Biochemical and pharmacological characterization of SSR69071, a novel orally active elastase inhibitor

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Abbreviations: α₁PI, α₁-protease inhibitor; HLE, human neutrophil elastase; PMNL, polymorphonuclear leukocyte; BAL, bronchoalveolar lavage; COPD, chronic obstructive pulmonary disease; ARDS, adult respiratory distress syndrome; MMP, Matrix Metalloproteinase.
Abstract

Human leukocyte elastase (HLE) is a proteinase, capable of degrading a variety of proteins. Under normal circumstances, the proteolytic activity of HLE is effectively controlled by its natural inhibitors. However, an imbalance between elastase and its endogenous inhibitors may result in several pathophysiological states such as chronic obstructive pulmonary disease, asthma, emphysema, cystic fibrosis and chronic inflammatory diseases. It is anticipated that an orally active HLE inhibitor could be useful for the treatment of these diseases.

SSR69071 is a potent inhibitor of HLE, the inhibition constant (Ki) and the constant for inactivation process (k_on) being $0.0168 \pm 0.0014 \text{ nM}$ and $0.183 \pm 0.013 \text{ 10}^6 \text{ /Msec}$, respectively. The dissociation rate constant, $k_{off}$ was $3.11 \pm 0.37 \text{ 10}^6 /\text{sec}$. SSR69071 displays a higher affinity for human elastase than for rat (Ki=3 nM), mice (Ki=1.8 nM) and rabbit (Ki=58 nM) elastases. Bronchoalveolar lavage fluid from mice orally treated with SSR69071 inhibits HLE (ex vivo), and in this model, SSR69071 has a dose-dependent efficacy with an ED$_{50}$=10.5 mg/kg po. SSR69071 decreases significantly the acute lung haemorrhage induced by HLE (ED$_{50}$=2.8 mg/kg po) in mice. Furthermore, SSR69071 prevents carrageenan (ED$_{30}$=2.2 mg/kg) and HLE – induced (ED$_{30}$=2.7 mg/kg) paw oedema in rats after p.o. administration.

In conclusion, SSR69071 is a selective, orally active and potent inhibitor of HLE with good penetration in respiratory tissues.
Human leukocyte elastase (HLE) belongs to the chymotrypsin family of serine proteinases. The optimum pH of this enzyme is close to neutrality and the catalytic site is composed of three hydrogen-bonded amino acid residues, His57, Asp102 and Ser195 (in chymotrypsin numbering), which form the so-called catalytic triad. The enzyme is composed of a single peptide chain of 218 amino acid residues and four disulfide bridges. It shows 30-40% sequence identity with other elastinolytic or nonelastinolytic serine proteinases. HLE preferentially cleaves the oxidized insulin B chain with Val at the P1 position, but it also hydrolyzes bonds with Ala, Ser or Cys in the P1 position (Skiles and Jeng, 1999).

HLE is located in the azurophilic granules of polymorphonuclear leukocytes (PMNL), where the HLE concentration is rather high (3 µg enzyme per 10^6 cells) (Liou and Campbell, 1995). The major physiological function is to digest bacteria and immune complexes and to take part in the host defense process (Travis et al., 1991). HLE aids in the migration of neutrophils from blood to various tissues such as the airways in response to chemotactic factors (Banda et al., 1988). HLE also takes part in wound healing, tissue repair and in the apoptosis of PMNL (Trevani et al., 1996).

In addition to elastin (highly flexible and highly hydrophobic component of lung connective tissue, arteries, skin and ligaments), elastase cleaves many proteins with important biological functions, including different types of collagens (Kittelberger et al., 1992), membrane proteins and cartilage proteoglycans (Janusz and Doherty, 1991). HLE also indirectly favors the breakdown of extracellular matrix proteins by activating procollagenase, prostromelysin and progelatinase (Rice and Banda, 1995). HLE inactivates a number of endogenous proteinase inhibitors such as α2-antiplasmin, α1-antichymotrypsin, antithrombin and tissue inhibitor of metalloproteinases (Higushi et al., 1992).

Extracellular elastase activity is tightly controlled in the pulmonary system by α1-Protease inhibitor (α1PI), responsible for protection of the lower airways from elastolytic damage, while the secretory leukocyte proteinase inhibitor, protects mainly the upper airways (Vogelmeier et al., 1991). In a number of pulmonary pathophysiological states e.g. pulmonary emphysema (Fujita et al., 1990), chronic bronchitis (Fujita et al., 1990) and cystic fibrosis (Griese, 2001), endogenous elastase inhibitors are inefficient in regulating HLE activity.

HLE is considered to be the primary source of tissue damage associated with inflammatory diseases such as pulmonary emphysema (Groutas, 1987), adult respiratory distress syndrome.
(ARDS) (McGuire et al., 1982), chronic bronchitis (Llewellyn-Jones et al., 1996), chronic obstructive pulmonary disease (COPD) (Piccioni et al., 1992), pulmonary hypertension (Cowan et al., 2000) and other inflammatory diseases (Adeyemi et al., 1985) as well as bronchopulmonary dysplasia in premature neonates (Stiskal et al., 1998).

HLE is involved in the pathogenesis of increased and abnormal airway secretions commonly associated with airway inflammatory diseases (Fujimoto et al., 1995). Thus, bronchoalveolar lavage (BAL) fluid from patients with chronic bronchitis and cystic fibrosis, had increased HLE activity. Furthermore, excessive elastase has been proposed to contribute not only to these chronic inflammatory diseases but also to acute inflammatory diseases such as ARDS and septic shock. These findings stimulated interest in the search for agents with elastase inhibitory activity and many synthetic inhibitors of HLE have been described and reviewed (Skiles and Jeng, 1999; Metz and Peet, 1999; Leung et al., 2000).

Recently we have synthesized a novel, elastase inhibitor with low molecular weight, SSR-69071, 2-(9-(2-Piperidinoethoxy)-4-oxo-4H-pyrido[1,2-a]pyrimidin-2-yloxymethyl)-4-(1-methylethyl)-6-methoxy-1,2-benzisothiazol-3(2H)-one-1,1-dioxide, C27H32N4O7S for the treatment of COPD, ARDS, cystic fibrosis, asthma and other inflammatory diseases. SSR69071 is a saccharine derivative with a molecular mass 565.64 Da (Fig. 1a). In the present study, we report on the biochemical and pharmacological properties of SSR69071. For comparison ZD8321, a selective and orally active elastase inhibitor (Veale et al., 1997), was synthetized and used as a reference in the biochemical and pharmacological studies (Fig. 1b).
Materials and methods

Animals Male NMRI mice approximately 6 weeks old and weighing between 18-20 g and CDBR male rats weighing 98-204 g were obtained from Charles-River Hungary Kft. Male New Zealand rabbits (3.5 – 5 kg) were obtained from LabNyul Kft, Hungary. All experimental and housing procedures were approved by the Institutional Animal Care and Use Committee of Sanofi-Synthelabo Research Budapest and by the Hungarian Animal Ethic Committee. Animals were acclimatized to housing conditions for at least one week before the experiments.

Reagents and Drugs Human sputum elastase (875 U/mg) and elastin – congo red were obtained from Elastin Product Co. Inc. (Owensville, Missouri, USA). Brij-35, Casein, Glycerolformal, Dimethicon, CMC, Tween 80, Cremophor EL, Meo-Suc-Ala-Ala-Pro-Val-pNa, porcine pancreatic elastase (5U/mg) and carrageenan were purchased from SIGMA-Aldrich Kft, (Budapest, Hungary). SSR69071 and ZD8321 were synthesized in the chemistry laboratory of Sanofi-Synthelabo Research Budapest. Their purity, determined by HPLC and TLC, were > 95 %.

In vitro studies

Elastase assay

Enzyme sources

Human leukocyte elastase Human sputum elastase was used as human leukocyte elastase (Green et al, 1991). The enzyme was dissolved in assay buffer (50 mM HEPES/NaOH, pH=7.8 containing 0.5 M NaCl and 0.1 mg/ml BSA) at a 656 U/ml (30 μM) concentration. This solution was diluted in assay buffer 10 times and was stored in aliquots (80 μl) at –80 °C.

Preparation of murine and rat neutrophil extracts Peritoneal neutrophils were obtained from mice and rats pretreated for 16 hours with a 1 % casein (100 ml/kg) solution, i.p. After peritoneal lavage the neutrophils were centrifuged (2000 g for 10 min) and resuspended in
Tris-HCl buffer (0.1 M, pH=7.5) containing 0.1 % Brij-35 and 1 M of MgCl₂ and lysed by sonication. The lysate was used as the source of neutrophil elastase (Kawabata et al., 1991).

**Preparation of rabbit neutrophil extracts** Rabbit PMNLs were obtained from peripheral blood of New Zealand rabbits after removal of erythrocytes by sedimentation at unit gravity through dextran (Boyum, 1976). After brief ultrasonic homogenization and alternate freezing and thawing, the suspension was centrifuged (12000 g, 10 min) and the supernatant was used as enzyme source.

**Determination of elastase inhibitory activity** The elastase activity was monitored using the specific chromogenic substrate Meo-Suc-Ala-Ala-Pro-Val-pNa. The assay mixture contained 130 µl (8.5 U) elastase, 100 µl substrate (final concentration: 400 µM), 20 µl inhibitor or DMSO and 50 µl assay buffer. Final volume of the assay was 300 µl. The assay was performed in microtiter plates placed in a kinetic plate reader (Vmax kinetic plate reader, Molecular Devices Corp.). The assay was started by addition of elastase and the change in absorbance at 410 nm was continuously monitored at 25 °C. Since both SSR69071 and ZD8321 are slow tight binding inhibitors of human leukocyte elastase, the assay reaction was monitored for 8.5 hours (read interval: 90 sec, number of readings: 340) (Williams et al., 1991).

**Determination and calculation of kinetic inhibitory constants for human leukocyte elastase inhibitors** Slow tight binding competitive inhibitors exhibited time-dependent inhibition (Cha 1975; Williams and Morrison, 1979). Three kinetic constants describe this type of inhibition, \( K_i \), inhibition constant, \( k_{on} \), the second-order kinetic constant describing the inactivation process and \( k_{off} \), the rate constant for the dissociation of enzyme inhibitor complex. The progress curve for the enzyme reaction in the presence of a slow tight binding inhibitor does not display a simple linear product versus time relationship (Morrison and Walsh, 1988; Williams and Morrison, 1979): as a result of slow onset of inhibition, product formation over time will be a curvilinear function.

The progress curves at different inhibitor concentrations can be described as follows:

Equation 1. \[ [A] = v_s t + \left( \frac{V_o - V_s}{k_{obs}} \right) (1-e^{-k_{obs}t}) + A_0 \]
where
Ao and A are absorbances at time 0 and time t, respectively
vo and vs are initial and steady state velocities in $\Delta A/\Delta t$ units, respectively
k_{obs}: pseudo-first-order rate constant of inhibition
Ao: absorbance (product) at time = 0

The control assay (without inhibitor) gave a linear progression curve, with the control velocities ($v_o$) being calculated by linear regression (Softmax for Windows (Version 2.31) (Molecular Devices Corp.). The nonlinear progress curves observed for the slow tight binding inhibitors of elastase were fitted to Equation 1; $v_s$, and $k_{obs}$ parameters were calculated with non-linear least squares (NLSQ) regression method using SigmaPlot for Windows (Jandel Scientific).
After analysis of progress curves, $K_i$ was calculated from Equation 2, where I and S are the concentrations of inhibitor and substrate in the reaction mixture, respectively. $K_m$ is the Michaelis constant for the substrate. In this analysis, $K_m = 57 \mu M$ for HLE.

Equation 2. $(v_o - v_s)/v_s = [I] [K_i(1 +[S]/K_m)]$

Re-arranging equation 2, we get

$$\frac{v_0}{v_s} = \frac{[I]}{1 - \frac{v_s}{v_o}} K_i \left(1 + \frac{[S]}{K_m}\right)$$

Plotting $v_o/v_s$ against $\frac{[I]}{1 - \frac{v_s}{v_o}}$ yields a straight line with a slope, where

$$K_i = \frac{slope}{1 - \frac{[S]}{K_m}}$$

The data for $k_{obs}$ versus $[I]$ is fitted via linear regression to yield a plot with a slope and the $k_{on}$ inactivation rate constant was calculated from this slope:

$k_{on}/slope = 1 + [S]/K_m$.

The reactivation rate constant ( $k_{off}$) was calculated from $K_i = k_{off}/k_{on}$. 
Determination of IC50 values for human leukocyte elastase – catalyzed hydrolysis of insoluble elastin

The ability of SSR69071 to inhibit the hydrolysis of insoluble elastin by human leukocyte elastase was determined spectrophotometrically using elastin congo-red as a substrate according to the method of Naughton and Sanger (Naughton and Sanger, 1961) with minor modifications. Elastin congo-red (final concentration: 10 mg/ml) and human elastase (final concentration: 20 nM) were incubated with various concentration of inhibitors in 1.2 ml of 0.1 M Hepes buffer pH 7.8, containing 0.2 M of NaCl and 0.1 mg/ml of BSA at 37 °C for 20 hours. After the incubation the reaction was stopped by centrifugation at 3000 rpm for 15 min at room temperature. Finally, absorbance of the supernatant at 495 nm was measured with spectrophotometer (Biochrom 4030, LKB-Pharmacia).

Inhibition percentage was calculated as follows:

\[
\text{Inhibition \%} = \frac{(At - Ab) - (Ai - Ab)}{At - Ab} \times 100
\]

Where:
At = Absorbance of supernatant in the absence of inhibitor
Ab = Absorbance of supernatant in the absence of enzyme and inhibitor
Ai = Absorbance of supernatant in the presence of inhibitor

Ex vivo experiments

Ex vivo inhibition of human leukocyte elastase activity in mice bronchoalveolar lavage fluid following oral administration SSR69071 was suspended in cremophor LE – distilled water = 1:3 (v/v) and administered in a volume of 10 ml/kg. Animals in the control group received the vehicle alone. In the case of dose-dependence studies, the animals were treated orally with SSR69071 (3-6-10-20 mg/kg doses) or ZD8321 (10-20-50-100 mg/kg doses) one hour before BAL fluid collection. In time dependence studies, animals were treated orally with SSR69071 (20 mg/kg) or ZD8321 (100 mg/kg) 10, 30, 60, 120, 240, 480 or 1440 min before BAL collection. Following oral treatment, the animals were euthanized and the trachea was...
exposed and a small incision made for insertion of a polyethylene cannula. A needle attached to a 1.0 ml syringe was inserted into the cannula and 0.5 ml of air was withdrawn from the airways. One-milliliter sterile physiologic saline was then instilled into the airways and the chest was briefly and gently massaged. Finally, the syringe was removed from the cannula and the BAL fluid was collected.

Elastase activity was monitored using the method described above with the following modification: BAL fluid was diluted (10 times) with the assay buffer in order to decrease the effect of natural elastase inhibitors. 150 µl diluted BAL was used in the assay. The enzymatic reaction was initiated by adding the elastase enzyme (6.5 nM, 50 µl). The change in absorbance (i.e. p-nitroaniline product formation) was then continuously monitored over 8.5 hours (read interval: 90 sec, number of readings: 340, T = 25 °C) using a plate reader (Vmax kinetic plate reader, Molecular Devices Corp.). Finally, data were processed through a Softmax program (Version 2.31, Molecular Devices Corp.).

The product formation inhibition, given as a percentage, was calculated as follows:

\[
\text{Inhibition} \% = \left( \frac{v_o - v_i}{v_o} \right) \times 100
\]

\(v_o\) : product formation rate in the presence of BAL fluid from control animals

\(v_i\) : product formation rate in the presence of BAL fluid from treated animals

**In vivo experiments**

**HLE-induced lung hemorrhage in mice** To test the effectiveness of HLE inhibitors, overnight fasted NMRI mice were treated orally via gastric tube. Investigated compounds were suspended in Cremophor EL – distilled water [1 : 3 (v/v)] and animals were treated orally in a volume of 10 ml/kg with SSR69071 (0.3-1-3-10 mg/kg doses or with ZD8321 (1-3-10-30 mg/kg doses), 30 minutes before the intra-tracheal instillation of HLE. Animals were anesthetized, and the trachea was exposed by an 5 mm incision of the neck. Animals received 10 IU HLE dissolved in 0.025 ml of ice-cold sterile physiologic saline. The solution was very slowly injected into the trachea. Three hours after HLE instillation, animals were euthanized with an overdose of
urethane. BAL fluid was collected as described above. This procedure was repeated three times and the total volume of BAL was recorded. Triton X100 was then added to the collected BAL fluid (final concentration 0.2 % v/v) to ensure cell disruption. The optical density of the supernatant was determined by spectrophotometer at 540 nm and it was correlated with haemoglobin content (Corteling et al., 2002). Values are given as mean ± SEM. The ID$_{50}$ (and 95% confidence intervals) was determined from the dose – response relationship using linear regression.

**Paw edema models on rats** To test the effectiveness of HLE inhibitors, overnight fasted rats were treated orally via the gastric tube or intravenously through the jugular vein. Animals were treated intravenously 10 minutes before or orally 120 minutes prior to intraplantar injection of HLE or carrageenan. SSR69071 was suspended in Tween80/CMC/dimethicon/distilled water (0.2:0.2:0.01:ad 100 ml) and the animals received either drug or vehicle in a volume of 1 ml/kg. For intravenous administration, SSR69071 was dissolved in glycerol - formal in a volume of 1 ml/kg. The paw edema was evoked by the intraplantar injection of 0.9 % saline solution of HLE (50U/ 0.1 ml/right hind paw) or intraplantar injection of 0.9 % saline solution of carrageenan (0.1 ml/right hind paw ) both in control and drug treated groups. The paw volume was measured immediately (control value) and at 0.5-1-2-3-4-5 hours after HLE or carrageenan injection by a plethysmograph (Ugo Basile Type 7150).

From the individual paw-volumes and post-HLE time courses, area-under-the-curve (AUC) values were calculated and expressed as mean ± SD. The edema values calculated in drug treated groups were compared to those obtained in vehicle control groups and percentage inhibition of paw edema values were calculated, ID$_{30}$ values (doses producing 30 % inhibition of paw edema) were then determined.

In order to determine the time-dependence effects of SSR69071 and ZD8321, both compounds were administered at the dose of 3 mg/kg, p.o. at different pre-treatment times (30, 60, 120, 180 and 300 minutes). The dose-dependence (0.1, 0.3, 1, 3, 10 and 30 mg/kg p.o.) study was performed, using a 2-hour pre-treatment time since the maximal effect was observed after 2 hours.
Statistical analysis *In vitro* IC$_{50}$ values were calculated with an un-weighted method for least-square fit of data. (Grafit). Statistical analysis was performed using a SAS/STAT and RS/1 software packages. The significance of differences were obtained using the Kruskal-Wallis test and Student’s test. A value of p $<$ 0.05 (*), p $<$ 0.01 (**) or p $<$ 0.001 (***) was considered as statistically significant (SAS, Version 6.12).
Results:

In vitro studies The elastase activity was measured with the help of a synthetic chromogenic substrate. Biphasic reaction progress curves were obtained during the inhibition of HLE by SSR69071 and ZD8321 as illustrated in Fig.2. The progress curve for the enzyme reaction in the presence of a slow tight binding inhibitor does not show a simple linear product versus time relationship. Product formation over time will be a curvilinear function because of the slow onset of inhibition for these compounds. As shown in Fig. 2, SSR69071 is a more potent elastase inhibitor than ZD8321. Inhibition constant (K_i) and the constant for inactivation process (k_on) were 16.8 ± 1.4 pM and 0.183 ± 0.013 10^6 /Msec for SSR69071. The dissociation rate constant of enzyme-inhibitor complex (k_off) of SSR69071, calculated as described in the Materials and Methods was 3.11 + 0.37 10^{-6}/sec. In the same experimental conditions the Ki value for ZD8321 was 5.57 ± 0.18 nM (Table 1).

Based on these results, SR69071 is a potent, competitive and slow tight binding type inhibitor of HLE. Both k_on and k_off values suggested that the enzyme – inhibitor complex has a fast association and a slow dissociation rate.

The effects of SSR69071, ZD8321 and α1-Antitrypsin were compared on the basis of their IC_{50} values. 5 nM HLE was incubated for 10 minutes with different concentrations of inhibitors and the remaining HLE activity was determined on the basis of hydrolysis of synthetic substrate. Inhibitory activities were determined and the IC_{50} values were calculated. SSR69071, ZD8321 and α1-antitrypsin inhibited HLE dose dependently with IC_{50} values of 3.9 nM, 99 nM and 10.9 nM, respectively.

The ability of SSR69071 and ZD8321 to inhibit HLE hydrolysis of insoluble elastin was also evaluated. The IC_{50} values of SSR69071 and ZD8321 to inhibit insoluble elastin degradation were 13 and 350 nM, respectively.

In order to determine species specificity of SSR69071, its elastase inhibitory effect was investigated on leukocyte elastase isolated from different species. SSR69071 showed high species specificity displaying a 2 log lower Ki for the human compared to the rat and mouse enzyme and 3 log lower for the rabbit enzyme. Ki values for mice, rat and rabbit elastase enzyme were 1.7 nM, 3 nM and 58 nM, respectively (Table 2).
SSR69071 is highly specific inhibitor of elastase type endopeptidases since it had no effect on the various receptors and enzymes (71 receptors and 27 enzymes tested by CEREP (France) and MDS Pharma Services (USA)) up to 10 µM in vitro (data not shown). The enzyme selectivity profile include the inhibition of other proteolytic enzymes, eg. serine proteinases: cathepsin G, tryptase and thrombin, cysteine peptidases: cathepsin B and metallopeptidases: endothelin converting enzyme, angiotensin converting enzyme, collagenase IV, neutral endopeptidase, MMP-2, MMP-3, MMP-7 and MMP-9.

**Ex vivo results** The activity of SSR69071 was determined in the BAL fluid after oral administration in mice. The elastase inhibitory potency of BAL was compared before and after oral treatment with SSR69071. We determined the dose dependence and the time dependence of this activity. Diluted BAL from vehicle treated mice did not significantly inhibit HLE (average HLE activity: 10.65 ± 0.46 mOD/min). SSR69071, administered orally, dose-dependently inhibited the human leukocyte elastase in the BAL fluid from 3 to 20 mg/kg (Fig. 3). This effect was not significant at the dose of 3 mg/kg (6 ± 5 %) but reached a statistical level of significance as from 6 mg/kg (27 ± 6 %, p<0.05). The maximal *ex vivo* enzyme activity inhibition was observed at the dose of 20 mg/kg (87 ± 3 %, enzyme activity = 1.1 ± 0.26 mOD/min, p<0.05). The calculated ID₅₀ was 10.5 mg/kg p.o. In the same experimental conditions, ZD8321 showed little *ex vivo* inhibitory potency since the highest dose tested (100 mg/kg) was only effective with a 44 ± 7 % inhibition of the elastase activity (Fig.3).

SSR69071 was orally administered (20 mg/kg) at different pre-treatment times (10, 30, 60 minutes and 2, 4 6 and 24 hours). Data obtained showed that HLE activity was significantly inhibited up to 6 hours with a maximum inhibitory activity observed at 30 min (90 ± 3 %, 1.2 ± 0.36 mOD/min) with even some significant inhibition seen after 10 minutes. Furthermore, HLE activity was still decreased by 42 ± 9 % (p<0.05) after a 24 hour pretreatment time (Fig. 4).

Conversely, ZD8321, administered at 100 mg/kg, displayed a moderate efficacy with a maximum HLE activity inhibition observed at 30 minutes (44 ± 9 %). This activity decreased, but still remained statistically significant after a 2 hour pretreatment time (26 ± 12 %, p<0.05) (Fig. 4.).
In vivo activity

1) Lung haemorrhage in mice The ability of SR69071 to protect animals from HLE induced lung hemorrhage was evaluated in mice. Intratracheal instillation of HLE (10 U, 10 – 15 µg) caused a severe lung haemorrhage in mice. Oral administration of SSR69071 dose-dependently and potently inhibited lung haemorrhage with an ID₅₀ value of 2.8 mg/kg (Fig. 5). Prevention of lung injury was statistically significant from the dose of 1 mg/kg (30 %, p≤0.05) and reached almost 90% at 30 mg/kg (p≤0.01). In the same experimental conditions, ZD8321 also caused a marked inhibition of haemorrhage with an ID₅₀ value of 1.6 mg/kg (Fig.5). BAL haemoglobin content was reduced by 45.6 % (p<0.01) at 1 mg/kg and by 89.3 % at the highest dose (10 mg/kg, p≤0.01).

2) HLE induced paw edema in rats The paw edema of this model is thought to reflect an increase in the permeability of the peripheral capillaries caused by elastase. Both SSR69071 and ZD8321 dose-dependently inhibited the HLE-induced paw edema after intravenous administration, with SSR69071 being more effective than ZD8321. The greatest inhibition was observed at 30 mg/kg (74.7%, p≤0.001 and 53%, p≤0.001 for SSR69071 and ZD8321 respectively). The calculated ID₃₀ values were 0.03 mg/kg i.v. for SSR69071 and 0.57 mg/kg i.v. for ZD8321 (Fig. 6A). Both SSR69071 and ZD8321 dose-dependently inhibited the HLE-induced paw edema after p.o. administration. SSR69071 displayed a maximal edema inhibition of 57.9 % (p≤0.001) at the dose of 30 mg/kg p.o. The calculated ID₃₀ values were 2.7 mg/kg p.o. for SSR69071 and 4.2 mg/kg p.o. for ZD8321 (Fig. 6B). The 3 mg/kg doses were selected from the dose – response study to determine the kinetics of inhibition on HLE induced paw edema in rats for both compounds. Both compounds were given from 0.5 to 5 hours prior to HLE in order to determine both the peak effect and the time duration efficacy (Fig. 6C). SSR69071 and ZD8321 at the doses of 3 mg/kg showed a maximal effect after a 2 hour pre-treatment time (32.4%, p≤0.01 and 28.4% p≤0.001 respectively). In addition, edema prevention remained statistically significant for at least 5 hours with both compounds (SSR69071, 15.2 %, p≤0.05 and ZD8321, 6.8 %, p≤0.05).

3) Carrageenan induced paw edema in rats Both SSR69071 and ZD8321 dose-dependently inhibited the carrageenan-induced paw edema after intravenous administration.
SSR69071 was more effective than ZD8321, maximal edema inhibition being 59 % (P≤0.001) and 47.4 % (p≤0.001) respectively. The calculated ID$_{30}$ values of SSR69071 and ZD8321 were 1.0 mg/kg and 2.5 mg/kg, respectively (Fig. 7A).

Both SSR69071 and ZD8321 from 1 to 30 mg/kg also dose-dependently inhibited the carrageenan-induced paw edema after oral administration. SSR69071 was slightly more effective than ZD8321 since maximal edema inhibitions were 51.1 % (p≤0.001) for SSR69071 and 46.1 % (p≤0.001) for ZD8321 at 30 mg/kg p.o. doses. The calculated ID$_{30}$ values of SSR69071 and ZD8321 were 2.2 mg/kg and 3.1 mg/kg, respectively (Fig. 7B).

The 3 mg/kg doses were selected from the dose–response study to determine the kinetics of inhibition on carrageenan induced paw edema in rats for both compounds. SSR69071 and ZD8321 were given orally from 0.5 to 5 hours prior to carrageenan in order to determine both the peak effect and the time duration efficacy (Fig. 7C). Both compounds showed a maximal edema inhibition after a 2 hour pre-treatment time (SSR69071 : 41.3 %, p≤0.001 and ZD8321 : 31.2 %, p≤0.05). In addition, the pharmacological activity remained statistically significant for at least 5 hours with both compounds.
Discussion

SSR69071 is a potent, competitive and slow tight binding inhibitor of HLE in vitro with a $K_i$ value of 16.8 pM (Table 1.). SSR69071 is more effective than the FK706 (Shinguh et al., 1997), ONO-5046 (Kawabata et al., 1991) and GW-311616A (Norman, 1998) according to the $K_i$ values. SSR69071 has a fast association rate and slow dissociation rate, resulting in a stable, slowly-reversible [HLE – Inhibitor] complex. The low value of $k_{off}$ suggests that the [SSR69071 – enzyme] complex is barely dissociated, resulting in the slow reversibility of inhibition. These properties of SSR69071 suggest an extremely high activity and long duration of action in humans.

The activity of SSR69071 was compared to that of $\alpha_1$PI, as it is responsible for protection of the lower airways from elastolytic damage (Vogelmeier et al., 1991). SSR69071, using methoxysuccinyl-Ala-Ala-Pro-Val-$p$-nitroanilide as a substrate, was approximately three times more potent than $\alpha_1$-Proteinase inhibitor, IC$_{50}$ values being 3.9 nM and 10.9 nM, respectively. Moreover, a more than 1000-fold higher concentration of $\alpha_1$PI on a weight basis was needed to inhibit enzyme activity because of its very high molecular mass (SSR69071: molecular mass=0.56 kDa, IC$_{50}$=0.0022 µg/ml, $\alpha_1$PI: molecular mass=54 kDa, IC$_{50}$=5.9 µg/ml). In this respect, low molecular weight inhibitors, which are therapeutically effective at low doses would have an advantage over high molecular mass inhibitors such as $\alpha_1$PI.

We examined the ability of SSR69071 to inhibit elastases from different sources by using a synthetic substrate. SSR69071 inhibited all types of elastase in a dose dependent manner, but at a much higher concentration compared to HLE. The $K_i$ values of SSR69071 for elastase-type endopeptidases such as rat, mouse, rabbit neutrophil elastase and porcine pancreatic elastases were 3 nM, 1.7 nM, 58 nM and $>>$100 nM, respectively. Thus, SSR69071 has a weak activity on porcine pancreatic type elastase and showed high species specificity displaying a 100 – 1000 fold lower Ki for human than for the rat, mouse and rabbit enzyme. The strong species selectivity of SSR69071 (human vs. rodent elastase) should be kept in mind when analyzing the effects of the compound on pharmacological animal models. The results in rats and mice may underestimate the expected in vivo potency of SSR69071 in humans.

The inhibitory activity of SSR69071 was not confined to synthetic peptide substrates but extended to the degradation by HLE of macromolecular substrates such as elastin. The elastin
congo-red derivative was used because of easy detection. Since, it was insoluble, it is not suitable for kinetic studies. When using insoluble elastin as the substrate of HLE, much longer incubation times (20 hours) were required than those used in the steady-state experiments, in order to gather sufficient reaction products for quantitative analysis. Similar to the study with a synthetic substrate, SSR69071 produced a dose-dependent inhibition of HLE-mediated elastolysis which yielded an IC$_{50}$ value of 13 nM.

The activity of SSR69071 was investigated in various in vivo animal models. The acute hemorrhagic assay conducted in the hamster is a widely used model for the assessment of in vivo activity of HLE inhibitors (Fletcher et al, 1990, Veale et al, 1997, Williams et al., 1991). This model is thought to be predictive for efficacy in emphysema (Fletcher et al, 1990). As the pharmacokinetic properties of SSR69071 were not compatible with studies in hamster, we set up and validated animal models in mice for the assessment of in vivo activity of SSR69071. The activity of SSR69071 was determined after oral administration in mice, in the BAL fluid. SSR69071 showed a dose-dependent efficacy (ED$_{50}$ was 10.5 mg/kg p.o. after 1 hour pre-treatment time). SSR69071 appeared in the BAL after oral treatment with an apparent fast absorption rate, as 10 minutes after the oral treatment significant HLE inhibitory activity (73 %) was observed (Fig. 4.). This activity demonstrated a very good penetration in the lungs from the systemic circulation. A maximum inhibitory activity was observed at 30 min (90 %), the activity being still decreased by 42 ± 9 % (p<0.05) after 24 hour pretreatment time which indicated a long duration of action.

The ability of SSR69071 to protect animals from HLE induced lung hemorrhage was evaluated in mice. Intratracheal instillation of HLE (10 IU) caused a severe lung haemorrhage. Oral administration of SSR69071 dose-dependently and potently inhibited the lung haemorrhage with an ID$_{50}$ value of 2.8 mg/kg.

The good oral activity of SSR69071 is very important because only a limited number of published elastase inhibitors show oral activity (Herbert et al., 1992; Skiles and Jeng, 1999; Metz and Peet, 1999; Leung et al., 2000) and their active doses are quite high, between 10 – 50 mg/kg (Herbert et al., 1992; Veale et al., 1997; Skiles and Jeng, 1999; Metz and Peet, 1999; Leung et al., 2000).

HLE is considered to play a crucial role in many inflammatory conditions where leukocytes infiltrate the site of inflammation and are activated by various stimuli (Fujie et al., 1999).
Shinguh et al. showed that selective elastase inhibitors could prevent edema formation induced by elastase in an experimental model in mice (Shinguh et al., 1997). Human neutrophil elastase also elicited paw edema as did other irritants such as zymosan, carrageenan and bradykinin. The paw edema in this model is thought to reflect increases in permeability of the peripheral capillaries (Shinguh et al., 1997). Nakagawa et al. showed that a selective elastase inhibitor attenuated the vascular permeability increase, leukocyte cell migration and development of granulated tissue induced by carrageenan (Nakagawa et al., 1986).

Intravenous and oral treatments with SSR69071 were effective on HLE- and carrageenan-induced paw edema in rats in a dose and time dependent manner. The maximal edema inhibition was obtained 2 hours following oral SSR69071 administration. Furthermore a significant inhibitory effect was observed up to 5 hours after the drug administration. SSR69071 dose-dependently inhibited HLE – and - carrageenan induced paw edema in rats. The calculated ID$_{30}$ value of SSR69071 was 2.7 mg/kg on HLE- induced and 2.2 mg/kg on carrageenan – induced paw edema in rats.

The oral activity of SSR69071 was demonstrated in mice and rats, in two different animal models, two HLE-dependent models and in an inflammatory model in rats (carrageenan induced edema formation).

In view of possible clinical development, we have performed a preliminary safety study in rats administered SSR69071 at a dose of 50 mg/kg/day orally for 14 days (~ 20 fold the pharmacological dose in this species). After two weeks period we could observe neither mortality, nor clinical signs, nor changes in haematology or biochemical parameters and nor histological alteration including liver (data not shown).

In conclusion, SSR69071 has been shown to be a potent and selective inhibitor of HLE exhibiting good oral activity in various rodent models (despite its lower potency on rat and mouse elastase) with a potential in the treatment of inflammatory bronchopulmonary diseases such as COPD and chronic bronchitis.
References


Legends for figures

**Fig. 1a.** Chemical structure of SSR69071. 2-(9-(2-Piperidinoethoxy)-4-oxo-4H-pyrido[1,2-a]pyrimidin-2-yloxyethyl)-4-(1-methylethyl)-6-methoxy-1,2-benzisothiazol-3(2H)-one-1,1-dioxide, C$_{27}$H$_{32}$N$_4$O$_7$S

**Fig. 1b.** Chemical structure of ZD8321. (S)-1-[(S)-2-(methoxycarbonylamino)-3-methylbutyryl]-N-[(S)-2-methyl-1-(trifluoroacetyl)propyl]pyrrolidine-2-carboxamide

**Fig. 2.** Progress curves for the inhibition of HLE by SSR69071 and ZD8321, regarding hydrolysis of the synthetic substrate, methoxysuccinyl-Ala-Ala-Pro-Val-p-nitroanilide. SSR69071 and ZD8321 were shown to be slow tight binding inhibitors of HLE.

**Fig. 3.** Dose dependence of the *ex vivo* activity of SSR69071 and ZD8321 in mice. Animals were treated orally 1 hour before the BAL collection. HLE was added exogenously into the BAL, and elastase activity was determined against a synthetic substrate (as indicated in the Materials and Methods section) and the elastase inhibitory activity was calculated. Values are means ± SEM for 10 – 15 mice. Student’s t test, * p<0.05, ** p<0.01, *** p<0.001

**Fig. 4.** Time dependence of the *ex vivo* activity of SSR69071 and ZD8321 in mice. Animals were treated orally at the specified time before the BAL collection. HLE was added exogenously into the BAL, and elastase activity was determined with synthetic substrate (as indicated in the Materials and Methods section) and the elastase inhibitory activity was calculated. Values are means ± SEM for 10 – 15 mice. Student’s t test, * p<0.05, ** p<0.01, *** p<0.001

**Fig. 5.** Effect of ZD8321 and SSR69071 on HLE – induced lung hemorrhage in mice. SSR69071 was administered orally 30 minutes before HLE (10 U/animal) instillation. Values are means ± SEM for 9 -12 mice. Kruskal-Wallis test, * p<0.05, ** p<0.01, *** p<0.001
**Fig. 6A.** Inhibitory effect of ZD8321 and SSR69071 on HLE-induced paw edema in rats. Compounds were administered intravenously 10 minutes before 50U/paw of HLE injection. The ID$_{30}$ values are 0.03 mg/kg i.v. for SSR69071 and 0.57 mg/kg i.v. for ZD8321, respectively. Values are means for 5 rats. Student’s t test, n.s. not significant, * p<0.05, ** p<0.01, *** p<0.001

**Fig. 6B.** Inhibitory effect of ZD8321 and SSR69071 on HLE-induced paw edema in rats. Compounds were administered orally 2 hours before 50U/paw of HLE injection. The ID$_{30}$ values are 2.7 mg/kg p.o. for SSR69071 and 4.2 mg/kg p.o. for ZD8321, respectively. Values are means for 5 rats. Student’s t test, n.s. not significant, * p<0.05, ** p<0.01, *** p<0.001

**Fig. 6C.** Time course effect of ZD8321 and SSR69071 on HLE-induced paw edema in rats. Compounds in a dose of 3 mg/kg were administered orally at given times before 50U/paw of HLE injection. Values are means for 5 rats. Student’s t test, n.s. not significant, * p<0.05, ** p<0.01, *** p<0.001

**Fig. 7A.** Inhibitory effect of ZD8321 and SSR69071 on carrageenan-induced paw edema in rats. Compounds were administered intravenously 10 minutes before 0.9% solution of carrageenan injection (0.1 ml/paw). The ID$_{30}$ values are 1.0 mg/kg i.v. for SSR69071 and 2.5 mg/kg i.v. for ZD8321, respectively. Values are means for 5 rats. Student’s t test, n.s. not significant, * p<0.05, ** p<0.01, *** p<0.001

**Fig. 7B.** Inhibitory effect of ZD8321 and SSR69071 on carrageenan-induced paw edema in rats. Compounds were administered orally 2 hours before 0.9% solution of carrageenan injection (0.1 ml/paw). The ID$_{30}$ values are 2.2 mg/kg p.o. for SSR69071 and 3.1 mg/kg p.o. for ZD8321, respectively. Values are means for 5 rats. Student’s t test, n.s. not significant, * p<0.05, ** p<0.01, *** p<0.001
Fig. 7C. Time course effect of ZD8321 and SSR69071 on carrageenan-induced paw edema in rats. Compounds were administered orally in 3 mg/kg p.o. doses at given times before 0.9% solution of carrageenan injection (0.1 ml/paw). Values are means for 5 rats. Student’s t test, n.s. not significant, * p<0.05, ** p<0.01, *** p<0.001
<table>
<thead>
<tr>
<th>Compound</th>
<th>Ki (nM)</th>
<th>Kinact=Kon (10^6/Msec)</th>
<th>Kreact=Koff (10^-6/sec)</th>
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<tbody>
<tr>
<td>SSR69071</td>
<td>0.0168 ± 0.00143</td>
<td>0.183 ± 0.013</td>
<td>3.11 ± 0.37</td>
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<tr>
<td>ZD8321</td>
<td>5.57 ± 0.176</td>
<td>0.0055 ± 0.0003</td>
<td>30.5 ± 0.88</td>
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**Table 1.** Kinetic constants for the inhibition of HLE by SSR69071 and ZD8321. Values are expressed as mean ± SEM (n = 5 – 6)
<table>
<thead>
<tr>
<th>Species</th>
<th>Elastase inhibition [ Ki (nM)]</th>
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</thead>
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<tr>
<td></td>
<td>SSR69071</td>
</tr>
<tr>
<td>human</td>
<td>0.0168 ± 0.001</td>
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<tr>
<td>mice</td>
<td>1.695 ± 0.4</td>
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<tr>
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<td>rabbit</td>
<td>58 ± 8</td>
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</table>

**Table 2.** Species specificity of the elastase inhibitory effect of SSR69071 and ZD8321. Values are expressed as mean ± SEM (n = 2 – 5)
Figure 1A

Figure 1B
SSR-69071

ZD-8321

Product (Absorbance)

Time (min)

0 100 200 300 400 500 600

0.0 0.2 0.4 0.6 0.8 1.0

I=0

I=5 nM

I=10 nM

I=25 nM

I=50 nM

I=75 nM

I=0

I=125 nM

I=250 nM

I=500 nM

I=1000 nM

I=2000 nM

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Figure 3
Figure 4
Figure 5
Figure 6A
Figure 6B
Figure 6C
Figure 7A
Figure 7B
% inhibition of paw edema (AUC)

pretreatment time (min)

SSR69071
ZD8321

Figure 7C