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

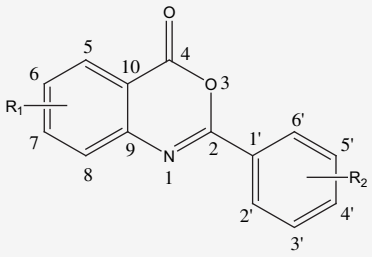
directly modulate NE responses in clinical practice. Therefore, research and development of a new generation of anti-inflammatory agents are needed and are in progress.

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.