Bioorganic & Medicinal Chemistry Letters, Vol.1, No.10, pp. 545-550, 1991
Printed in Great Britain

0960-894X/91 $3.00 + .00

PREVENTION OF HUMAN LEUKOCYTE ELASTASE-MEDIATED LUNG DAMAGE BY 3-ALKYL-4-AZETIDINONES

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(*Received* 22 *August* 1991)

Abstract - Simple 3-alkyl-4-azetidinones have been previously reported as potent inhibitors of human leukocyte elastase (HLE). Further modification of these simple monocyclic β-lactams has led to development of substituted 4-azetidinones that both inhibit HLE in a time dependent manner and, like previously reported modified cephalosporin sulfones, prevent HLE-induced lung damage in hamsters.

Human leukocyte elastase (EC 3.4.21.37; HLE) is a serine proteinase isolated from the azurophilic granules of polymorphonuclear leukocytes and is implicated in the tissue destruction associated with pulmonary emphysema[1,2], rheumatoid arthritis[3], cystic fibrosis[4], and glomerulonephritis[5]. Considerable research efforts have focused upon the development of potent and selective inhibitors of this enzyme in hopes of reducing the degradation of connective tissues associated with these chronic and degenerative diseases.[6-10] We discovered that a variety of β-lactam antibiotics may be chemically modified to inhibit HLE.[11] Many of these, notably the cephalosporin sulfones, exhibit potent time dependent inhibition indicating possible further chemical modification of the enzyme. Indeed, in one case of a cephalosporin sulfone ester having leaving groups at the C-7 and C-3' positions, the x-ray crystal structure of the inhibitor with the closely related porcine pancreatic elastase (PPE) unequivocally shows a novel double hit mechanism.[12] Serine-195 of PPE was acylated by the β-lactam carbonyl and the N-ε of histidine-57 was alkylated by Michael addition to the C-3' position via intermediates reminiscent of the mechanistic actions of cephalosporin antibiotics.[13]

Several HLE inhibitors from the cephalosporin class of compounds were chosen for further evaluation in *in vivo* models of HLE mediated-lung damage.[14-15] When administered intratracheally, these compounds prevented lung damage in hamsters treated with HLE. Compound **2** (L-659,286) was found to persist in the lung for several hours and was active in preventing HLE-mediated lung damage when administered intratracheally. Prolinamide **1** (L-658,758) was developed further for evaluation as a possible clinical candidate in the treatment of pulmonary diseases where HLE is implicated.[16]

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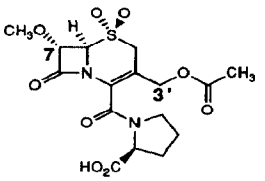
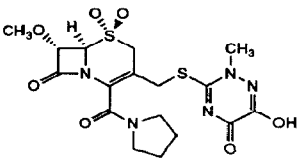


1 (L-658,758)



2 (L-659,286)

The inhibition of HLE by simple monocyclic β-lactams has been reported.[17] The structure-activity relationship developed at C-3 was consistent with that observed at C-7 of the cephalosporins. Compounds having small alkyl groups at C-3 were most potent. HLE preferentially cleaves peptide bonds adjacent to small hydrophobic amino acids and it is proposed that the substituents at the C-3 position are binding in the P_1 specificity pocket. While the inhibitory potencies of these 4-azetidinones against HLE were impressive (IC_{50}'s ≤ 0.1μg/mL), they were devoid of any *in vivo* efficacy. Herein we report the further development of the 3-alkyl-4-azetidinones as inhibitors of HLE with *in vivo* activities in preventing HLE-mediated lung damage in hamsters. These relatively simple molecules have modest time dependent inhibitory activities versus HLE yet have *in vivo* potencies comparable to **1**.

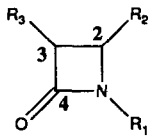
Materials. Compounds **1** , **2**, and the monocyclic β-lactams **3-31** were synthesized in our laboratories by modifications of methods previously described.[17-20] Acid ethers **3-9, 11-12, 16, 19** were prepared by ruthenium oxide oxidation[21] of the appropriate allyl ethers. All substituted 4–azetidinones were characterized by 200 MHz ^{1}H-NMR spectroscopy and mass spectrometry. The structures of the substituted 4-azetidinones are shown in Table 1.

Inhibition of HLE. Inhibition of HLE was assayed spectrophotometrically as described[22] at 25° C by continuous monitoring of the release of *p*-nitroaniline at 410 nm from the substrate succinyl-Ala-Ala-Pro-Ala-*p*-nitroanilide. Results are listed in Table 1 and are expressed in terms of the bimolecular rate constant $k_{obs}/[I]$ in $M^{-1}sec^{-1}$.[23]

Prevention of HLE-mediated damage in the hamster lung. The assay to evaluate the inhibition of HLE-mediated lung damage in hamsters has been published in detail.[15] In summary, HLE (50 units) in saline (200 μL) was injected in the trachea of anesthetized hamsters, causing hemorrhage into the air spaces of the lung. Inhibitors were initially screened at 100 μg in saline (200 μL) and were injected intratracheally 30 minutes prior to instillation of enzyme. Hemorrhage was determined 3 hours after treatment with HLE by spectrophotometric evaluation of hemoglobin content in lung lavage fluid. Initial screening results are listed in Table 1. Dose responses (ED_{50}'s) and time courses of inhibitor instillation at 1 to 4 hours prior to enzyme treatment for selected compounds are shown in Table 2.

Inhibition of HLE by substituted 3-alkyl-4-azetidinones. The second order rate constants for inactivation of HLE by N-acyl β-lactams are shown in Table 1. These values are quite modest when compared to the activities versus HLE for some of the cephalosporin sulfones; eg. $k_{obs}/[I] > 50{,}000\ M^{-1}sec^{-1}$.[22] The (±)-trans-isomer **3** was less potent than its corresponding (±)-cis-isomer **4**, isolated as a minor reaction product from the preparation of **3**. The majority of the activity in the trans series resides in the (R,R)-isomer **5**. As observed previously [17], the C-3 n-propyl analog **7** was more potent than the C-3 ethyl compound **3**.

Table 1. Inhibition of Human Leukocyte Elastase (HLE) and HLE-induced Lung Hemorrhage in the Hamster by Intratracheal Administration of Monocyclic β-Lactams



Compd. Number	R1	R3	R2	Relative Stereochem. of R3 and R2	k_{obs}/[I] (±SD) ($M^{-1}sec^{-1}$)		Inhib. @ 100 μg (%) (±SD)	
3	$COOC_2H_5$	C_2H_5	OCH_2COOH	t [a]	467	(35)	98	(1)
4	$COOC_2H_5$	C_2H_5	OCH_2COOH	c	796	(41)	81	(9)
5	$COOC_2H_5$	(R)-C_2H_5	(R)-OCH_2COOH	t	918	(19)	99	(1)
6	$COOC_2H_5$	(S)-C_2H_5	(S)-OCH_2COOH	t	136	(13)	86	(8)
7	$COOC_2H_5$	n-C_3H_7	OCH_2COOH	t	1504	(200)	88	(8)
8	$COOC_2H_5$	C_2H_5	$OCH(CH_3)COOH$ (isomer A)	t	346	(21)	71	(28)
9	$COOC_2H_5$	C_2H_5	$OCH(CH_3)COOH$ (isomer B)	t	158	(1)	81	(15)
10	$COOC_2H_5$	C_2H_5	$OCH_2CONHCH_2COOH$	t	1000	(53)	72	(15)
11	$COOC_2H_5$	C_2H_5	$O(CH_2)_2COOH$	t	560	(18)	31	(21)
12	$COOC_2H_5$	C_2H_5	$O(CH_2)_3COOH$	t	1247	(234)	23	(25)
13	$COOC_2H_5$	C_2H_5	SCH_2COOH	t	1086	(103)	56	(21)
14	$COOC_2H_5$	C_2H_5	$S(O)CH_2COOH$	t	1673	(69)	71	(19)
15	$COOC_2H_5$	C_2H_5	$S(O)_2CH_2COOH$	t	5615	(942)	70	(13)
16	$COOCH_2Ph$	C_2H_5	OCH_2COOH	t	955	(14)	51	(4)
17	$COOC_2H_5$	C_2H_5	OPh-4-COOH	t	6350	(1145)	-54	(63)
18	$COOC_2H_5$	C_2H_5	OPh-4-CH_2COOH	t	4102	(108)	-20	(18)
19	$CONHCH_2Ph$	C_2H_5	OCH_2COOH	t	129	(33)	50	(4)
20	$CONHCH_2Ph$	(S)-C_2H_5	(S)-OPh-4-COOH	t	339	(11)	17	(42)
21	$CONHCH_2Ph$	(R)-C_2H_5	(R)-OPh-4-COOH	t	3842	(17)	2	(8)
22	$CONHCH_2Ph$	C_2H_5	OPh-4-CH_2COOH	t	2074	(54)	-38	(40)
23	SO_2Ph-4-CH_3	C_2H_5	$CON(CH_3)CH_2COOH$	c	165	(1)	84	(7)
24	SO_2Ph-4-CH_3	(R)-C_2H_5	(R)-$CON(CH_3)CH_2COOH$	c	110	(1)	61	(23)
25	SO_2Ph-4-CH_3	(S)-C_2H_5	(S)-$CON(CH_3)CH_2COOH$	c	732	(105)	94	(5)
26	SO_2Ph-4-CH_3	(S)-C_2H_5	(R)-$CON(CH_3)CH_2COOH$	t	398	(72)	74	(6)
27	SO_2Ph-4-CH_3	(R)-C_2H_5	(S)-$CON(CH_3)CH_2COOH$	t	271	(10)	99	(0)
28	SO_2Ph-4-CH_3	C_2H_5	$COOCH_2COOH$	c	1554	(16)	73	(14)
29	SO_2Ph-4-CH_3	C_2H_5	$COOCH_2COOH$	t	9423	(665)	14	(27)
30	SO_2Ph-4-CH_3	C_2H_5	COOH	c	93	(8)	80	(1)
31	SO_2Ph-4-CH_3	(R)-C_2H_5	(S)-COOH	t	377	(11)	98	(1)

[a] t = trans; c = cis.

The nature of the substituent at C-2 has a significant effect on the potency versus the enzyme. Lactate ethers **8** and **9** were less active than the glycolate, while the glycine amide **10** was more active. The 3-propionic ether **11** was comparable to **3** while the 4-butyric ether **12** and the sulfur-containing acids **13-15** were better inhibitors than **3**. Replacement of the ethoxycarbonyl at N-1 with benzyloxycarbonyl (**16**) or benzylaminocarbonyl (**19**) enhanced or decreased the enzyme activity, respectively. Phenolic acid leaving groups at C-2 greatly enhanced inhibitory potency in **17-18** and in **21-22**.

The inhibition of HLE by N-*p*-toluenesulfonyl β-lactams is also shown in Table 1. The structural considerations for efficient enzyme inhibition are different from those seen for the N-acyl β-lactams. The (±)-cis-sarcosinamide **23** is a modest inhibitor of HLE. All four stereoisomers **24-27** had some activity against the enzyme with the most potent being the (S,S)-cis isomer **25**. Within this series, it is interesting to note that (S,R)-trans isomer **26** and (R,S)-trans isomer **27** differ only slightly whereas in the N-acyl compounds, (R,R)-trans isomer **5** is much more potent than the (S,S)-trans isomer **6**. A large difference is seen in the (±)-cis glycolic ester **28** and the (±)-trans glycolic ester **29**. The C-4 carboxylic acids **30** and **31** retained inhibitory activity even though HLE is an endoproteinase and might not be expected to tolerate a carboxylate in close proximity to the active site.[11]

Prevention of HLE-mediated lung damage in hamsters by 3-alkyl-4-azetidinones. Compounds were initially screened at 100 μg in 200 μL saline administered intratracheally (i.t.) to hamsters 30 minutes prior to the i.t. instillation of HLE (50 units) in saline (200 μL). These results are also shown in Tables 1 for the two classes of inhibitors. Within a series of stereoisomers, each inhibitor generally prevented nearly all HLE-mediated lung damage. Compounds **3-7** were essentially equipotent in this assay despite differences in their inhibitory capacities. Diasteromeric lactate ethers **8** and **9** had nearly the same effect. The ethers **11** and **12** and the phenyl ethers **17-18, 20-22** were considerably less active *in vivo* than the glycolate ethers. The thioethers **13-15** and the more hydrophobic N-acyls **16** and **19** gave 50-70% inhibition at this dose.

Each of the stereoisomers of **23** displayed some efficacy in preventing lung damage. Compounds **24-27** prevented HLE-mediated damage although not in proportion to their potency against the enzyme. The least potent inhibitor, (R,R)-cis isomer **24**, was not significantly less potent than the slightly more active inhibitor, the (R,S)-trans isomer **27**, and the most active compound, the (S,S)-cis isomer **25**, with this dosing regimen. The glycolate esters **28** and **29** also displayed *in vivo* activity in inverse proportion to their relative enzyme inhibitory potencies. Finally, the C-4 carboxylic acids **30** and **31** obviated most of the HLE-induced lung hemorrhage in the initial screening.

In order to more accurately assess the relative potencies of these compounds in this assay, both dose response and time course data were obtained for selected compounds. Compounds were administered at varying doses (100 to 3 μg) 30 minutes prior to the instillation of HLE and ED_{50}'s obtained. As shown in Table 2, monocyclic β-lactams **3**, **5-7**, and **31** and cephalosporin sulfone **1** gave ED_{50}'s ≈ 3-11 μg. There appears to be very little separation of compound efficacy based upon *in vitro* potency in these results.

The duration of action of these compounds in the hamster lung was measured and the results shown as a time course in Table 2. Compounds (200 μg) were administered i.t. 1 to 4 hours prior to instillation of enzyme into the lung. The relative potencies of the compounds perhaps become more evident in this experiment. Compound **5** still gives good inhibition at a 3 hour predose, whereas the less active isomer **6** has no activity at

Table 2. Dose Response and Time Course for Inhibition of HLE-induced Lung Hemorrhage in Hamsters by Selected Substituted 4-Azetidinones

	Compound Number							
	1	**3**	**5**	**6**	**7**	**23**	**28**	**31**
ED_{50}[a]	3.1 (0.6)	6.3 (2.8)	3.9 (0.3)	11.3 (3.2)	6.3 (1.2)	18.7 (5.2)	24.6 (5.2)	3.0 (0.5)
Time Course:[b]								
1 hr	98 (0)	96 (4)	100 (1)	92 (8)	83 (12)	78 (11)	66 (21)	78 (15)
2	85 (20)	63 (4)	82 (14)	49 (29)	31 (28)	68 (6)	32 (25)	33 (30)
3	74 (14)	36 (8)	59 (19)	6 (15)	-17 (48)	60 (11)	11 (7)	1 (31)
4	-23 (24)	-5 (43)	-14 (36)	-3 (40)	-2 (17)	4 (25)	30 (2)	16 (8)

[a] Dose (μg) of compound (± SE) administered intratracheally required to give 50% inhibition at 30 minutes prior to instillation of HLE (n = 4). [b] % Inhibition (± SE) at 200 μg by intratracheal administration at 1-4 hours prior to instillation of HLE (n = 4).

this timepoint. The racemate **3** has intermediate activities between its two resolved enantiomers, **5** and **6**. The mere addition of another carbon to the C-3 alkyl in compound **7** significantly decreases its efficacy at the later timepoints despite increased enzyme inhibition. The N-*p*-toluenesulfonyl β-lactam **23** has good activity out to 3 hours. The efficacies of the glycolate ester **28** and the acid **31** dropped off much more quickly.

The development of simple monocyclic β-lactams that inhibit HLE and prevent HLE-mediated lung damage in the hamster has now been demonstrated. However, it is obvious from Tables 1 and 2 that *in vitro* potency against HLE does not necessarily directly translate into relative *in vivo* activity in the described assay. The potencies of the monocyclic β-lactams listed in Table 1 are indeed modest. The time dependent inhibition of HLE by the cephalosporin sulfones was certainly more impressive; in some cases greater than three orders of magnitude more potent.[18,22] The sensitivity of HLE to inhibitor structure is not apparent with these C-3 mono-alkyl β-lactams. Molecular modeling studies with the HLE X-ray structure suggest that the close proximity of the carboxylate of the glycolates to the enzyme surface may be contribute to the low activity.[24] Thus, those compounds with the carboxylate extended further away from C-4 (eg., **10, 12, 17, 18**) tend to be more active inhibitors, but not always (**11**).

The relative *in vitro* and *in vivo* activities support the advantages of time dependent or irreversible enzyme inhibitors. Once the enzyme-inhibitor complex is formed, enzyme activity is reduced by the permanent removal of active enzyme. A potent reversible HLE inhibitor was reported to actually exacerbate HLE-induced lung damage.[25] Weak inhibitors such as **23** may only be effective because the compound survives in the lung long enough to still be available to completely neutralize the instilled enzyme. Detailed pharmacokinetic analysis of these compounds recovered from the lung would address the issue of bioavailability. Activity in the lung would be dependent upon the clearance of the inhibitor from the lung which may be related to polarity of the compound.[26] The objective for the development of inhibitors of human leukocyte elastase is to reduce the elasteolytic activity of the enzyme below levels at which permanent damage to connective tissue occurs. In diseases where HLE activity is intimated to be the cause of tissue damage, synthetic inhibitors such as the ones described herein could restore the "protease-antiprotease" balance.[1]

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