a curve obtained from the MPO standard.

5.2.9. Statistical analysis

Results are expressed as the mean ± S.E.M. Data were analyzed using the GraphPad Prism software (GraphPad Software, San Diego, CA). Statistical analysis was performed using Student's *t*-test or one-way analysis of variance (ANOVA) followed by Tukey's multiple-comparison test. A value of $p < 0.05$ was considered statistically significant.

Acknowledgment

The investigation was supported by research grants to P.W. Hsieh and T.L. Hwang from the National Science Council of the Republic of China in Taiwan.

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