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Inhibition of human sputum elastase by 7-substituted 5-methyl-2-isopropylamino-4*H*-3,1-benzoxazin-4-ones

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Abstract—7-Substituted 5-methyl-2-isopropylamino-4*H*-3,1-benzoxazin-4-ones (BOZNs) were prepared and tested as inhibitors of human sputum elastase (HSE). The BOZNs with certain amino acid residues at the 7-position proved to be potent inhibitors of HSE. Some of the compounds also showed a high selectivity for HSE versus chymotrypsin. In a hamster model in which acute injury was induced by intratracheal administration of HSE (1.0 mg/kg), these compounds, when administered intratracheally (1.0 mg/kg) either 30 or even 240 min before challenge with HSE, significantly suppressed pulmonary hemorrhage. These findings suggest that 7-substitution of BOZN by amino acid residues can produce strong and HSE-specific inhibitors, with potential use in elastase-mediated disorders.

Key words: benzoxazin-4-one; chymotrypsin; elastase inhibitor; human leukocyte elastase; human sputum elastase; pulmonary injury

HLE* is considered to play a critical role in various diseases with tissue degradation [1] and, thus, inhibitors of HLE may exert potent therapeutic effects on these diseases. Certain 4*H*-3,1-benzoxazin-4-ones have been reported to be potent inhibitors of several serine proteinases [2]. Because of broad chemical variation and optimization of this class of compounds, many 2- and/or 5-substituted 4*H*-3,1-benzoxazin-4-one derivatives have been prepared in the search for useful HLE inhibitors [3–5]. However, no attempts have been made to prepare such derivatives with amino acid residues at the 7-position. Therefore, 7-substituted BOZN derivatives were synthesized in our laboratories for the purpose of increasing the affinity and specificity to HLE. As a result, potent and highly specific HLE inhibitors were obtained by introducing several kinds of amino acid residues at the 7-position. Some of the 7-substituted BOZNs were effective in a hamster model of acute injury induced by intratracheal administration of HSE which is equivalent to HLE [6], suggesting their potential use in elastase-mediated disorders.

Materials and Methods

7-Substituted BOZN derivatives were synthesized in our laboratories (Kokubo M *et al.*, EP 0317645B1). HSE (875 U/mg protein) and bovine pancreatic α-chymotrypsin (62 U/mg protein) were purchased from the Elastin Products Co., Inc. (Pacific, MO) and the Sigma Chemical Co. (St. Louis, MO), respectively. Inhibition of these enzymes by the synthesized BOZNs was assayed routinely using their synthetic substrates as described previously [7]. The IC_{50} value and selectivity were defined as the concentration of inhibitor giving rise to 50% inhibition of the hydrolysis and as the ratio of IC_{50} for chymotrypsin to that for HSE, respectively. An acute pulmonary injury model was caused by intratracheal administration of HSE (1.0 mg/kg) to anesthetized golden hamsters (8 weeks of age; Charles River Inc., Atsugi, Japan). 7-Substituted BOZNs (1.0 mg/kg) were also intratracheally administered to the same hamsters, 30 or 240 min before the HSE challenge. Four hours later, bronchoalveolar lavage was performed with saline to determine pulmonary hemorrhage [7]. Animals administered vehicle at 30 min before HSE administration were used as the positive control (100%). The data were analyzed for statistical significance by one-way ANOVA, and intergroup comparison was performed by Scheffe's F-test.

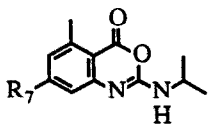
Results and Discussion

Thirty-five 7-substituted BOZN derivatives were synthesized and tested for their ability to inhibit HSE and their selectivity for HSE. Most of the compounds have amino acid residues at the 7-position, such as glutamic acid, glycine, lysine, phenylalanine, phenylglycine and/or serine. Since BOZN was hardly soluble in water, these amino acid moieties were chosen in order to give increased solubility to this compound in addition to increased affinity to HSE. Introduction of these amino acid residues at the 7-position produced strong inhibitors of HSE with IC_{50} values ranging from 1.3×10^{-10} to 5.2×10^{-7} M (Table 1). The compounds, whose amino acid residues were protected by hydrophobic groups such as a *t*-butyloxycarbonyl group or a benzyloxycarbonyl group, had a tendency to have lower IC_{50} values for HSE. It should be mentioned that the IC_{50} values obtained in the present investigation lie in the same magnitude as K_i values determined by kinetic measurement of acylation and deacylation constants (e.g. compounds 10 and 15 in Table 1, and the values given in Ref. 7). This is due to the fact that the concentration of the substrate used for the reaction was close to its K_m value [8]. Under these conditions, IC_{50} values are considered to be roughly related to K_i values [9] and, therefore, should be useful for assessment of inhibitor potential.

α-1-Proteinase inhibitor, a major elastase inhibitor in the lung and blood [10], has a k_{ass} value of 6.5×10^7 M^{-1} sec^{-1} for HLE, which is closest to that for bovine chymotrypsin (5.9×10^6 M^{-1} sec^{-1}) or human chymotrypsin (5.4×10^6 M^{-1} sec^{-1}), compared with other serine proteinases [11], suggesting that the substrate specificity of HLE seems to closely resemble that of chymotrypsin among serine proteinases. Therefore, the ratio of an IC_{50} value for chymotrypsin to that for HSE was taken as a parameter for the selectivity for HLE. Some of the synthesized compounds showed their selectivity for HSE versus chymotrypsin with more than 100-fold in favor of HSE, whose HLE selectivity could overcome that of the α-1-

* Abbreviations: BOZN, 5-methyl-2-isopropylamino-4*H*-3,1-benzoxazin-4-one; HLE, human leukocyte elastase; and HSE, human sputum elastase.

Table 1. Elastase inhibitory capacity and selectivity of 7-substituted BOZNs



No.	R_7 =	IC_{50}* (nM)	Selectivity†
1	Ac-NH-	120	4
2	PhO-Ac-NH-	10	19
3	Pivaloyl-NH-	64	11
4	i-Butoxycarbonyl-NH-	61	6
5	p-Phenylbenzoyl-NH-	16	9
6	1-Naphthoyl-NH-	16	32
7	Z-Gly-NH-	2.3	7
8	HCl·Glu-NH-	28	229
9	Z-Glu-NH-	2.1	523
10	CPS-Glu-NH-	2.9	1690
11	Z-Glu (O^{t}Bu)-NH-	1.1	172
12	Boc-Glu (O^{t}Bu)-NH-	1.8	67
13	CPS-Gly-Glu-NH-	2.0	690
14	HCl·Z-Lys-NH-	3.7	378
15	HCl·CPS-Lys-NH-	30	240
16	Z-Lys (Boc)-NH-	1.0	140
17	Z-Lys (CPS)-NH-	0.20	750
18	Z-Lys (4-PyCO)-NH-	1.3	540
19	Boc-Lys (Z)-NH-	1.8	83
20	HCl·Lys (Z)-NH-	19	63
21	HCl·Phe-NH-	520	19
22	Z-Phe-NH-	1.8	25
23	HCl·Phe-Phe-NH-	28	8
24	Boc-Phe-Phe-NH-	1.2	2
25	Z-Glu-Phe-NH-	0.30	800
26	Z-Glu (O^{t}Bu)-Phe-NH-	0.13	277
27	HCl·Z-Lys-Phe-NH-	18	39
28	HCl·Lys (Z)-Phe-NH-	7.0	53
29	Boc-Lys (Z)-Phe-NH-	0.67	57
30	Z-Lys (Boc)-Phe-NH-	0.23	100
31	HCl·Phg-NH-	11	10
32	Boc-Phg-NH-	15	18
33	Boc-Ser (Bzl)-NH-	2.1	18
34	HCl·Ser (Bzl)-NH-	81	12
35	Z-Tyr-NH-	0.59	42

Abbreviations: Ac, acetyl; Boc, *t*-butyloxycarbonyl; Bzl, benzyl; CPS, 4-chlorophenylsulfonyl; Phg, *N*-phenylglycine; PhO-Ac, phenoxyacetyl; 4-PyCO, 4-pyridylcarbonyl; ^{t}Bu, *t*-butyl; and Z, benzyloxycarbonyl.

* IC_{50} for HSE.

† Ratio of IC_{50} for chymotrypsin to that for HSE.

proteinase inhibitor (10-fold in favor of HLE) [11] and even that of 2- or 5-substituted 4*H*-3,1-benzoxazin-4-ones previously reported (maximum 25-fold in favor of HLE) [3]. The derivatives having a glutamic acid or lysine residue had a tendency to be highly selective for HSE. Some of these compounds also showed weaker inhibition for other serine proteases as well as increased stability [7]. Used *in vivo*, both the low concentration required for effective inhibition of HSE and high selectivity for HSE may guarantee low toxicity [12], contributing to minimizing the side-effects.

The therapeutic efficacy of some of the 7-substituted BOZNs, which were chosen as the compounds with relatively higher selectivity for HSE, was further evaluated in a pulmonary injury model using hamsters. The administration of 7-substituted BOZNs via the tracheal route was effective in preventing acute pulmonary injury, when given 30 min before the HSE challenge (Table 2). When given 240 min before HSE administration, some compounds failed to suppress the hemorrhage of the lung, but others were still effective in preventing HSE-induced injury. This discrepancy may be due to the hydrophilic property of the compounds and their dispersion and/or metabolism in the lung. It should be noted that, in our preliminary experiments, an acute pulmonary injury induced by lipopolysaccharide in the hamster was also effectively prevented by intratracheal or intravenous administration of 7-substituted BOZNs (unpublished data). The *in vivo* efficacy of these compounds, together with *in vitro* elastase inhibitory capacity, suggests that 7-substituted BOZNs may become candidates to provide potential elastase-specific inhibitors for the treatment of elastase-mediated disorders.

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Table 2. Inhibition of elastase-induced pulmonary hemorrhage by 7-substituted BOZNs

No.	7-Substituted BOZN	N	Pulmonary hemorrhage (%) 30 min	240 min
	(vehicle)	20	100 ± 30.5	ND*
8	R_7 = HCl·Glu-NH-	10	45.6 ± 34.4†	102 ± 47.2
9	Z-Glu-NH-	9	44.4 ± 23.8†	ND
10	CPS-Glu-NH-	20	16.8 ± 13.2†	35.4 ± 21.3†
13	CPS-Gly-Glu-NH-	15	23.8 ± 25.5†	74.1 ± 52.7
14	HCl·Z-Lys-NH-	15	36.5 ± 49.7†	17.0 ± 14.0†
15	HCl·CPS-Lys-NH-	10	40.2 ± 28.6†	48.1 ± 30.2†

7-Substituted BOZNs were administered intratracheally to hamsters at a dose of 1.0 mg/kg, 30 or 240 min before the intratracheal administration of HSE (1.0 mg/kg). After 4 hr, bronchoalveolar lavage was performed with 4 mL of saline. Pulmonary hemorrhage was evaluated on the basis of the content of hemoglobin in the lavage fluid. Animals administered 0.01 M phosphate-buffered saline (pH 7.5) as vehicle at 30 min before HSE administration were used as the positive control. Data are expressed as means ± SD. Abbreviations: defined in Table 1.

* Not determined.

† $P < 0.05$ when compared with vehicle administration (30 min).

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