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Review Article

2-Spirocyclopropyl cephem sulfones: human neutrophil elastase inhibitors Syn-1390 and Syn-1396

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Introduction

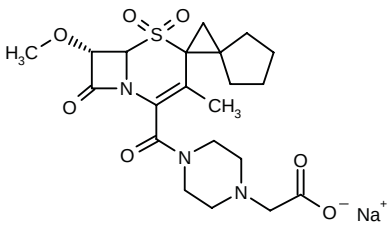
Human leukocyte elastase (HLE), also known as human neutrophil elastase (HNE), is a proteolytic enzyme. HNE is a serine protease present in the azurophilic granules of human polymorphonuclear leukocytes (PMN) and is secreted in response to a variety of inflammatory stimuli. HNE has important roles in the intracellular digestion of proteins after phagocytosis. In the extracellular environment, HNE is capable of degrading a variety of structural proteins, including elastin and collagen. The degradative capacity of HNE under normal circumstances is effectively controlled by its endogenous inhibitors α_1-proteinase inhibitor (α_1-PI) and α_2-macroglobulin (α_2-M) present in the plasma, as well as secretory leukocyte protease inhibitor (SLPI) on mucosal surfaces (1-3). However, stimulated PMNs produce a burst of active oxygen metabolites (*e.g.*, hypochlorous acid) which are capable of oxidizing an important amino acid residue (methionine, Met^{358}) present in α_1-PI (4). Oxidized α_1-PI is unable to bind effectively with HNE and thus has limited potency as an HNE inhibitor (5). It has been suggested that this protease-antiprotease imbalance permits HNE to perform its degradative functions.

Improper modulation of HNE activity has been implicated as a contributing factor in a number of human disease states such as pulmonary emphysema, rheumatoid arthritis, adult respiratory distress syndrome (ARDS) and cystic fibrosis (CF) (6-9). The burden of free active HNE within the airway secretions of most CF patients is very high (10). Although α_1-PI (also known as α_1-antitrypsin, α_1-AT) is present in the CF lung, it is inactive in most patients (11). The mechanism by which α_1-AT is inactivated probably involves both oxidative and proteolytic processes. The massive number of neutrophils present in the CF lung release H_2O_2 and myeloperoxidase, a neutrophil enzyme that converts Cl^- to OCl^- in the presence of H_2O_2, thus suggesting a large oxidant burden is present in the airways (12). In addition, bronchial secretions from CF patients can directly inactivate α_1-AT through elastase-mediated proteolysis (13). Therefore, individuals with CF have a functional deficiency of airway α_1-AT, a situation thought to render patients particularly susceptible to the early development of lung disease (14).

It has been suggested that low-molecular weight synthetic and selective HNE inhibitors that can be delivered to the site of unregulated PMN elastase activity can be potentially useful in the treatment of CF and related diseases. In 1986, a communication from Merck, Sharp and Dohme revealed that cephalosporin antibiotics can be modified to elicit potent inhibitory activity against HNE (15). Since then, much work has been reported in this area. Considerable chemical efforts were addressed to the modification of the C-7, C-2 and C-3 positions of the cephalosporin moiety (16-18). Some of these inhibitors prevent lung damage in hamsters treated intratracheally with HNE (19). Functionalization of the C-4 carboxyl, *e.g.*, esters, amides, thiolesters and ketones, were extensively investigated (16, 20-23). We anticipated that appropriate modification at C-2 position in the dihydrothiazine ring might give compounds which would show HLE inhibitory activity. Indeed, introduction of a spiro system at C-2 position of 1,1-dioxocephem *tert*-butyl esters gave a new series of potent and selective HLE inhibitors (24-27).

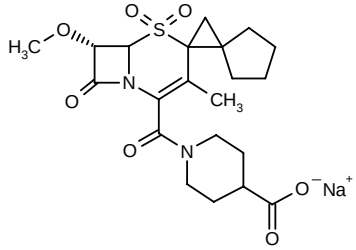
Here we will give a comprehensive account of Syn-1390 and Syn-1396, two members of a newly developed series of HLE inhibitors (Fig. 1). We will demonstrate that Syn-1390 and Syn-1396 have potent biological activity, preventing the hemorrhage induced in murine (C57BL/6) lung by HNE. Furthermore, in the rat agar bead model of

Syn-1390



$C_{21}H_{28}N_3NaO_7S$ Mol wt: 489.58

Syn-1396



$C_{21}H_{27}N_2NaO_7S$ Mol wt: 474.56

Fig. 1. Structures, molecular formulas and molecular weights of the 2-spirocyclopropyl cephem sulfones, Syn-1390 and Syn-1396.

Pseudomonas aeruginosa chronic lung infection, aerosol administration of Syn-1390 and Syn-1396 significantly decreased elastase activity (p <0.05) and lung neutrophil counts (p <0.05).

Synthesis

The target molecules, Syn-1390 and Syn-1396, can be obtained by two different ways:

1) Treatment of 6-azopenicillanate (I) with boron trifluoroetherate in a mixture of methanol and methylene chloride gives 6α-methoxy penicillanate (II), which on oxidation with peracetic acid gives the corresponding 1β-oxide (III). Ring opening of the oxide (III) by heating with 2-mercaptobenzothiazole in toluene gives the intermediate (IV), which on cyclization with bromine in methylene chloride gives 2β-bromomethyl penicillanate (V). Rearrangement of (V) with pyridine in dimethyl sulfoxide gives the cephem intermediate (VI). The sulfone derivative (VII) is obtained by oxidizing (VI) with peracetic acid in methylene chloride. Introduction of the double bond at C-2 position of the sulfone (VII) is achieved by heating with dimethylamine hydrochloride in a mixture of *tert*-BuOH, methylene chloride and formaldehyde. Reaction of the intermediate (VIII) with diazo cyclopentane, generated *in situ* by treatment of cyclopentyl hydrazone with silver (I) oxide, gives 2,2,2,-tricholoroethyl-7α-methoxy-2-spiro-(2'-spirocyclopentyl)cyclopropyl-3-methyl-3-cephem-4-carboxylate-1,1-dioxide (IX). Removal of the trichloroethyl group by treatment with Zn/glacial acetic acid gives the corresponding acid (X). Reaction of the acid (X) with oxalyl chloride in methylene chloride gives the acid chloride (XI), which is condensed with 4-(1-butoxycarbonyl methyl)piperazine to afford the product (XII). Deprotection of the *tert*-butyl group with formic acid gives the free acid (XIII). Finally, this compound is converted into the desired sodium salt (XIV) of Syn-1390 by treatment with sodium carbonate in water. In a similar manner, reaction of the acid (X) with oxalyl chloride followed by treatment with 4-*tert*-butoxycarbonyl piperidine in methylene chloride gives the intermediate (XV). Hydrolysis of the *tert*-butyl ester with formic acid gives the free acid (XVI). Treatment with sodium carbonate in water gives the corresponding sodium salt of Syn-1396 (XVII) (Scheme 1).

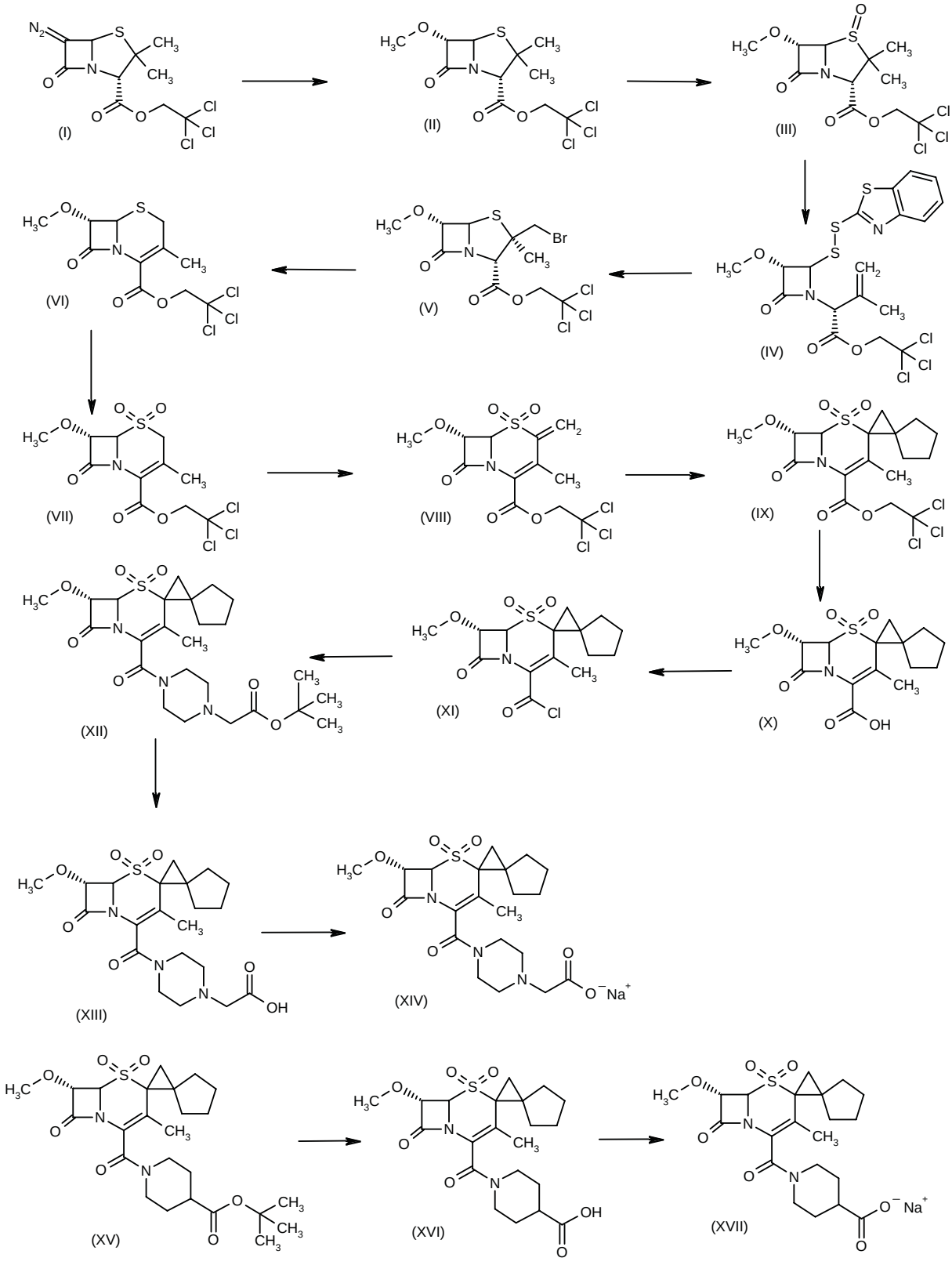
2) 7-ADCA (XVIIII) is reacted with isobutylene and concentrated H_2SO_4 in DME to afford the corresponding *tert*-butyl ester (XIX), which on oxidation with Na_2WO_4 in presence of H_2O_2 gives the sulfone (XX). The sulfone (XX) on treatment with $NaNO_2$ and 2.5 (N) H_2SO_4 in the presence of MeOH gives the 7α-methoxy cephem intermediate (XXI). Heating of compound (XXI) with dimethylamine hydrochloride and formaldehyde in a mixture of DMF and dioxane gives the 2-exomethylene cephem intermediate (XXII). Cycloaddition of (XXII) with diazo cyclopentane generated *in situ* by treatment of cyclopentyl hydrazone with silver (I) oxide, gives 7α-methoxy-2-spiro (2'-spirocyclopentyl)cyclopropyl cephem derivative (XXIII). Deprotection of the *tert*-butyl ester group with formic acid gives the free acid (X) (Scheme 2).

From the intermediate acid (X), the target molecules Syn-1390 and Syn-1396 and their corresponding sodium salts can be prepared, as described in Scheme 1.

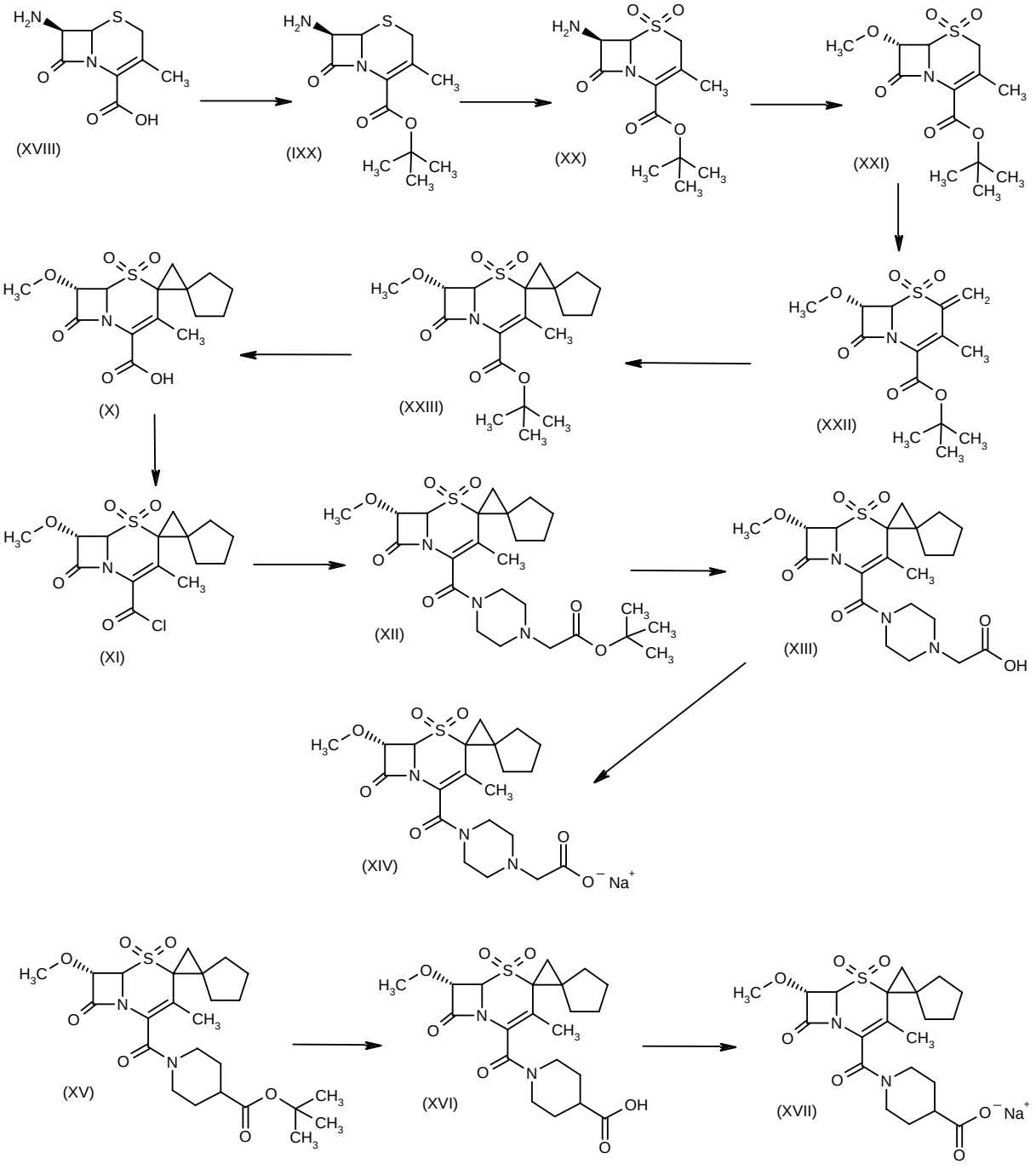
In vitro activity

Neutrophil elastase, purified from human sputum (HNE, Elastin Products Corporation, St. Louis, MO, USA), hydrolyzed the synthetic chromogenic substrate methoxy succinyl ala-ala-pro-val *p*-nitroanilide (MeOSAAPV*p*NA) (28). Both Syn-1390 and Syn-1396 inhibited the hydrolysis of MeOSAAPV*p*NA by 20 nM HNE in a dose-dependent fashion (Fig. 2). The IC_{50}s for Syn-1390 and Syn-1396 were 91 and 34 nM, respectively. *In vitro* elastase inhibition by Syn-1390 and Syn-1396 was reversible, as elastase activity could be partially recovered when incubation times were prolonged to 4 h.

Scheme 1: Synthesis of Syn 1390 and Syn 1396



Scheme 2: Synthesis of Syn 1390 and Syn 1396



In vivo activity

In vivo elastase activity was determined using a model of human neutrophil elastase-mediated hemorrhage in the murine lung (29). C57BL/6 mice received 50 µl inhibitor and/or HNE at the nares, followed 1 h later by bronchoalveolar lavage to determine the extent of elastase-induced lung hemorrhage. This method of instillation results in homogeneous distribution of drug throughout the airways and lung parenchyma. Baseline lung injury was determined by instilling 9 µM HNE alone. Inhibitors were added at different concentrations, followed 30 min

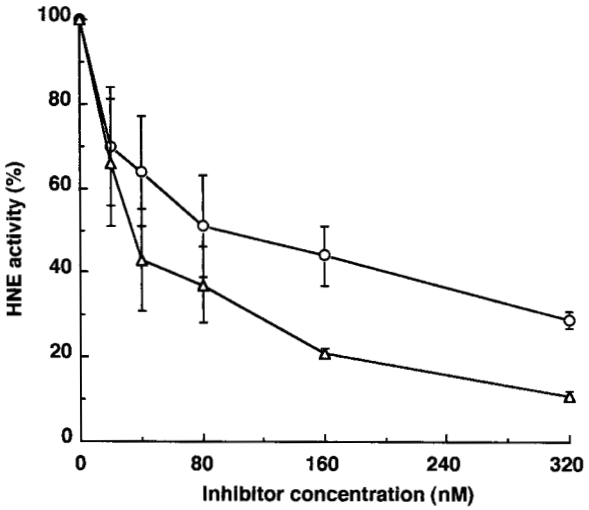


Fig. 2. Human neutrophil elastase (HNE) inhibition by Syn-1390 (○) and Syn-1396 (Δ). Inhibitors were incubated with 20 nM HNE at room temperature for 1 h, followed by the addition of the chromogenic substrate, MeSAAPV*p*NA. Hydrolysis of the substrate was monitored in a spectrophotometer at 410 nm for 2 min. Results are expressed as percent elastase activity in the absence of inhibitor. Each data point is the mean of triplicate determinations.

later by 9 μM HNE instillation. Table I presents results reported as a function of molar ratios of inhibitor to HNE (133:1, 40:1, 13:1 and 4:1). Clearly, both Syn-1390 and Syn-1396 can protect the lung against HNE-mediated injury, with significant protection being observed at inhibitor:HNE concentrations as low as 4:1. No adverse effects of either compound were observed in mice.

To determine the *in vivo* duration of protection against elastase-mediated lung hemorrhage, each inhibitor was instilled at a molar ratio of inhibitor to HNE of 266:1, either 1, 2 or 4 h before HNE instillation. The *in vivo* protection provided by both compounds could be observed for up to 4 h after each inhibitor had been instilled (Table I). These results indicate that the biological $t_{1/2}$ is sustained, an important characteristic in determining the potential clinical usefulness of neutrophil elastase inhibitors.

Although both inhibitors demonstrate similar *in vitro* activity against HNE, it is important to note that Syn-1390 provides significantly more protection against HNE-mediated lung injury *in vivo* than does Syn-1396. The differences of *in vitro* as compared to *in vivo* efficacy are likely related to the better availability of Syn-1390 than Syn-1396 at the site of HNE deposition in the lung.

Aerosol delivery

We utilized a state-of-the art aerosol delivery chamber in the present studies. The aerosol chamber is housed in a Biosafety Cabinet in a Level 2 containment facility with the air flow isolated for additional safety. The chamber is designed to accept animals ranging in size from 20-250 g. The aerosol chamber is equipped with sampling ports for a Met One Model 237 Portable Airborne Particle Counter which utilizes a solid-state laser diode resulting in increased accuracy and reliability in continuously monitoring the size of the particles. The aerosol is removed through HEPA filtered adjustable vacuum flow which allows us to control the flow rates. This is an important consideration in that adjustable flow rates are required for aerosolizing animal species with different breathing rates. The aerosol flow rates are calibrated using fluorescent microspheres obtained from Duke Scientific Corporation and this is done prior to each aerosol treatment.

Chronic rat lung experiments

The chronic rat lung model of *Pseudomonas aeruginosa* infection originally described by Cash *et al.* (30) is one of the most used animal models developed to date to

Table I: In vivo *protection against HNE-mediated lung hemorrhage.*

Inh.: HNE[1] molar ratio Time	% Inhibition* 133:1/40:1/13:1/4:1 30 min	% Inhibition* 266:1 1h/2h/4h
Syn-1390	91/75/63/53	92/88/81
Syn-1396	70/33/49/39	47/45/50

[1]Inh: inhibitor. HNE: human neutrophil elastase. *Results are expressed as % inhibition of lung hemorrhage. Times correspond to the intervals between inhibitor and HNE instillation. Results are the means of 3-7 samples.

Table II: Total cell counts and % neutrophils in bronchoalveolar lavage fluid from control and treated animals (n = 3 at each time point).

	Total cell counts (x 10^6)			Neutrophils (%)		
	Day 0	Day 3	Day 7	Day 0	Day 3	Day 7
Control	1.2 ± 0.5	4.9 ± 0.3	3.1 ± 0.1	59 ± 8	49 ± 3	48 ± 5
Syn-1390	0.4 ± 0.06	0.4 ± 0.04	0.3 ± 0.09	60 ± 9	22 ± 6*	6 ± 1*
Syn-1396	0.8 ± 0.04	0.3 ± 0.1	0.2 ± 1.06	58 ± 8	15 ± 9*	3 ± 2*

*Significantly different from control ($p < 0.01$).

Table III: Total elastase concentration in bronchoalveolar lavage fluid from control and treated animals (n = 3 at each time point).

	Total elastase concentration (nM)		
	Day 0	Day 3	Day 7
Control	64 ± 3.5	79 ± 3.0	70 ± 7.1
Syn-1390	58 ± 4.6	47 ± 4.0*	33 ± 9.3*
Syn-1396	68 ± 4.7	43 ± 5.1*	32 ± 6.9*

*Significantly different from control ($p < 0.01$).

study the pathogenesis of chronic *P. aeruginosa* lung infections. The model involves the incorporation of *P. aeruginosa* into agar beads followed by intratracheal deposition into the lungs of rats. A chronic infection state ensues, and animals have been studied up to 1 year following inoculation and found to be chronically infected (31). A number of clinical correlates to cystic fibrosis have been demonstrated in the model, including similar, if not identical, lung pathology (30, 32), immune complex disorders (33) and conversion to the mucoid phenotype by *P. aeruginosa* strains colonizing the airways (31).

The chronic rat lung model was used to assess the efficacy of aerosolized 2-spirocyclopropyl cephalosporin sulfone derivatives on the progression of chronic *P. aeruginosa* lung infection in experimental animals. The end points were the examination of the lungs of animals for the presence or absence of *P. aeruginosa*, elastase concentrations, total cell counts and neutrophil levels in bronchoalveolar lavage fluid, and lung histopathological changes following aerosol treatment with protease inhibitor.

Eighty-one animals were utilized in the experiments. All animals were inoculated intratracheally with 10^4 *P. aeruginosa* strain PAO in agar beads. Two days following inoculation, rats were exposed to aerosol preparations from an Aero-Tech II nebulizer (CIS-US, Bedford, MA, USA). The nebulizer was operated at 45 psi, with a flow rate of 10 l/min and contained 10 ml of the preparation to be aerosolized. The 10 ml volume was dispensed in 25-30 min. Animals were treated daily for 7 days; control animals received daily exposure to normal saline. Animals were sacrificed on days 0, 3 and 7. Three animals from treatment and control groups were subjected to bronchoalveolar lavage consisting of 10 ml normal saline at 37 °C on days 0, 3 and 7. Lavage fluids were examined for total cell counts by hemacytometer, neutrophil counts by differential counts following Wrights staining, and total elastase in lavage fluid was measured using the synthetic chromogenic elastase substrate MeOSAAPV*p*NA. On days 0, 3 and 7, the left lungs of the 3 treatment and control animals were removed for quantitative culture and the left lungs of 3 treatment and control animals were removed and processed for histopathological examination.

The data in Table II demonstrates that Syn-1390 and Syn-1396 reduced neutrophil counts in *P. aeruginosa* infected animals at 3 and 6 days following initiation of infection. When compared to neutrophil counts in control (untreated) animals, neutrophil counts in bronchoalveolar lavage fluid obtained from Syn-1390 and Syn-1396 treated animals demonstrated a significant (p <0.05) decrease. Total cell counts were not significantly different between control and treated animals.

As shown in Table III, Syn-1390 and Syn-1396 reduced the elastase concentrations in *P. aeruginosa* infected animals at 3 and 7 days following initiation of infection. When compared to elastase concentrations in control (untreated) animals, elastase concentrations in bronchoalveolar lavage fluid obtained from Syn-1390 and Syn-1396 treated animals demonstrated a significant (p <0.05) decrease.

No differences were noted between control and treated animals in lung pathology or bacterial numbers (data not shown).

Conclusions

The 2-spirocycopropyl cephem sulfones represent novel and potent human neutrophil elastase inhibitors as determined by both *in vitro* and *in vivo* analyses. These inhibitors have high specificity towards HNE with minimal activity against other serine proteases. High concentrations of the inhibitors could be delivered to the airways without evidence of adverse effects. This is consistent with the well-known safety profile of cephalosporins in general. Since these inhibitors are of low molecular weight, it is possible to deliver high inhibitor to elastase molar ratios at relatively low inhibitor concentrations.

The protection provided by Syn-1390 against human neutrophil elastase-mediated lung injury was significant and sustained. Both Syn-1390 and Syn-1396 were also potent inhibitors of rat neutrophil elastase. Aerosol delivery of Syn-1390 in rats chronically infected with *P. aeruginosa* led to inhibition of inflammation. These data suggest that the 2-spirocyclopropyl cephem sulfones Syn-1390 and Syn-1396 may represent an effective therapeutic alternative to protect the lung from neutrophil elastase-mediated lung damage during respiratory bacterial infections.

Acknowledgements

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