JPET Fast Forward. Published on July 26, 2011 as DOI: 10.1124/jpet.111.182139

AZD9668: pharmacological characterisation of a novel oral inhibitor of neutrophil elastase

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Running title: Preclinical pharmacology of AZD9668, a novel NE inhibitor

Word counts
Number of text pages: 28 (abstract to end of references)
Number of tables: 6
Number of figures: 5
Number of references: 40
Words in abstract: 248
Words in introduction: 681
Words in discussion: 1268
Running title: 57 characters (including spaces)

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List of non-standard abbreviations:

α_1-AT, α_1-antitrypsin
BAL, broncho-alveolar lavage
catG, cathepsin G
fMLP, fmet-leu-phe
DBA, direct binding assay
CCK, cholecystokinin
DMSO, dimethyl sulphoxide
EDC, N-ethyl (2-dimethylaminopropyl)-carbodiimide
IL, interleukin
LC-MS/MS, liquid chromatography combined with tandem mass spectrometry
LPS, lipopolysaccharide
NE, neutrophil elastase
NHS, N-hydroxysuccinimide
PMN, polymorphonuclear
Pr-3, proteinase-3
RU, refractive units
SLPI, secretory leukoprotease inhibitor
TRIS-HCl, tris(hydroxymethyl)aminomethane hydrochloric acid

Recommended section assignment: Drug Discovery and Translational Medicine

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Abstract

AZD9668 is a novel, oral inhibitor of neutrophil elastase (NE), an enzyme implicated in the signs, symptoms and disease progression in NE-driven respiratory diseases such as bronchiectasis and chronic obstructive pulmonary disease via its role in the inflammatory process, mucus overproduction and lung tissue damage. *In vitro and in vivo* experiments were evaluated the binding kinetics, potency and selectivity of AZD9668, its effects in whole-blood and cell-based assays, and its efficacy in models of lung inflammation and damage. In contrast to earlier NE inhibitors, the interaction between AZD9668 and NE was rapidly reversible. AZD9668 was also highly selective for NE over other neutrophil-derived serine proteases. In cell-based assays, AZD9668 inhibited plasma NE activity in zymosan-stimulated whole blood. In isolated human polymorphonuclear cells, AZD9668 inhibited NE activity on the surface of stimulated cells and in the supernatant of primed, stimulated cells. AZD9668 showed good cross-over potency to NE from other species. Oral administration of AZD9668 to mice or rats prevented human NE-induced lung injury, measured by lung haemorrhage, and an increase in matrix protein degradation products in broncho-alveolar lavage (BAL) fluid. In an acute smoke model, AZD9668 reduced the inflammatory response to cigarette smoke as indicated by a reduction in BAL neutrophils and interleukin-1β. Finally, AZD9668 prevented airspace enlargement and small airway wall remodelling in guinea pigs in response to chronic tobacco smoke exposure whether dosed therapeutically or prophylactically. In summary, AZD9668 has the potential to reduce lung inflammation and the associated structural and functional changes in human diseases.

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Introduction

Neutrophils are major cellular mediators of inflammation and play an important role in a number of chronic inflammatory lung diseases, including chronic obstructive pulmonary disease (COPD), bronchiectasis, acute lung injury and acute respiratory distress syndrome. For example, in COPD, the percentage of airways with neutrophils in bronchial tissues relates to the severity of airflow obstruction measured by forced expiratory volume in 1 second (FEV_1) (Hogg et al., 2004). Furthermore, sputum neutrophil numbers are correlated with peripheral airway dysfunction measured by high-resolution computed tomography (O'Donnell et al., 2004).

Neutrophil elastase (NE), an enzyme stored in the azurophilic granules of neutrophils, is an aggressive and cytotoxic 29-kDa serine protease (Sinha et al., 1987). The high intracellular concentration of NE (5 mM) is contained by its tight sub-cellular compartmentalisation. Extracellular NE activity is regulated by endogenous protease inhibitors including α_1-antitrypsin (α_1-AT), secretory leukoprotease inhibitor (SLPI), and α_2-macroglobulin (Rubin, 1996). When the extracellular free enzyme concentration exceeds the buffering capacity of endogenous inhibitors, NE becomes implicated in the signs, symptoms and disease progression in inflammatory lung disorders via its role in the inflammatory process (Bergin et al., 2008), mucus overproduction (Caldwell et al., 2005) and lung tissue damage (Wright et al., 2002).

A number of clinical observations indicate that targeting NE might be beneficial for treating some inflammatory lung diseases. In COPD, the high prevalence of emphysema in patients with α_1-AT deficiency, particularly in smokers, is believed to be caused by the unchecked action of serine proteases on lung tissue (Laurell and Eriksson, 1963). Lung lavage studies reveal that these patients have an increased number of neutrophils, increased levels of plasma

and urinary desmosine (a biomarker of elastin tissue breakdown) correlated to carbon monoxide diffusion capacity (Stolk et al., 2005), and a greater NE burden in the lower airways (Morrison et al., 1987). There is also a positive correlation between the local distribution of NE in contact with alveolar interstitial elastin and the presence of emphysematous changes in COPD lung tissue (Damiano et al., 1986). In broncho-alveolar lavage (BAL) fluid from patients with COPD, increased NE and reduced endogenous anti-protease levels are correlated with emphysema severity, with high levels of NE expressed in patients with rapidly declining lung function (Betsuyaku et al., 1996).

As well as COPD, there is accumulating evidence that NE may also be important in the pathophysiology of other chronic inflammatory lung diseases, including bronchiectasis and cystic fibrosis (CF). In bronchiectasis, a disorder primarily characterised by neutrophilic inflammation, varying degrees of fixed airway obstruction and acute infective exacerbations, high levels of NE activity have been reported in BAL (Chan et al., 2003). In CF, lung destruction is caused by obstruction of the airways due to dehydrated, thickened secretions, resultant endobronchial infection and an exaggerated inflammatory response leading to the development of bronchiectasis. The inflammatory response in the airways in CF is characterised by high amounts of interleukin-8 (IL-8), which attracts neutrophils to the lung, with a resulting excessive release of NE (Weldon et al., 2009).

In animal models, genetic deficiency or pharmacological intervention with small-molecule or physiological inhibitors of NE affords significant protection (approximately 60% reduction) against the pro-inflammatory and emphysematous effects of chronic cigarette smoke exposure (Wright et al., 2002). In addition, a broader role for NE modulating mucus hypersecretion and mucociliary clearance has been reported in several *in vitro* and *in vivo* pharmacological studies (eg.Voynow et al., 1999).

With the exception of α_1-AT protein agents, only one other NE inhibitor has received clinical registration. Sivelestat is currently used in Korea and Japan for the treatment of acute respiratory distress syndrome-related respiratory failure (Hayakawa et al., 2010). However, the use of sivelestat is limited by its poor pharmacokinetics, necessitating administration by infusion, and the risks of organ toxicity imposed by its irreversible interaction with the target enzyme (Kawabata et al., 1991).

AZD9668, (International Nonproprietary Name, alvelestat [Figure 1; Quality Assurance and Safety Medicines {QSM}, 2010]), is a novel, orally bioavailable NE inhibitor, with potential to be useful in neutrophil-driven inflammatory lung diseases. In this paper, we report the preclinical pharmacology of AZD9668 from studies used to support the drug's clinical development.

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Methods

Binding kinetics of AZD9668

The binding kinetics between AZD9668 and human NE were analysed using a Biacore T100 instrument and a Direct Binding Assay (DBA). NE (100 µg/mL in 10 mM acetate buffer pH 4.5), pre-incubated for 10 minutes with AZD9668 (1 µM) to maintain active site availability, was immobilised to a CM5 sensor chip surface by amine coupling. An activated and deactivated CM5 chip surface using amine coupling was used as a control surface.

After equilibration with running buffer (0.1 M Tris-HCl pH 7.4 containing 0.5 M NaCl buffer pH 7.4, with 1% dimethyl sulphoxide [DMSO]), AZD9668 was injected over the immobilised enzyme at a flow rate of 50 µL/min and the association rate determined. After 1 minute, running buffer was applied to the surface and the dissociation rate was determined over 5 minutes. The rate of complex (AB) formation between AZD9668 (A) and the immobilised NE (B) during the sample injection was given by:

$$d[AB]/dt = k_{on}[A][B] - k_{off}[AB] \qquad \text{(Equation 1)}$$

The interaction data were evaluated using T100 Evaluation software and the on- (k_{on}) and off- (k_{off}) rates and K_D (k_{off}/ k_{on}) were determined using global fit.

Potency, selectivity and species cross-over of AZD9668

The potency and selectivity of AZD9668 were determined by measuring the cleavage of peptide substrates to products by a range of serine proteases: human NE, proteinase-3 (Pr-3), cathepsin G (catG), pancreatic elastase, trypsin and chymotrypsin. Species cross-over was assessed in the same way using NE from mouse, rat, guinea pig and dog. Substrate concentrations were chosen to be at or below their calculated K_m (i.e. the concentration of

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substrate that permits half-maximal rate of reaction). The final substrate concentrations were as follows: MeOSuc-Ala-Ala-Pro-Val 7-amido-4-methylcoumarin 100 μM; Boc-Ala-Ala-Nva-SBzl 60 μM; N-a-Benzoyl-L-Arginine 7-amido-4-methylcoumarin 150 μM; Suc-Ala-Ala-Pro-Phe 7-amido-4-methylcoumarin 200 μM; Abz-Glu-Pro-Phe-Trp-Glu-Asp-Glu-EDDn 8 μM. Enzymes were pre-incubated with inhibitors for 15 minutes before the addition of substrate and the amount of product was measured 90 minutes later (60 minutes for porcine pancreatic elastase) after addition of a low-pH stopping buffer (acetate buffer, pH 4.3 containing 0.20 M sodium monochloroacetate, 0.06 M sodium acetate and 0.14 M acetic acid).

Potency (IC_{50}) was defined as the molar concentration of AZD9668 required to inhibit protease activity (i.e. generation of product) by 50%. pIC_{50} was calculated as the $-\log^{10}$ of the IC_{50}. The Cheng-Prusoff equation below was then used to calculate K_i (i.e. the enzyme inhibitor dissociation constant) using predetermined values for K_m (data not shown):

$$K_i = IC_{50}/(1+[S]/K_m) \qquad \text{(Equation 2)}$$

For comparison, the selectivity of AZD9668 was compared with two reference compounds, ONO6818 and ONO5046 (sivelestat).

A broader analysis of AZD9668 selectivity was made by an external company (MDS Pharma) in over 300 ligand-binding, enzyme and ion-channel assays using a single concentration of AZD9668 (10 μM). Activity in these assays was flagged by ≥50% inhibition. Full IC_{50} determinations were then performed in assays where activity was observed.

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Potency of AZD9668 in whole-blood and cell-based assays

The activity of AZD9668 was measured in human whole-blood and cell-based assays (cell-associated and explosive NE assays) and in a model of epithelial cell injury.

In the whole-blood assay, citrate anti-coagulated human whole blood was incubated with AZD9668 for 15 minutes before the addition of opsonised zymosan (final concentration 0.75 mg/ml). NE activity was measured in cell-free plasma following addition of NE substrate (71 μM final concentration in 0.1 M Tris-HCl pH7.4 containing 0.5 M NaCl) and incubation for 60 minutes at room temperature.

In the cell-associated NE assay, polymorphonuclear (PMN) cells (>90% neutrophils) isolated by centrifugation of citrate anticoagulated blood through Polymorphprep (Axis-Shield, Oslo, Norway), were resuspended at a concentration of 3×10^6/ml in Hanks balanced salt solution and then incubated sequentially with lipopolysaccharide (LPS) (1 μg/ml) and then fmet-leu-phe (fMLP) (10 nM) to maximise the expression of cell-associated NE, as described by Owen et al (Owen et al., 1995). After extensive washing in phosphate-buffered saline to remove soluble enzyme, cell-surface NE activity was measured in the presence or absence of AZD9668 (15 minute pre-incubation) by the addition of NE substrate (final concentration 71 μM) for 60 minutes.

In the explosive NE assay (Yurewicz and Zimmerman, 1977), Polymorphprep-purified PMN (0.75×10^6/ml) were pre-incubated with cytochalasin B (2.5 μg/ml) for 15 minutes at 37°C and then substrate (66 μM final concentration), inhibitor and 30 nM fMLP were added simultaneously. NE activity was then measured after 3 minutes as described above. Percent inhibition was calculated by comparing NE activity with and without AZD9668. Potency was expressed as pIC_{50} as described above.

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Activity of AZD9668 in *in vivo* assays

Animals

Mice (18-20 g) were kept at 5-10 animals per type III macrolon wire top cage (Tecniplast, Italy) in a room with a 12-hour light-dark cycle, at 20-22°C and 50-60% humidity. Mice were fed with Lactamin R70 pellets (Lantmannen, Sweden) and tap water *ad libitum*.

Rats (180-220 g) were kept at 4-6 animals per type IV macrolone cage (Tecniplast, Italy) with 12-hour light dark-cycle at 22°C with 50-60% relative humidity and fed with Lactamin R70 pellets (Lantmannen, Sweden) and given tap water *ad libitum*. All animals were allowed to acclimatise to the standard housing conditions for 1 week before use. Health monitoring was done, without positive findings according to Federation of European Laboratory Animal Science Associations guidelines.

Guinea pigs (Hartley) were purchased from Charles River, Montreal, and were housed with 12-hour light dark-cycle at 22°C. They were fed standard guinea pig chow supplemented with hay bales, and water *ad libitum*. All procedures were approved by the University of British Columbia Animal Care Committee.

The *in vivo* efficacy of AZD9668 was determined in three acute and one chronic animal models. All studies were carried out in accordance with standards established by Council of Europe ETS123 App A, AstraZeneca Global R&D Standards for animal care and according to EU Directive 86/609 and Swedish legislation. The studies were approved by the regional Ethic's Board, Malmoe/Lund in Sweden.

In a model of acute lung injury (based on a model developed by Tremblay et al) (Tremblay et al., 2002), human NE administered as a bolus into the trachea of anaesthetised (isoflurane, Forene, Abbott, Sweden) mice causes haemorrhage over a period of hours and the

accumulation of red blood cells in BAL fluid. Human NE (250 U/ml and 1 ml/kg body weight), dissolved in 9 mg/ml NaCl, was given to female C57BL/6JBomTac mice (Taconic Europe, Denmark) intra-tracheally 1 hour after oral administration of AZD9668 in drug vehicle (0.5% hydroxypropyl methylcellulose in citrate buffer). Non-drug-treated control and NE-treated animals were administered either 9 mg/ml NaCl or human NE as appropriate 1 hour after administration of drug vehicle. Four hours later, the mice were sacrificed by an overdose of pentobarbital and subjected to BAL. The BAL fluid was then centrifuged and the cell pellet resuspended in 1 mL deionised water to lyse the red blood cells. Haemorrhage was defined as the concentration of haemoglobin in BAL cell lysate and was calculated by determining the absorbance at 412 nm and extrapolating the values from a haemoglobin reference curve.

In a similar model in rats (female HanTac: WH, Taconic Europe, Denmark), human NE (250 U/ml and 1 ml/kg body weight) was administered 1 hour after oral dosing with AZD9668 in drug vehicle (Migloyl 812). Non-drug-treated control and NE-treated animals were administered either 9 mg/ml NaCl or human NE as appropriate 1 hour after administration of drug vehicle. BAL samples were taken from different animal groups at 2 and 4 hours for measurement of hydroxyproline and desmosine, respectively. Hydroxyproline is a marker for collagen breakdown and desmosine is a marker of elastin tissue breakdown.

The concentration of total desmosines (sum of desmosine and isodesmosine) in BAL fluid was measured by a method based on liquid chromatography combined with tandem mass spectrometry (LC-MS/MS). Quantification was made from a standard curve in the range 0.1 to 10 nmol/L. At the lower limit of quantification (0.1 nmol/L), the precision of the assay was 5.6% and at the upper limit (10 nmol/L) it was 8.4%.

The hydroxyproline concentration in BAL fluid was measured by a method based on LC-MS/MS. Quantification was made from a standard curve in the range 0.33–13.3 μmol/L. At the lower end of the standard curve, the precision of the assay was 2.0% and at the upper end it was 2.5%.

In an acute smoke model (van der Vaart et al., 2004), mice were allowed to acclimatise to the standard housing conditions for approximately 1 week. In the second week, all mice were placed in the automated smoke exposure box system (ProMech Lab AB, Malmö, Sweden) for increased periods of time, starting with 10 minutes on day 1 increasing to 50 minutes on day 3 to acclimatise to the new conditions.

Female BALB/cJBomTac mice (Taconic Europe, Denmark) were exposed alternately to cigarette smoke (12 Kentucky Research cigarettes 1R3F) and fresh air for 50 minutes twice daily for 4 days in a whole-body box exposure system with positions randomised. AZD9668 in drug vehicle (20% glucose, 0.1% polysorbate in water) was administered orally 45 minutes before each smoke exposure. Non-drug-treated air and smoked control animals were exposed to drug vehicle only 45 minutes before exposure to fresh air or smoke as appropriate. Animals were sacrificed (as described above) 16 hours after the last smoke exposure and subjected to BAL. Total and differential BAL cell counts were performed. IL-1β in undiluted BAL samples was analysed using enzyme-linked immunosorbance assay (ELISA) (R&D systems, Oxon, UK).

In a chronic smoke model which reproduces many of the pathophysiological aspects of human COPD, the effects of AZD9668 were evaluated in guinea pigs exposed to the tobacco smoke from Kentucky R21 cigarettes (2 hours, nose only exposure) once daily, 5 days a week for a duration of 24 weeks.

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Groups of 6–7 female Hartley strain guinea pigs (Charles River, Montreal, Quebec), initially weighing approximately 350 g, were exposed to either air (control animals) or smoke. The smoking apparatus and details of the exposure methods have been described previously (Churg et al., 2007). AZD9668 (100 mg/kg and 1 ml/kg) or vehicle only (corn oil) was given orally by gavage according to prophylactic and therapeutic dosing regimens. In the prophylactic group, AZD9668 was given once daily 45 minutes prior to each smoke exposure for the duration of the model. In the therapeutic group, animals were treated with vehicle 45 minutes prior to smoke exposure for the first 12 weeks and then transferred to oral AZD9668 (as described above) for the final 12 weeks. At 24 Weeks, the animals were euthanised and BAL and tissue samples were removed for analysis of inflammatory indices and structural changes as described (Churg et al., 2007).

Statistical analysis

Data from the acute animal models are represented as mean values ± standard error of the mean×2. Group sizes were decided by 80% power calculation. Statistical significance was calculated using a one-sided Student's t-test for a decreasing effect using pooled inter-animal variability from the ANOVA.

In the chronic smoke model (data represented as mean ± standard error of the mean) differences between air, smoked vehicle and smoke plus AZD9668 groups were analysed by ANOVA followed by Tuckey's post test for multiple comparisons made using SYSTAT (SYSTAT, Evanston, IL).

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Materials

Human NE and human catG, purified from human sputum, were obtained from Merck-Calbiochem (Darmstadt, Germany). Human NE used in the kinetic binding studies and Pr-3 were obtained from Elastin Products (Owensville, USA). Dog and guinea pig NE (purified from dog neutrophils) and recombinant rodent NE were prepared in house.

Coupling reagents N-ethyl (2-dimethylaminopropyl)-carbodiimide (EDC), N-hydroxysuccinimide (NHS), 1 M ethanolamine, HBS-P buffer (10 mM Hepes, pH 7.4, 150 mM NaCl, 0.005% p20) and CM5 Biacore sensor chip were all obtained from GE Healthcare Biosciences AB, Sweden. DMSO was obtained from Sigma-Aldrich, Sweden (Cat number D2650).

Porcine pancreatic elastase, chymotrypsin, trypsin, cytochalasin B, fMLP, LPS (LPS-*EColi* serotype 0-111B4) and tris(hydroxymethyl)aminomethane hydrochloric acid (TRIS-HCl) were obtained from Sigma Chemical Company (Stockholm, Sweden). The substrates for NE (human and other species) and porcine pancreatic elastase (MeOSuc-Ala-Ala-Pro-Val 7-amido-4-methylcoumarin), trypsin (N-a-Benzoyl-L-Arginine 7-amido-4-methylcoumarin) and chymotrypsin (Suc-Ala-Ala-Pro-Phe 7-amido-4-methylcoumarin) were obtained from VWR International (Stockholm, Sweden).

The Pr-3 substrate was obtained from Elastin Products (Owensville, USA). The catG substrate (Abz-Glu-Pro-Phe-Trp-Glu-Asp-Glu-EDDn), ONO6818, sivelestat (ONO5046), roflumilast, dexamethasone, salbutamol and tiotropium were synthesised in house (Department of Medicinal Chemistry, AstraZeneca R&D, Lund, Sweden).

Zymosan A from *Saccharomyces Cerevisiae* was from Fluka Laboratories (Buchs, Switzerland). Opsonised zymosan was prepared by incubating sterile (boiled) zymosan with

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pooled fresh human serum (50 mg zymosan/mL serum) at 37°C for 20 minutes. The opsonised zymosan was washed in phosphate-buffered saline and stored frozen at a concentration of 30 mg/ml.

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Results

Binding kinetics

AZD9668 had a high binding affinity for human NE (K_D = 9.5 nM) and potently inhibited NE activity (Table 1; Figure 2). The calculated pIC_{50} (IC_{50}) and K_i values for AZD9668 for human NE were 7.9 (12 nM) and 4.9 nM, respectively. Compared with and in contrast with ONO6818, an inhibitor that binds covalently to NE, AZD9668 exhibited a more rapid association and dissociation rate, and its interaction with NE was fully reversible.

Potency, selectivity and species cross-over

AZD9668 was at least 600-fold more selective for human NE compared with other serine proteases (Table 2). Compared with reference NE inhibitors, AZD9668 generally showed greater specificity than ONO6818 or sivelestat for NE over other neutrophil-derived serine proteases, particularly Pr-3 and pancreatic elastase (Table 3).

In a panel of 319 *in vitro* radioligand-binding, ion-channel and enzyme assays, significant inhibition (greater than 50% inhibition at 10 μM) by AZD9668 was detected at only three targets, the adenosine transporter (IC_{50}, 3.2 μM), noradrenaline transporter (7.2 μM) and the cholecystokinin (CCK) receptor (4.6 μM) (data not shown). AZD9668 showed good cross-over potency for NE from other species (Table 4).

Potency in whole-blood and cell-based assays

The pIC_{50} (IC_{50}) values for the whole-blood, cell-associated, and explosive-release assays were 7.36 (44 nM), 7.32 (48 nM) and 7.30 (50 nM), respectively (Table 5).

Activity of AZD9668 in acute *in vivo* models

In the acute lung injury model, instillation of human NE into the trachea of mice increased BAL haemoglobin but not desmosine or hydroxyproline (data not shown). Oral administration of AZD9668 at doses >1.5 mg/kg was associated with a significant reduction in human NE-induced haemoglobin levels (Figure 3). In the rat, AZD9668 inhibited the increase in NE-induced BAL hydroxyproline in a dose-dependent manner with significant inhibition at and above 2.5 mg/kg. It also inhibited the increase in BAL desmosine at a dose of 10 mg/kg (Figure 4).

In the model of smoke-induced airway inflammation, AZD9668 significantly reduced the number of BAL neutrophils at doses of 6 mg/kg and above and BAL IL-1β at doses of 1 mg/kg and above (Figure 5).

Activity of AZD9668 in the guinea pig chronic smoke model

In a model of chronic smoke induced inflammation and emphysema in guinea pigs, AZD9668 given orally was equally effective at reducing signs of inflammation and structural change whether given prophylactically or therapeutically. With both treatments AZD9668 prevented smoke-induced increases in lavage neutrophils and macrophages at the 6-month sacrifice time. Similarly, both treatments completely prevented airspace enlargement (emphysema) and small airway remodelling (Table 6).

There were no drug-related adverse effects in any of the *in vivo* models.

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Discussion

The results of the studies presented show that AZD9668 is a specific, potent and rapidly reversible inhibitor of human NE. In accordance with the described pathophysiological role of NE in COPD and other respiratory conditions, AZD9668 has the potential to inhibit the inflammatory burden and associated insidious decline in lung function in these conditions in a formulation that is convenient and well tolerated. AZD9668 differs from other small-molecule NE inhibitors – as exemplified by sivelestat – in several respects. Firstly, the enzyme affinity (K_i) of AZD9668 was approximately 10-fold lower than that reported for sivelestat (Kawabata et al., 1991). Second, the pharmacokinetic properties of AZD9668 make it suitable for oral dosing; sivelestat is administered by intravenous infusion. Third, its interaction with NE is rapid. The association constant for AZD9668 (K_{on} 7.0×10^6 M-1S-1) is comparable to the endogenous inhibitor, α_1-AT, and therefore likely to be sufficient to inhibit elastase activity *in vivo* at sites of inflammation, where α_1-AT may have lost its serine protease inhibitor activity due to oxidative inactivation (Beatty et al., 1980). Fourth, the rapid dissociation of AZD9668 from its target NE (i.e. its reversibility) contrasts to other covalent NE inhibitors, which bind to the enzyme covalently and have the potential to form drug-protein adducts. These adducts may be associated with drug accumulation, hypersensitivity and increased risk of toxicity (Zhou et al., 2005), which limit their long-term use in the clinic.

The selectivity of AZD9668 for NE over other serine proteases, particularly Pr-3, was significantly greater than either ONO6818 (another NE inhibitor) or sivelestat. Whether the potential safety advantages associated with high selectivity occur at the expense of efficacy is as yet unknown. Pr3 and catG, the two other granule-associated serine proteases in neutrophils, individually have the potential to contribute to human COPD by multiple mechanisms often with significant overlap to NE (Maryanoff et al., 2010), but in contrast to

NE (Shapiro et al., 2003), their role in human COPD or in the relevant smoke-driven animal models has yet to be demonstrated.

The potent inhibitory activity of AZD9668 on NE in biochemical assays was confirmed in whole-blood and cell-based systems. In the whole-blood assay, high levels of NE are released on stimulation, which overcome the serine protease inhibitor capacity of blood. In this assay, AZD9668 inhibited zymosan-stimulated NE activity in plasma with a pIC_{50} of 7.36 (46 nM). This translated to an actual potency of 29 nM when adjusted for plasma protein binding. The whole-blood assay has been useful in predicting appropriate therapeutic dosing and exposure in man and in a recent clinical trial it was shown that AZD9668 inhibited *ex vivo* zymosan-stimulated NE activity after oral dosing in human subjects (Gunawardena et al., 2010).

The cell-associated NE assay was developed to confirm inhibition of NE activity expressed specifically on the cell surface rather than the soluble form of the enzyme. Recent studies have suggested that the surface-bound catalytically active enzyme may be as important as the soluble form in mediating pathological effect (eg.Young et al., 2007; Owen, 2008) and may be more resistant to endogenous antiprotease inhibitors (Owen et al., 1995). However, direct evidence in man for a specific pathophysiological role of the cell-associated enzyme is still lacking (Tang et al., 2004). Nevertheless, similar to its activity in whole blood, AZD9668 proved to be a potent inhibitor of cell-associated NE with a pIC_{50} of 7.31 (48 nM).

Aside from the NE activity expressed on the surface of activated cells, quantal release of soluble NE has also been reported (Liou and Campbell, 1995). The rapid, localised release of high concentrations of NE that result may lead to a zone of destruction around the neutrophil (Aboussouan and Stoller, 2008) where interaction with physiological substrates and inhibitor may occur under non-equilibrium conditions. In this context, most efficient inhibition will likely be achieved with inhibitors with rapid enzyme association rates (e.g. α_1-AT). In the

explosive assay, AZD9668, with its rapid association constant, inhibited NE activity potently, with an pIC_{50} of 7.30 (50 nM).

As well as its effects *in vitro,* AZD9668 reduced levels of human NE-induced BAL haemoglobin in a mouse model of acute lung injury and reduced human NE-induced BAL desmosine and hydroxyproline in an equivalent rat model. We have not observed any significant increase in BAL hydroxyproline and desmosine after instillation of human NE in the mouse, perhaps due to sample volumes being insufficient for analysis.

The better efficacy observed in the mouse reflects a lower AZD9668 plasma protein binding compared to rat and therefore improved exposure of the drug after a given oral dose (data not shown).

Desmosine is an atypical amino acid that cross-links and provides structure to elastin; in COPD, urinary desmosine is regarded as a biomarker of lung injury and levels are increased according to disease severity and the rate of decline of lung function (Gottlieb et al., 1996; Luisetti et al., 2008). AZD9668 has also been shown to significantly reduce urinary desmosine levels after 4 weeks' oral administration to patients with CF (Elborn et al., 2011). By measuring effects on markers relevant to human lung disease, these data show that AZD9668 inhibits NE-mediated lung injury. Similarly, 6 weeks' treatment with MR889, a synthetic cyclic thiolic NE inhibitor, reduced desmosine levels in a subgroup of COPD patients with short disease duration (Luisetti et al., 1996).

The role of inflammatory cytokines (e.g. IL-6 and IL-8) in COPD is well established, particularly during disease exacerbations (Bhowmik et al., 2000). In the *in vivo* model of acute smoke-induced lung inflammation, treatment with AZD9668 was associated with a significant reduction of neutrophils and IL-1β in BAL. These results are consistent with

previous studies demonstrating that NE has the capacity to stimulate diverse pro-inflammatory signalling pathways through multiple mechanisms (Bedard et al., 1993; Wiedow and Meyer-Hoffert, 2005).

The effects of AZD9668 in the chronic smoke model were consistent with those observed in the more acute models. Both prophylactic and therapeutic treatment prevented increases in lavage inflammatory cells and structural changes in the form of emphysema and small airway remodelling. We had previously reported that another NE inhibitor ZD0892 (Wright et al., 2002) reduced lavage inflammatory cells and partially prevented emphysema in a similar model (airway remodelling was not examined) when given prophylactically, but was not effective in preventing emphysema when started at 4 months of smoke exposure, although it did reduce inflammatory cells with this regime. Whether these differences are an effect of dose, or timing, or specificity and fast onset mechanism of AZD9668 compared with ZD0892 (add ref no) is not clear. It is interesting that AZD9668 was able to completely prevent emphysema even though it does not inhibit proteinase 3 or cathepsin G (at least in humans), suggesting that those proteases may not play a role in the genesis of emphysema. This is also the first demonstration that a NE inhibitor can prevent small airway remodelling, an observation of clinical importance, since there is increasing evidence that small airway remodelling is a major cause of airflow obstruction in COPD.

Taken together, these findings suggest that NE inhibition has the potential to have a beneficial effect in inflammatory lung diseases by modulating inflammatory mediators and lung destruction. In a recent exploratory study in patients with bronchiectasis, 4 weeks' treatment with AZD9668 significantly improved some lung function measures and reduced the inflammatory mediators IL-6 and RANTES (Stockley et al., 2010).

JPET Fast Forward. Published on July 26, 2011 as DOI: 10.1124/jpet.111.182139
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To conclude, AZD9668 is a potent, selective and reversible inhibitor of human NE with good efficacy in preclinical models. Its pharmacological profile suggests that it represents a significant advance versus previous NE inhibitors and it has the potential to be effective for neutrophil-driven inflammatory lung diseases, such as bronchiectasis and COPD.

Acknowledgments

We would like to thank Dr Henrik Lindberg and Dr Claes Lindberg for their assistance with the mass spectroscopic analysis of desmosine and hydroxyproline. Editorial support was provided by Dr Nicky French from Complete Medical Communications, funded by AstraZeneca.

Authorship contributions

Participated in research design: TS, KE, MG, CJ, TO, HL, AS, AC, JLW

Conducted experiments: TS, KE, MG, ML, VK, CJ, TO, HF-H, AC, JLW

Performed data analysis: TS, KE, MG, VK, CJ, TO, HL, AS, AC, JLW

Wrote or contributed to the writing of the manuscript: TS, KE, MG, ML, VK, CJ, TO, HF-H, HL, AS, AC, JLW

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Footnotes

a) Financial support

The studies were funded by AstraZeneca.

b) Citations for meeting abstracts

Sanfridson A, Ottosson T, Ekholm K, Jungar C, Granse M, Kozma V, Falk Hakansson H, Lal H, Stevens T. AZD9668: Pharmacological characterisation of a novel oral inhibitor of neutrophil elastase (NE). Eur Respir J 2010; 942s.

c) Reprint requests

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JPET Fast Forward. Published on July 26, 2011 as DOI: 10.1124/jpet.111.182139
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Figure legends

Figure 1

- **Chemical structure of AZD9668 (alvelestat; Quality Assurance and Safety Medicines [QSM], 2010)**.

Figure 2

- **Biacore assay of AZD9668 binding to human NE.** Sensorgram showing the association and dissociation kinetics of AZD9668 and ONO6818 with immobilised human NE. The y-axis indicates the extent of interaction in Biacore refractive units (RU) and the x-axis is time in s. After equilibration with running buffer (phosphate buffer pH 7.4), AZD9668 or ONO6818 were injected over the immobilised enzyme at a flow rate of 50 µL/min and the association rate determined. After 1 minute, running buffer was applied to the surface and the dissociation rate was determined over 5 minutes. The rate of complex (AB) formation and resultant on-rate (k_{on}), off-rate (k_{off}) and K_D (k_{off}/ k_{on}) were determined as described in the methods. Data represent 1 of 2 separate experiments.

Figure 3

- **Effects of AZD9668 on human NE-induced acute lung injury in mice.** Dose response for AZD9668 measuring BAL haemoglobin. Values are mean +/- SEM ×2 (n=6-15 animals/group). Statistical significance was calculated using a one-sided Student's t-test for a decreasing effect using pooled inter-animal variability from the ANOVA. $^{\dagger\dagger\dagger}P$ <0.001 vs. control; $^{**}P$ <0.01 vs. human NE; $^{***}P$ <0.001 vs. human NE.

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Figure 4

- **Effects of AZD9668 on human NE-induced acute lung injury in rats.** Dose vs. response data for AZD9668 is shown, measuring breakdown products in BAL: hydroxyproline (left) and desmosine (right). Values are mean +/- SEM ×2 (n=4-10 animals/group). Statistical significance was calculated using a one-sided Student's t-test for a decreasing effect using pooled inter-animal variability from the ANOVA. $^{\dagger\dagger}P$ <0.01 vs. control values; $^{\dagger\dagger\dagger}P$ <0.001 vs. control values; $^{*}P$ <0.05 vs. human NE; $^{**}P$ <0.01 vs. human NE; $^{***}P$ <0.001 vs. human NE.

Figure 5

- **Effects of AZD9668 on tobacco smoke-induced airway inflammation in mice.** Dose vs. response data for AZD9668 measuring BAL neutrophils (left) and IL-1β (right). Values are mean +/- SEM ×2 (n=16-19 animals/group). Statistical significance was calculated using a one-sided Students t-test for a decreasing effect using pooled inter-animal variability from the ANOVA. $^{\dagger\dagger\dagger}P$ <0.001 vs. air; $^{*}P$ <0.02 vs. smoke; $^{**}P$ <0.002 vs. smoke; $^{***}P$ <0.001 vs. smoke.

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Tables

Table 1. Binding kinetics and affinity of AZD9668 for human NE

Compound	**N**	**k_{on} (1/M·s)**	**k_{off} (1/s)**	**off-rate $t_{½}$ (sec)**	**K_D (nM)**
AZD9668	2	7.0×10^{6}	5.7×10^{-2}	12	9.5

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Table 2. Potency of AZD9668 for human NE and other serine proteases

Serine protease	**Replicates**	**pIC_{50}** **Mean ± SEM**
NE (human)	5	7.9 ± 0.12
Proteinase-3 (human)	4	<4.6
Cathepsin G (human)	5	<5.1
Chymotrypsin (bovine)	3	<4.5
Pancreatic elastase (porcine)	5	<4.6
Trypsin (bovine)	5	<4.6

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Table 3. Comparative selectivity of AZD9668, ONO6818 and sivelestat

Serine protease	**AZD9668 (pIC_{50}=7.9)**	**ONO6818 (pIC_{50}=8.0)**	**Sivelestat (pIC_{50}=8.0)**
NE	1	1	1
Proteinase-3	1900	63	3
Cathepsin G	680	>2000	>2000
Chymotrypsin	>2000	1000	199
Pancreatic elastase	>2000	79	79
Trypsin	>2000	>2000	>2000

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Table 4. Potency of AZD9668 for NE across species

Species	**Replicates**	**pIC_{50}** **Mean ± SEM**	**Selectivity for human NE (fold)**
Human	5	7.9 ± 0.12	1
Rat	6	6.7 ± 0.03	18
Mouse	6	6.5 ± 0.02	24
Guinea pig	2	6.5 ± 0.03	24
Dog	9	6.4 ± 0.03	33

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Table 5. Potency of AZD9668 in whole-blood and cell-based assays

Assay	**Replicates**	**pIC_{50}** **Mean ± SEM**
Whole-blood	6	7.36 ± 0.04
Cell-associated	6	7.31 ± 0.04
Explosive	6	7.30 ± 0.05

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Table 6. Effect of AZD9668, dosed prophylactically or therapeutically, on lung inflammation and emphysema in guinea pigs following chronic exposure to smoke

	Readout at 24 weeks (Mean ± SEM)			
	Air + vehicle	**Smoke + vehicle**	**Smoke + AZD9668 (prophylactic)**	**Smoke + AZD9668 (therapeutic)**
BAL neutrophils x 10^3	4.4 ± 1.1*	28.0 ± 11.0	6.9 ± 0.9*	9.2 ± 1.7*
BAL macrophages x 10^6	3.7 ± 0.6**	9.5 ± 1.9	4.2 ± 0.4**	4.2 ± 0.4**
Airspace size - mean linear intercept, µm	49.7 ± 1.1#	57.6 ± 1.0	47.7 ± 0.8#	48.2 ± 0.9#
Airway wall thickness, µm	29.6 ± 2.1#	56.4 ± 0.6	26.7 ± 1.3#	24.1 ± 1.7#

*P <0.03 compared with smoke exposure

**P <0.002 compared with smoke exposure

#P <0.0000 compared with smoke exposure

FIGURE.01

N-{[5-(methanesulfonyl)pyridin-2-yl]methyl}-6-methyl-5-(1-methyl-1H-pyrazol-5-yl)-2-oxo-1-[3-(trifluoromethyl)phenyl]-1,2-dihydropyridine-3-carboxamide

$C_{25}H_{22}F_3N_5O_4S$

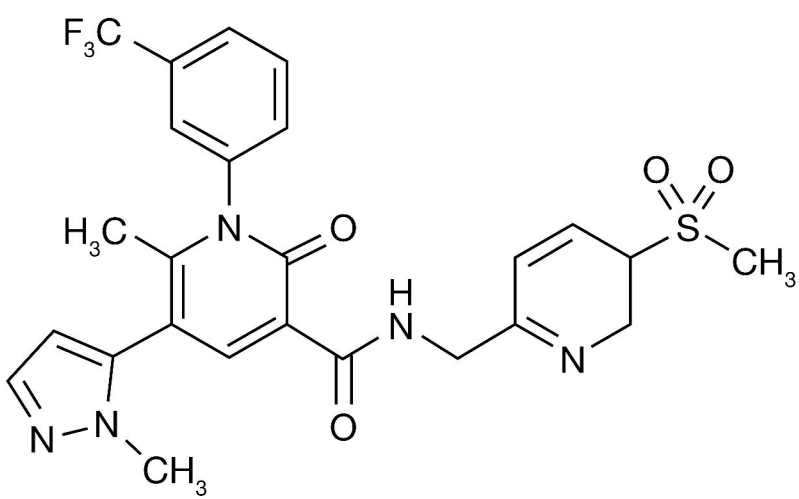

FIGURE.02

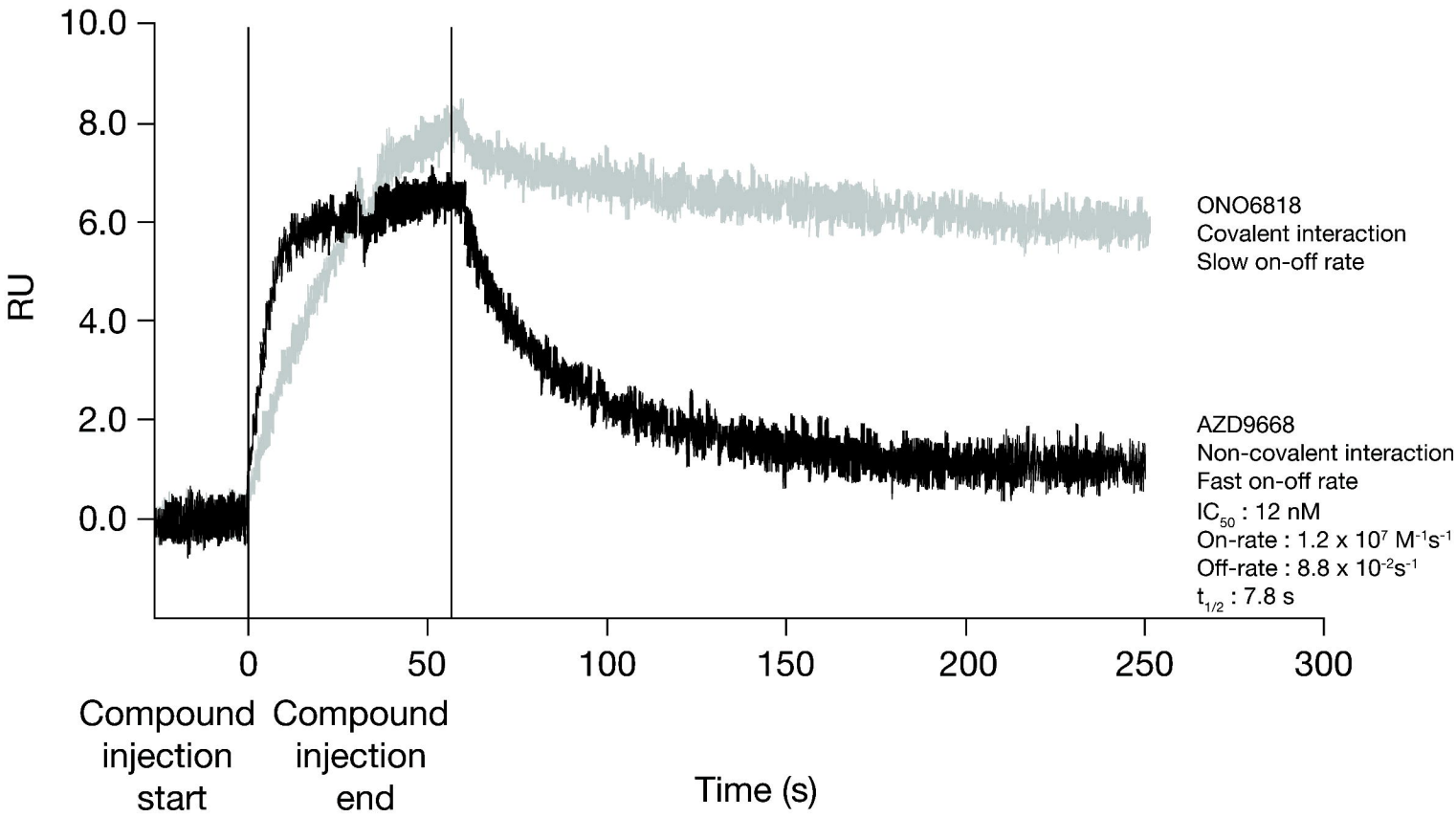
10.0
8.0
6.0
4.0
2.0
0.0
RU
0
50
100
150
200
250
300
Compound injection start
Compound injection end
Time (s)
ONO6818
Covalent interaction
Slow on-off rate
AZD9668
Non-covalent interaction
Fast on-off rate
IC50 : 12 nM
On-rate : 1.2 x 10^7 M^-1s^-1
Off-rate : 8.8 x 10^-2s^-1
t1/2 : 7.8 s

FIGURE.03

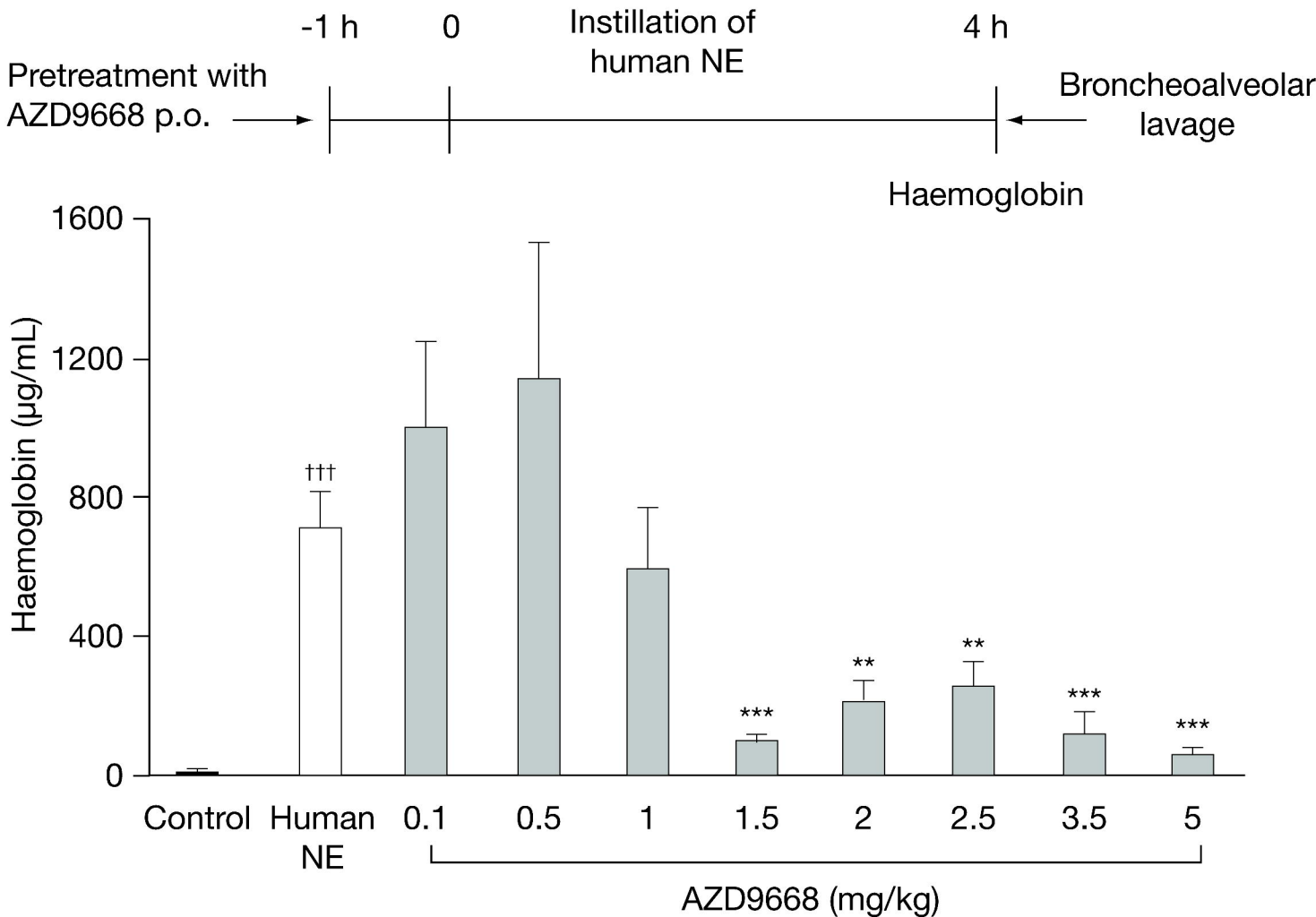
-1 h
0
Instillation of human NE
4 h
Pretreatment with AZD9668 p.o.
Broncheoalveolar lavage
Haemoglobin
Haemoglobin (µg/mL)
1600
1200
800
400
0
†††
***
**
**
***
***
Control
Human NE
0.1
0.5
1
1.5
2
2.5
3.5
5
AZD9668 (mg/kg)

FIGURE.04

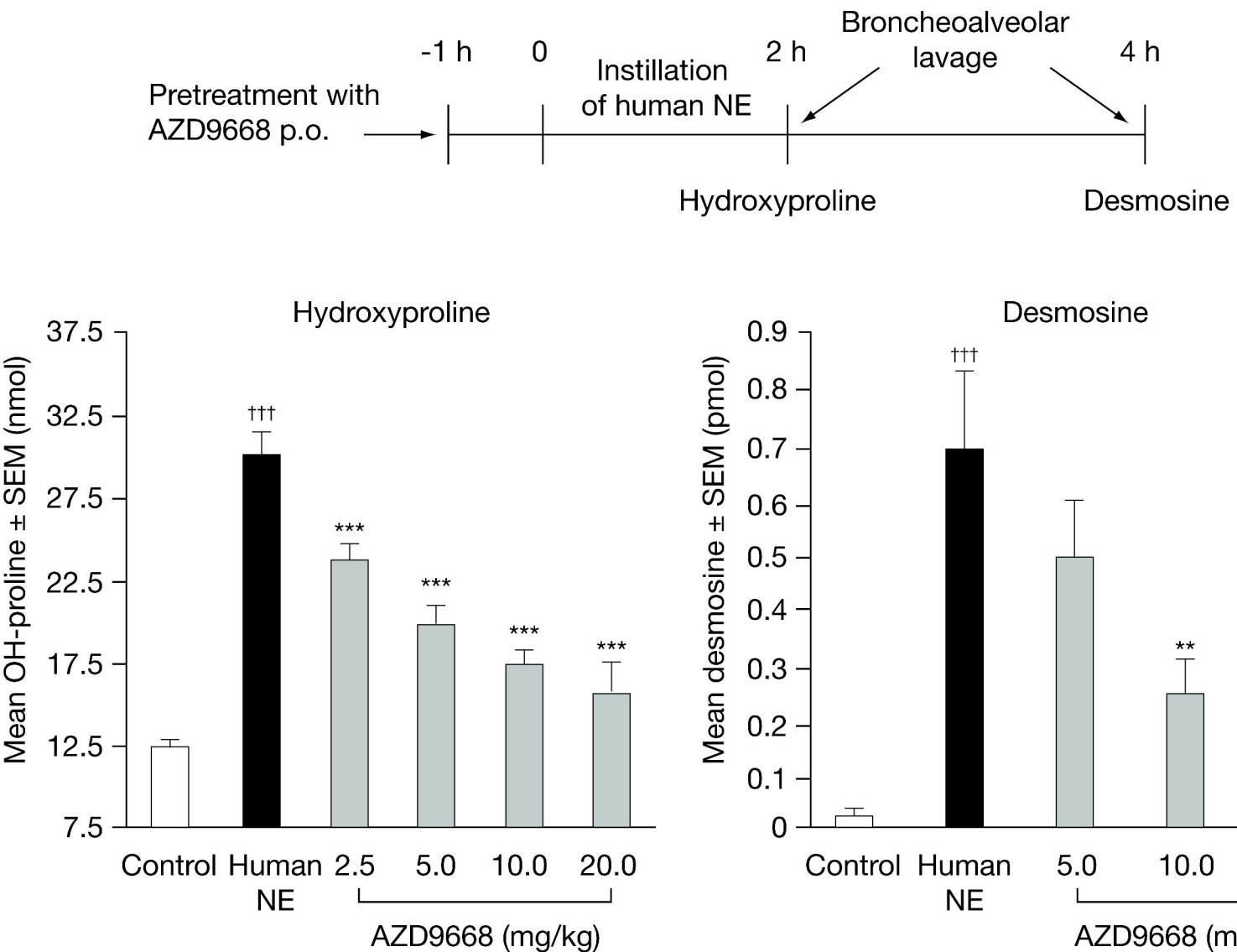

FIGURE.05