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AZD7624, an Inhaled p38 Inhibitor, Demonstrates Local Lung Inhibition of LPS-Induced TNFα with Minimal Systemic Exposure

Rikard Pehrson, Tove Hegelund-Myrbäck, Danen Cunoosamy, Sara Asimus, Paul Jansson, Naimish Patel, Annika Borde, and Sofia Lundin

AstraZeneca, Mölndal, Sweden

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ABSTRACT

Inhaled drugs generally aim to drive a local pharmacological effect in lung, at the same time minimizing systemic exposure, in order to obtain efficacy in lung disease without unwanted systemic effects. Here, we demonstrate that inhaled delivery of a p38 inhibitor (AZD7624) can provide superior pharmacokinetic exposure and superior pharmacodynamic lung effects. In rats, inhaled AZD7624 had a five times higher dose-adjusted lung exposure compared with intravenous dosing. In healthy volunteers, lipopolysaccharide (LPS)-induced tumor necrosis factor α (TNFα) in sputum has been shown to be significantly reduced (85%) by means of inhaled AZD7624. Here, we demonstrate that this effect is associated with a mean unbound plasma concentration, gained from in vitro and ex vivo LPS-challenge protocols, significantly below potencies obtained for AZD7624, suggesting that lung exposure is probably much higher than systemic exposure. This assessment was made for the unbound potency ($pIC_{50}u$), e.g., the potency remaining after adjustment for plasma protein binding and blood plasma ratio. Hence, the unbound potency of AZD7624 to inhibit LPS-induced TNFα in human mononuclear cells, in whole blood as well as in alveolar macrophages in vitro, was 8.4, 8.7 (full inhibition), and 9.0 (partial inhibition), respectively. The $pIC_{50}u$ in whole blood ex vivo was 8.8, showing good in vitro/in vivo potency correlations. Thus, a mean unbound AZD7624 plasma concentration of 0.3 nmol/l, which was associated with a decrease in LPS-induced sputum TNFα by 85%, is much lower than the $pIC_{50}u$. This demonstrates that AZD7624 can achieve significant local lung pharmacodynamic effects with concomitant sub-pharmacological systemic exposure.

Introduction

In the development of inhaled drugs for the treatment of lung disease the goal is to achieve sufficient local unbound concentration at the effect site in the lung (Cooper et al., 2012) at the same time minimizing systemic concentrations, so that likelihood of unwanted systemic side effects is also minimized (Lötvall, 1997). Such a separation has been demonstrated for inhaled budesonide in asthmatic subjects (Toogood et al., 1990).

Chronic obstructive pulmonary disease (COPD) is a major cause of morbidity and mortality worldwide (Rycroft et al., 2012; Ford et al., 2013). The p38 mitogen-activated protein kinase (MAPK) plays a central role in the regulation and activation of key proinflammatory mediators (Saklatvala, 2004; Chung, 2011). Patients with COPD have a higher percentage of p38 positive macrophages obtained from both alveoli and sputum compared with healthy smokers and nonsmokers (Gaffey et al., 2013), and p38 MAPK activation correlates with the degree of lung function impairment and alveolar wall inflammation (Renda et al., 2008).

Inhibition of p38 thus represents an attractive target (Chung, 2011), and several p38 inhibitors have been under development either for oral or inhalation delivery (Norman, 2015). For the confirmation of target engagement with these therapeutic agents, the use of lipopolysaccharide (LPS) challenge in vitro, ex vivo, and also in vivo in both humans and laboratory animals has been reported. Such data allow an analysis of the translatability of in vitro data to expected in vivo findings, in particular an evaluation of whether inhaled delivery can provide efficacy in the lung with less systemic exposure compared with oral dosing for p38 inhibitors. Of the adverse events associated with p38 inhibition, skin rash figures prominently (Cohen et al., 2009; MacNee et al., 2013) and potentially could be avoided via inhaled delivery.

LPS inhalation is a well established model of inflammation, and cigarette tobacco contains high concentrations of LPS (Kitz et al., 2008; Zielen et al., 2015). Following inhalation of LPS there is an increase in sputum tumor necrosis factor α (TNFα) (Michel et al., 1997; Nightingale et al., 1998; Korsgren et al., 2012).

In the present studies, the inhaled p38 inhibitor AZD7624 (Brough et al., 2008) was tested in several LPS challenge models: 1) peripheral blood mononuclear cells (PBMC) in

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ABBREVIATIONS: AUC, area under the curve; COPD, chronic obstructive pulmonary disease; FLISA, fluorescence-linked immunosorbent assay; LC-MS/MS, liquid chromatography–tandem mass spectrometry; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; PBMC, peripheral blood mononuclear cells; $pIC_{50}u$, unbound potency remaining after adjustment for plasma protein binding and blood plasma ratio; TNFα, tumor necrosis factor α.

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vitro, 2) human blood ex vivo, 3) human alveolar macrophages in vitro (Patel et al., 2018), and 4) inhaled LPS in healthy volunteers (Patel et al., 2018). The extent of pharmacokinetic lung targeting was investigated in rats. The LPS challenge models allowed comparison of potency from in vitro to ex vivo. Subsequently, pharmacodynamic lung targeting was investigated by comparing ex vivo potency to in vivo effect after inhalation.

Materials and Methods

The animal study was approved by the Animal Ethics Committee of Gothenburg (134-2013) and was carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health.

For the studies involving human subjects, these have been carried out in accordance with the Declaration of Helsinki and with approval by relevant ethics committees.

In Vitro

In Vitro LPS Challenge in Human Alveolar Macrophages. Human lung tissue was obtained from lung cancer resection sections from four patients (Midlands Lung Tissue Consortium) and was flushed with phosphate-buffered saline to isolate macrophages. phosphate-buffered saline washings were pooled and centrifuged at 400*g* for 10 minutes at room temperature prior to resuspension of the pellet in 10 ml of assay buffer (RPMI 1640 medium containing 100 IU/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine). Cells were seeded in assay buffer at 50,000 cells per well in 96-well plates. Plates were incubated under standard tissue culture conditions for 24 hours. Macrophages were preincubated with AZD7624 (0.003–100 nM) for 4 hours at 37°C. The p38 MAPK pathway was induced with LPS (1 μg/ml, *Escherichia coli* 0111:B4,L4130, cat. no. H4522; Sigma-Aldrich, Poole, UK) overnight at 37°C, after which supernatants were collected. TNFα levels were determined using a fluorescence-linked immunosorbent assay [FLISA; human TNFα (standard for FLISA; cat no. 210-TA-010), monoclonal anti-TNFα (cat no. MAB610), biotinylated anti-TNFα polyclonal antibody (cat. no. BAF210) from R&D Systems, Abingdon, UK].

Human Peripheral Blood Mononuclear Cells. Human blood was obtained from healthy volunteers, and PBMC were separated by density gradient centrifugation and suspended in assay buffer (RPMI1640).

For inhibition experiments, cells were preincubated with compound for 20 hours. After the preincubation step, LPS (1 μg/ml, *E. coli* 0111:B4, cat. nos. L4130 and H4522; Sigma-Aldrich) was added for 4 hours to induce TNFα production. The amount of TNFα released was quantified using FLISA [human TNFα (standard for FLISA; cat no. 210-TA-010), monoclonal anti-TNFα (cat no. MAB610), biotinylated anti-TNFα polyclonal antibody (cat. no. BAF210) from R&D Systems].

Whole Blood. Human blood was obtained from healthy volunteers. For inhibition experiments, cells were preincubated with compound for 30 minutes. After the preincubation step, LPS (1 μg/ml, *E. coli* 0111:B4, cat. nos. L4130 and H4522; Sigma-Aldrich) was added for 4 hours to induce TNFα production. The amount of TNFα released was quantified using FLISA as above.

Plasma Protein Binding. The binding of AZD7624 to plasma proteins was investigated using equilibrium dialysis followed by liquid scintillation counting.

In Vivo

Rat Pharmacokinetic Study. Male Wistar rats (272–325 g; Harlan Netherlands B.V./Envigo, Huntingdon, UK) were used in the study. The rats were exposed to a single dose of AZD7624. The test formulations were administered intravenously to 28 animals and by dry powder inhalation to 26 animals. Blood and whole lung tissue specimens were collected up to 80 hours postdose, from two animals per time point, for both the intravenous and inhalation sample group.

Plasma and whole lung concentrations of AZD7624 were determined by liquid chromatography–tandem mass spectrometry (LC-MS/MS) and pharmacokinetic parameters were calculated. For intravenous dosing, 1 mg/kg (1 ml/kg as solution; 3 mM citric acid in saline, pH 6) was administrated to briefly anesthetized animals (isoflurane) as a single tail vein injection. Dry powder inhalation was conducted through a nose-only “flow-past” exposure system consisting of a semi-open inhalation chamber, restraining tubes for the rats, and a modified Wright dust feed mechanism (IH8; Promech Lab AB, Vintrie, Sweden). Measurements of the aerosol concentration in the inhalation chamber were performed by filter sampling at one of the inhalation ports (AP40, 47 mm; Merck Millipore, Burlington, MA). Measurements of the aerosol concentration in the inhalation chamber was performed by filter sampling at one of the inhalation ports (AP40, 47 mm; MilliporeSigma) during the time of animal dosing with a filtered sampling flow rate of 0.25 ml/min. Lung-delivered doses of AZD7594 (micrograms per kilogram) were calculated as described elsewhere (Phillips et al., 2017).

Assuming the same plasma clearances following intravenous dosing and inhalation, respectively, the delivered inhaled dose used for dose normalization was determined by plasma area-under-the-curve (AUC) values.

Ex Vivo LPS Challenge following Ascending Doses of AZD7624. This was a randomized, double-blind, placebo-controlled, single ascending study in healthy male subjects. The study was conducted at the Quintiles Drug Research Unit at Guy’s Hospital, Quintiles Ltd. By means of inhalation, predicted lung-deposited doses of 29, 101, 336, 631, and 1177 μg (each $n = 6$) plus placebo ($n = 12$) were given. Blood samples were taken from subjects prior to dosing and at 5 and 15 minutes after inhalation. Whole blood samples were stimulated with LPS (*E. coli* 0111:B4, L4391; Sigma-Aldrich) ex vivo for 4 hours at 37°C, and ex vivo plasma TNFα levels were measured using a sandwich immunoassay with capture antibodies immobilized onto MSD Small Spot plates (cat no. K151 BHC-1; MSD, Rockville, MD). Corresponding plasma concentration in each sample was measured by solid-phase extraction followed by LC-MS/MS and allowed a comparison of AZD7624 plasma concentrations versus inhibition of LPS-induced TNFα.

Human Inhaled LPS Challenge. A single-center, randomized, double-blind, placebo-controlled, two-way crossover phase I study was conducted with healthy volunteers to investigate the effect of single dose–inhaled AZD7624 versus placebo on inflammatory biomarkers in induced sputum and blood after inhalation of LPS. Parts of the results have already been reported for publication (Patel et al., 2018). Healthy male and female volunteers of nonchildbearing potential, aged 18–55 years, were screened within 28 days before the first administration of AZD7624, followed by a second pre-entry visit (7–14 days before start of dosing) for sputum induction (Pizzichini et al., 1996) and methacholine challenge [$PC_{20} > 16$ mg/ml required to enter the study (Crapo et al., 2000)].

Subjects received a single inhaled lung-deposited dose of AZD7624 (1200 μg) or placebo 30 minutes prior to LPS challenge (*E. coli* LPS serotype O26:B6; Sigma-Aldrich), followed by sputum induction 6 hours after LPS challenge (6.5 hours after inhalation) for measurement of inflammatory biomarkers. For the LPS challenge, 45,000 endotoxin units of LPS [approximately 9 μg, (Gorbet and Sefton, 2005) was delivered by a breath-activated Mefar dosimeter (Istanbul, Turkey]. Plasma concentrations were measured by solid-phase extraction followed by LC-MS/MS. The average plasma concentration of AZD7624 from the LPS challenge until the sputum induction was calculated by means of noncompartmental analysis.

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Pharmacokinetic-Pharmacodynamic Evaluation

Cell and Whole Blood Models. The potency of AZD7624 in inhibiting LPS-induced TNFα was evaluated in an E_{max} model (Phoenix 6.4.0.768; Pharsight/Certara, L.P., Princeton, NJ), where $E = E_0 + E_{max} \times (1 - I_{max} \times C/(C + IC_{50}))$.

$$E0 = \text{base level of TNF}\alpha \text{ in the absence of LPS} \quad (1)$$

E_{max} = maximal stimulation of TNFα production by LPS. E_{max} was allowed to differ using a population approach, e.g., $E_{max} = tvE_{max} \times e\hat{}nE_{max}$

$$\text{Imax} = \text{Maximal inhibition of LPS} - \text{induced TNF}\alpha \text{ production by AZD7624} \quad (2)$$

Experiments carried out on human material were analyzed using population modeling. For visualization in figures, the inhibitory potential of AZD7624 was expressed as percentage of maximal individual response to LPS adjusted for base line. In the case of ex vivo LPS challenge, the maximal individual response to LPS used as its basis the blood samples obtained prior to AZD7624 inhalation.

Human Inhaled LPS Challenge. The average total plasma concentration from onset of LPS challenge to sputum induction was determined for each subject by means of noncompartmental analysis (Phoenix 6.4.0.768; Pharsight/Certara L.C.). The relative individual sputum TNFα response to LPS (after AZD7624 treatment) compared with that after placebo was determined. The results are shown in Fig. 5.

Adjustment for Protein Binding and Blood/Plasma Ratio. All potency calculations obtained were transformed to estimated unbound concentration ($pIC_{50}u$) in plasma or media by using the unbound fraction in plasma (38.7%) as well as the measured human blood/plasma ratio (1.56). Hence, potency derived in plasma was adjusted for the unbound potency remaining after adjustment for plasma protein binding and blood plasma ratio ($pIC_{50}u$) (pIC_{50} increased by 0.41 in human) and potency derived in human whole blood was adjusted for both blood/plasma ratio and unbound fraction in plasma (pIC_{50} increased by 0.19 and 0.41, i.e., 0.60).

Results

TNFα Response in Cell Models after LPS Stimulation

Human Alveolar Macrophages. In human alveolar macrophages in vitro (n = 4), LPS resulted in a distinct increase in TNFα from 244.7 ± 44.0 to 469.9 ± 92.7 pg/ml. AZD7624 given in vitro could only partially suppress this increase (max inhibition 62% ± 5%) with a pIC_{50} of 9.0 ± 0.1 ($pIC_{50}u$ same as pIC_{50}, Fig. 1).

Human PBMC. In human PBMC (14 donors), baseline values of TNFα (175.0 ± 28.5 pg/ml) increased by LPS (3909.0 ± 351.0 pg/ml). AZD7624 fully suppressed this increase (see Fig. 2; Table 1) with a pIC_{50} of 8.4 ± 0.1 ($pIC_{50}u$ same as pIC_{50}).

Human Whole Blood with AZD7624 Given In Vitro. In human whole blood (11 donors), LPS resulted in a distinct increase in TNFα from 0.0 ± 0.0 to 544 ± 140.5 pg/ml. AZD7624 fully suppressed this increase (see Fig. 3; Table 1) with a pIC_{50} of 8.1 ± 0.1 and thus a $pIC_{50}u$ of 8.7 ± 0.1 (8.1 + 0.19 + 0.41).

Rat In Vivo Pharmacokinetic Study. The dose-adjusted lung and plasma concentrations are shown in Fig. 4, illustrating the higher lung AUC following inhalation compared with intravenous dosing. The plasma AUC_{0-inf} was 339 and 36.7 nmol/l*h after intravenous and inhalation administration, respectively. Since oral bioavailability in rats is very low (1%, data not shown), the dose administered via the lung can determined to be 1 mg/kg*36.7/339 = 0.11 mg/kg. The whole lung AUC_{0-inf} was 31,862 and 16,416 nmol/l*h, after intravenous and inhalation administration, respectively. The dose-adjusted lung AUC after inhalation was thus 16,416*1/0.11 = 149,236 nmol/l*h, which is approximately five times higher than after intravenous dosing.

Ex Vivo LPS Challenge following Ascending Doses of AZD7624. In human whole blood taken from donors who had inhaled AZD7624 (48 donors with 36 given AZD7624 and 12 given placebo), LPS resulted in a distinct increase in TNFα from 11 ± 1.0 to 2021.2 ± 124.9 pg/ml. AZD7624 suppressed this increase (I_{max} 95% ± 7%) with a pIC_{50} of 8.2 ± 0.1 and a $pIC_{50}u$ of 8.8 ± 0.1 (8.2 + 0.19 + 0.41, Fig. 5).

Human Inhaled LPS Challenge. The increase in sputum TNFα following LPS challenge was 4.44 ± 5.51 pg/ml after AZD7624 compared with 30.46 ± 33.29 pg/ml with placebo. The absolute difference between treatments was

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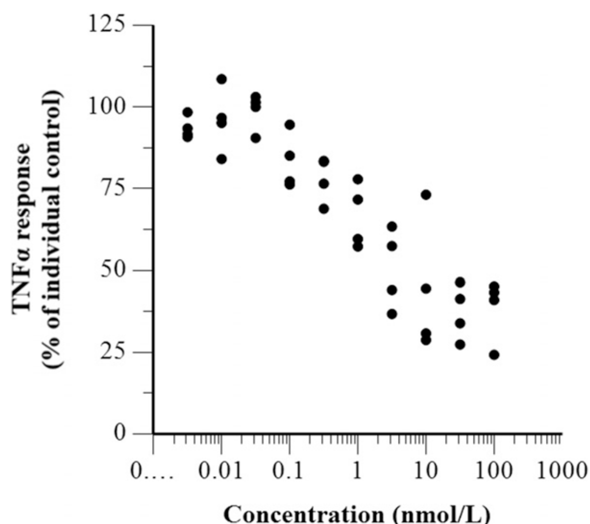

Fig. 1. Effect of AZD7624 on LPS-induced TNFα in human alveolar macrophages, expressed as percentage of individual maximal response corrected for base line.

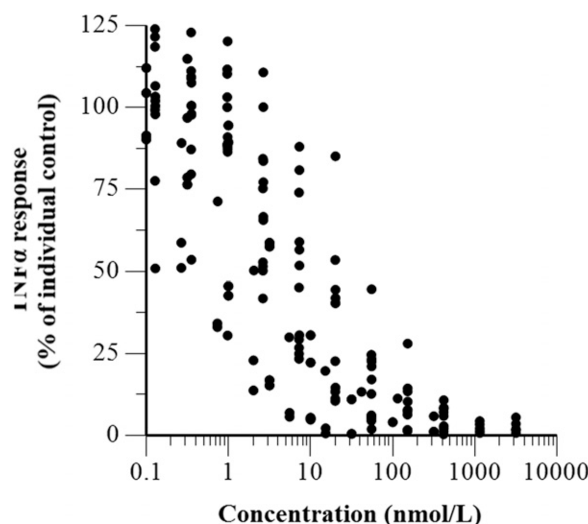

Fig. 2. Effect of AZD7624 on LPS-induced TNFα in PBMC, expressed as percentage of individual maximal response corrected for base line.

TABLE 1
Potency parameters in vitro and ex vivo for AZD7624

	$pIC_{50}u$	pIC_{50}	I_{max}	E_{max}	E_0	Cell Density	Number of Observations
			%	*pg/ml*	*pg/ml*	*10^5/ml*	
AM in vitro	9.0 ± 0.1	9.0 ± 0.1	62 ± 5	469.9 ± 92.7	244.7 ± 44.0	3.125	4
PBMC in vitro	8.4 ± 0.1	8.4 ± 0.1	100	3909.0 ± 351.0	175.0 ± 28.5	9.6	14
Whole blood in vitro	8.7 ± 0.1	8.1 ± 0.1	100	544.1 ± 140.5	0.0 ± 0.0	N/A	11
Whole blood ex vivo	8.8 ± 0.1	8.2 ± 0.1	95 ± 7	2021.2 ± 124.9	11 ± 1.0	N/A	36

AM, alveolar macrophages.

26.02 pg/ml ($P < 0.001$) corresponding to an 85.4% relative reduction with AZD7624 (Patel et al., 2018).

The plasma concentration of AZD7624 was measured up to 24 hours after inhalation; however, with respect to the effect on sputum TNFα response, the concentrations up to time of sputum induction are relevant, i.e., 6.5 hours after inhalation of AZD7624. The plasma concentration at time of LPS challenge was 2.8 ± 0.9 nmol/l, whereas the plasma concentration 30 minutes prior to sputum induction was 0.4 ± 0.1 nmol/l (Fig. 6). The average concentration during the LPS challenge was 0.9 ± 0.0 nmol/l, which becomes an unbound concentration of 0.3 ± 0.0 nmol/l when adjusted for plasma protein binding.

Plasma Protein Binding and Blood Plasma Ratio. The percentage unbound fraction at 10 nmol/l of AZD7624 was 38.7% ± 0.38, and no concentration-dependent change was observed in the range 1–1000 nmol/l. The blood/plasma ratio was determined to be 1.56 ± 0.11.

Discussion

The potency of AZD7624 as measured by inhibition of LPS-induced TNFα release was similar in PBMCs (in vitro) and in whole blood (in vitro and ex vivo). The same serotype of LPS was used in all assays. This suggests that AZD7624 potency measurement obtained in vitro in PBMC or both in vitro and ex vivo whole blood are all comparable. This correlation has previously been shown for another p38 inhibitor, SD006, which had a similar potency in inhibiting LPS-induced TNFα in human primary monocytes (79 nmol/l) as in whole blood (113 nmol/l) (Burnette et al., 2009). Another p38 inhibitor (PH797804) demonstrated good potency correlation with LPS-induced TNFα in human monocytes in vitro (unbound IC_{50} was 3.4 nmol/l) and in human whole blood in vitro (unbound IC_{50} was 2.9 nmol/l, when corrected for plasma protein binding) (Hope et al., 2009).

The pharmacokinetic results in rats demonstrate that inhalation of AZD7624 results in a higher (*dose adjusted*) lung concentration and a higher lung AUC compared with intravenous administration. It is important for an inhaled drug aimed at treating lung disease to have local lung effects in the absence of major systemic effects. Inhaled AZD7624 demonstrated in human a similar plasma pharmacokinetic profile as in rat, suggesting the potential to minimize systemic adverse effects with low systemic concentrations.

Following inhalation of AZD7624, the extent of inhibition of LPS-induced TNFα in sputum (85% at a mean unbound plasma concentration of 0.3 nmol/l) was higher than would be predicted on the basis of potency in PBMC and in whole blood in vitro and ex vivo. Furthermore, in the assay of human alveolar macrophages in vitro there was only partial inhibition of this cytokine (maximal inhibition 62%), and since the

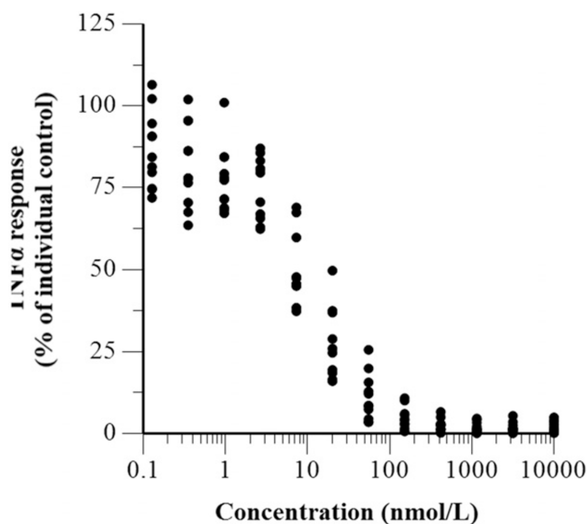

Fig. 3. Effect of AZD7624 on LPS-induced TNFα in whole blood, expressed as percentage of individual maximal response corrected for base line.

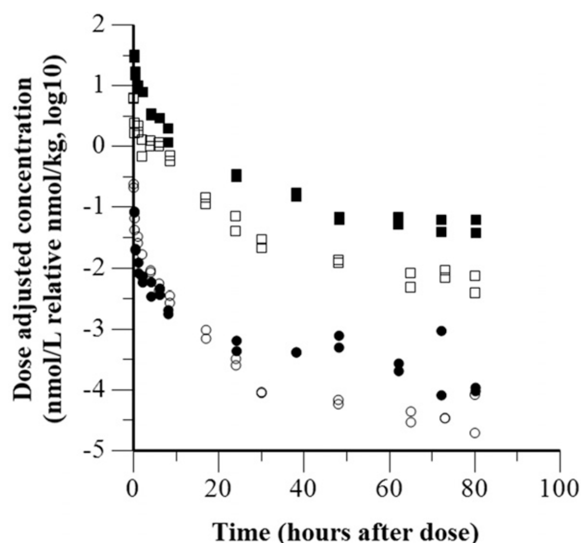

Fig. 4. Dose-adjusted concentrations in plasma and lung of AZD7624 in rats after intravenous or inhalation dosing. Squares represent lung concentrations after inhalation (filled) and intravenous dosing (open), whereas circles represent plasma concentrations after inhalation (filled) and intravenous dosing (open). Each dot represents a sample from an individual rat. Note the logarithmic concentration scale.

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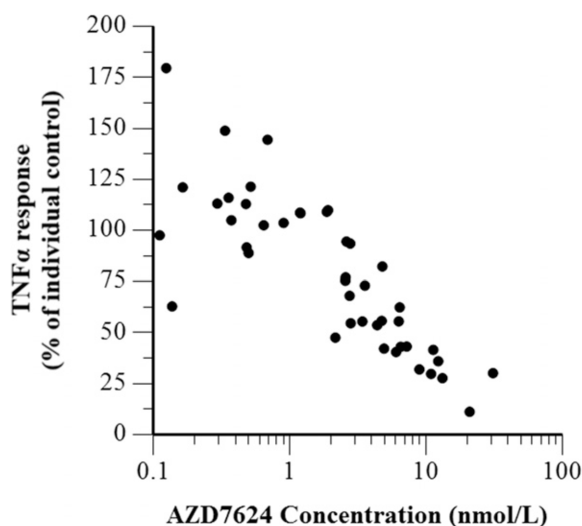

Fig. 5. Effects of AZD7624 on LPS-induced TNFα ex vivo after inhalation of ascending doses of AZD7624.

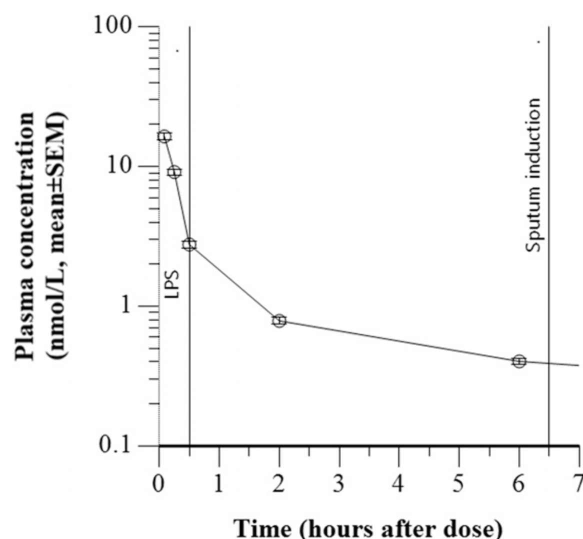

Fig. 6. Plasma concentration of AZD7624 after inhalation of 1200 μg AZD7624. Vertical lines mark performance of inhaled LPS challenge and subsequent induction of sputum.

mean unbound plasma concentration is close to the reported pIC_{50} (and $pIC_{50}u$) of 9.0 or 9.2 [as reported elsewhere in a similar assay (Patel et al., 2018)], the expected inhibition of LPS-induced TNFα in alveolar macrophages is far less than 85%. This may be because other cells besides alveolar macrophages may also be responsible for TNFα release in lung following LPS challenge.

Published data for the p38 inhibitor PH797804 suggests that potency in whole blood in vitro is comparable to plasma concentrations (following oral dosing) associated with a reduction in LPS-induced cytokines in vivo. Thus, PH797804 inhibits LPS-induced TNFα in whole blood in vitro with an unbound IC_{50} of 2.9 nmol/l (Hope et al., 2009). When 30 mg PH797804 was given orally to healthy subjects prior to inhaled LPS challenge, the LPS-induced increase in interleukin-6 in both sputum and plasma was markedly decreased compared with placebo (Singh et al., 2015). The associated total plasma concentration of PH797804 was 37.8–56.8 ng/ml (Singh et al., 2015), which, adjusted for plasma protein binding and molecular weight (Hope et al., 2009), corresponds to an estimated unbound concentration of 3–4 nmol/l. This correlates well with the measured sputum concentration of 4 nmol/l (Singh et al., 2015). The similar unbound potency in vitro compared with measured concentrations of PH797804 in both plasma and sputum suggests that following systemic dosing of a p38 inhibitor the same potency observed in vitro is also observed in vivo.

In the current study, inhalation of AZD7624 exerted a local anti-inflammatory effect, suggesting higher local lung exposure, at same time that systemic exposure was below $pIC_{50}u$. This indicates that inhaled delivery of AZD7624 would provide adequate target engagement or anti-inflammatory effect in the lung, at the same time avoiding potentially unwanted adverse effects of systemic p38 inhibition. However, in a study investigating whether AZD7624 was efficacious in suppressing exacerbations in COPD patients, inhaled AZD7624 failed to achieve clinical efficacy (Patel et al., 2018). It can be speculated that this was the result insufficient local target engagement for effective COPD exacerbation control.

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Authorship Contributions

Participated in research design: Pehrson, Hegelund-Myrbäck, Cunoosamy, Asimus, Patel, Lundin, Jansson, Borde.

Conducted experiments: Cunoosamy, Lundin, Borde.

Performed data analysis: Pehrson.

Wrote or contributed to the writing of the manuscript: Pehrson, Hegelund-Myrbäck, Cunoosamy, Lundin, Patel.

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Address correspondence to: Dr. Rikard Pehrson, AstraZeneca, B381, Pepparedsleden 1, 431 53 Mölndal, Gothenburg, Sweden. E-mail: Rikard.Pehrson@astrazeneca.com

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