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Title Page

Title:

AZD7624, an inhaled p38 inhibitor, demonstrate local lung inhibition of LPS-induced TNFα with minimal systemic exposure

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

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Running Title Page

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Demonstrating local lung inhibition of LPS-induced TNFα

Corresponding author:

Rikard Pehrson

Rikard.Pehrson@astrazeneca.com

Address

B381

AstraZeneca Gothenburg

Pepparedsleden 1

431 53 Mölndal

Telephone +46(0)317766376

Fax: +46(0)317763717

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Number of tables: 1

Number of figures: 6

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Number of words in

Abstract: 245

Introduction: 433

Discussion: 628

Nonstandard abbreviations:

RPMI 1640, Roswell Park Memorial Institute medium;

LPS, Lipopolysaccharide;

FBS, Fetal bovine serum;

pIC50u, unbound potency remaining after adjustment for plasma protein binding and blood plasma ratio

AM, alveolar macrophages

Recommended section assignment: Translational Medicine

Abstract

Inhaled drugs generally aim to drive a local pharmacological effect in the lung while 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 5 times higher dose-adjusted lung exposure compared to intravenous dosing. In healthy volunteers, LPS-induced 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 significantly below potencies obtained for AZD7624 gained from *in vitro* and *ex vivo* LPS-challenge protocols, suggesting that lung exposure is likely much higher than systemic exposure. This assessment was made for the unbound potency (pIC50u), 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 pIC50u 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 pIC50u. This demonstrates that AZD7624 can achieve significant local lung pharmacodynamic effects concomitant with 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) while minimizing systemic concentrations so that likelihood of unwanted systemic side effects is also minimized (Lotvall, 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 pro-inflammatory mediators (Saklatvala, 2004; Chung, 2011). Patients with COPD have a higher percentage of p38 positive macrophages obtained both from alveoli and sputum compared to 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 represent 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 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 *in vivo* findings, and in particular an evaluation as to whether inhaled delivery can provide efficacy in the lung with less systemic exposure compared to oral dosing for p38 inhibitors. Of the adverse events associated with p38 inhibition, skin rashes 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 TNFα (Michel et al., 1997; Nightingale et al., 1998; Korsgren et al., 2012).

In the current studies, the inhaled p38 inhibitor AZD7624 (Brough et al., 2008) has been tested in several LPS challenge models; i) human blood derived mononuclear cells (PBMC) *in vitro*, ii) human blood *ex vivo*, iii) human alveolar macrophages *in vitro* (Patel et al., in press) and iv) inhaled LPS in healthy volunteers (Patel et al., in press). 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 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 (PBS) to isolate macrophages. PBS washings were pooled and centrifuged at 400 g for 10 minutes at room temperature prior to re-suspension of the pellet in 10 mL assay buffer (RPMI 1640 medium containing 100 U/mL penicillin, 100 μg/mL streptomycin, 2 mM L-Glutamine). Cells were seeded in assay buffer at 50,000 cells per well in a 96-well plate. Plates were incubated under standard tissue culture conditions for 24 h. Macrophages were pre-incubated with AZD7624 (0.003–100 nM) for 4 h at 37°C. The p38 MAPK pathway was induced with LPS (1 μg/mL, E. coli 0111:B4,L4130, H4522 Sigma, Pole UK.) overnight at 37°C after which supernatants were collected. Tissue necrosis factor alpha (TNFα) levels were determined using a fluorescence-linked immunosorbent assay (FLISA, Human TNFα (Standard for FLISA, 210-TA-010), Monoclonal anti-TNFα (MAB610), Biotinylated anti-TNFα polyclonal Antibody (BAF210) from R&D Systems, Abingdon, UK).

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Human peripheral blood mononuclear cells (PBMC)

Human blood was obtained from healthy volunteers and PBMC were separated by density gradient centrifugation and suspended into assay buffer (RPMI1640).

For inhibition experiments, cells were pre-incubated with compound for 20 hours. After the pre-incubation step, LPS (1 μg/mL E. coli 0111:B4, L4130, H4522 Sigma, Pole UK) was added for 4 hours to induce TNFα production. The amount of TNFα released was quantified using FLISA (Human TNFα (Standard for FLISA, 210-TA-010), monoclonal anti-TNFα (MAB610) and biotinylated anti-TNFα polyclonal antibody (BAF210) from R&D Systems, Abingdon, UK).

Whole blood

Human blood was obtained from healthy volunteers. For inhibition experiments, cells were pre-incubated with compound for 30 minutes. After the pre-incubation step, LPS (1 μg/mL E. coli 0111:B4, L4130, H4522 Sigma, Pole UK) 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 grams, Harlan Laboratories, the Netherlands) 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

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tissue specimen were collected up to 80 hours post dose, from two animals per time point, for both the intravenous and inhalation sample group.

Plasma and whole lung concentrations of AZD7624 were determined by 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 anaesthetised 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 Holding Ab, Sweden). Measurements of the aerosol concentration in the inhalation chamber was performed by filter sampling at one of the inhalation ports (AP40, 47 mm, Millipore). Measurements of the aerosol concentration in the inhalation chamber was performed by filter sampling at one of the inhalation ports (AP40, 47 mm, Millipore) during the time of animal dosing with a filter sampling flow rate of 0.25 mL/min. Lung delivered doses of AZD7594 (µg/kg) were calculated as described elsewhere (Phillips et al., 2017).

Assuming the same plasma clearance following intravenous dosing and inhalation respectively, the delivered inhaled dose used for dose normalization was determined by plasma 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

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with LPS (E.coli 0111:B4, L4391, Sigma Aldrich) *ex vivo* for 4 hours at 37ºC and *ex vivo* plasma TNFα levels were measured using a sandwich immunoassay with capture antibodies immobilized onto MSD "Small Spot" plates (MSD Cat No. K151 BHC-1). 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

This was a single-centre, randomised, double-blind, placebo-controlled, 2-way cross-over phase I study in healthy volunteers to investigate the effect of single-dose inhaled AZD7624 vs placebo on inflammatory biomarkers in induced sputum and blood after inhalation of LPS. Parts of the results has already been published (Patel et al., in press). Healthy male and female volunteers of non-childbearing 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 [required PC20 >16 mg/mL 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 (Escherichia 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. 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 non-compartment analysis.

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PKPD evaluation

Cell and whole blood models

The potency of AZD7624 in inhibiting LPS-induced TNFα was evaluated in an Emax model (Phoenix 6.4.0.768, Pharsight, Princeton USA), where E=E0+Emax*(1-Imax*C/(C+IC50)).

E0=basal level of TNFα in the absence of LPS

Emax=maximal stimulation of TNFα production by LPS. Emax was allowed to differ using a population approach e.g. Emax=tvEmax*e^nEmax

Imax=Maximal inhibition of LPS-induced TNFα production by AZD7624

Experiments carried out on human material were analysed using population modelling. 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 was based on 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 non-compartmental analysis (Phoenix 6.4.0.768, Pharsight, Princeton USA). The relative individual sputum TNFα response to LPS (after AZD7624 treatment) compared to that after placebo, was determined. The results are shown in figure 5.

Adjustment for protein binding and blood/plasma ratio

All potency calculations obtained were transformed to estimated unbound concentration (pIC50u) 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 unbound fraction in plasma (pIC50 increased by 0.41 in man) and potency derived in human whole blood was adjusted for both blood/plasma ratio and unbound fraction in plasma (pIC50 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 pIC50 of 9.0±0.1 (pIC50u same as pIC50, Figure 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 Table 1 and Figure 2) with a pIC50 of 8.4±0.1 (pIC50u same as pIC50).

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 table 1 and figure 3) with a pIC50 of 8.1±0.1 and thus a pIC50u of 8.7±0.1 (8.1+0.19+0.41).

Rat in vivo pharmacokinetic study

The dose-adjusted lung and plasma concentration are shown in figure 4, illustrating the higher lung AUC following inhalation compared to intravenous dosing. The plasma $AUC_{0\text{-}inf}$ 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 be determined to be 1 mg/kg*36.7/339=0.11 mg/kg. The whole lung $AUC_{0\text{-}inf}$ was 31862 and 16416 nmol/L*h, after intravenous and inhalation administration, respectively. The dose-adjusted lung

AUC after inhalation was thus 16416*1/0.11=149236 nmol/L*h which is approximately 5 times higher than after intravenous dosing.

Ex vivo LPS challenge following ascending doses of AZD7624

In human whole blood taken from donors that 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 (Imax 95±7%) with a pIC50 of 8.2±0.1 and a pIC50u of 8.8±0.1 (8.2+0.19+0.41, figure 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 26.02 pg/mL ($p<0.001$) corresponding to an 85.4% relative reduction with AZD7624 (Patel et al., in press).

The plasma concentration of AZD7624 was measured up to 24 hours after inhalation, but regarding effect on the 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 (figure 6). The average concentration during the LPS challenge was 0.9±0.0 nmol/L, which adjusted for plasma protein binding becomes an unbound concentration of 0.3±0.0 nmol/L.

Plasma protein binding and blood plasma ratio

The percentage unbound fraction at 10 nmol/L 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*) or 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* in whole blood are all comparable. This correlation has previously been shown for another p38 inhibitor SD006 that 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 IC50 was 3.4 nmol/L) and in human whole blood *in vitro* (unbound IC50 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 to 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 man a similar plasma pharmacokinetic profile as in the 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 based on potency in PBMC and in whole blood *in vitro* and *ex vivo*. Furthermore, in the human alveolar macrophages *in vitro* assay there was only partial inhibition of this cytokine (maximal inhibition 62%) and since the mean unbound plasma concentration is close to the reported pIC50 (and pIC50u) of 9.0 or 9.2 [as reported elsewhere in a similar assay, (Patel et al., in press)], 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 IC50 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 IL-6 in both sputum and plasma was markedly decreased compared to placebo (Singh et al., 2015). The associated total plasma concentration of PH797804 was 37.8 to 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 to 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 to 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, exerts a local anti-inflammatory effect, suggesting higher local lung exposure, at same time that systemic exposure is below pIC50u. This would allow inhaled delivery of AZD7624 to provide adequate target engagement or anti-inflammatory effect in the lung, while avoiding potentially unwanted adverse effects of systemic p38 inhibition.

However, in a study investing whether AZD7624 was efficacious in suppressing exacerbations in COPD patients, inhaled AZD7624 failed to achieve clinical efficacy (Patel et al., in press). It can be speculated that this is due to that local target engagement is not sufficient for effective COPD exacerbation control.

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Authorship contribution

Participated in research design:

Pehrson, Hegelund-Myrbäck, Cunoosamy, Asimus, Patel, Lundin, Janson and Borde

Conducted Experiments:

Cunoosamy, Lundin and Borde

Performed data analysis:

Pehrson

Wrote or contributed to the writing of the manuscript:

Pehrson, Hegelund-Myrbäck, Cunoosamy, Lundin and Patel

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Financial disclosure

All authors have been employees at AstraZeneca.

Figure Legends

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

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

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

Figure 4: Dose-adjusted concentrations in plasma and lung of AZD7624 in rats after intravenous or inhalation. 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.

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

Figure 6: Plasma concentration of AZD7624 after inhalation of 1200 µg AZD7624. It is indicated when inhaled LPS challenge was performed and when subsequent induction of sputum was conducted.

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Table

Table 1: Potency parameters *in vitro* and *ex vivo* for AZD7624

	pIC50u	pIC50	Imax %	Emax pg/mL	E0 pg/mL	Cell density 10^5/ mL	Number of observations
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

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Figures

Figure 1

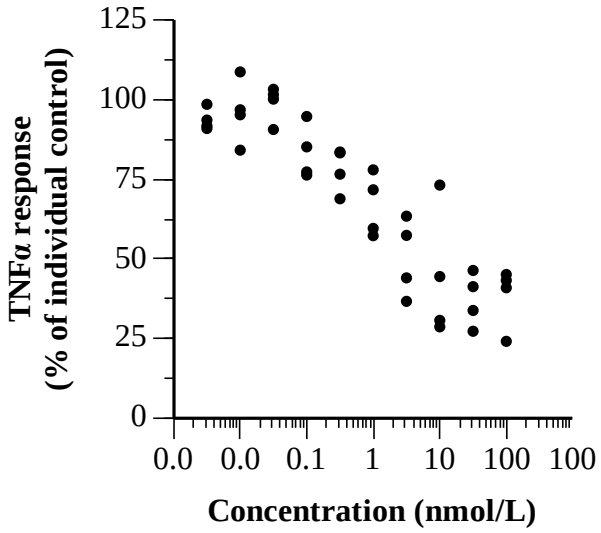

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Figure 2

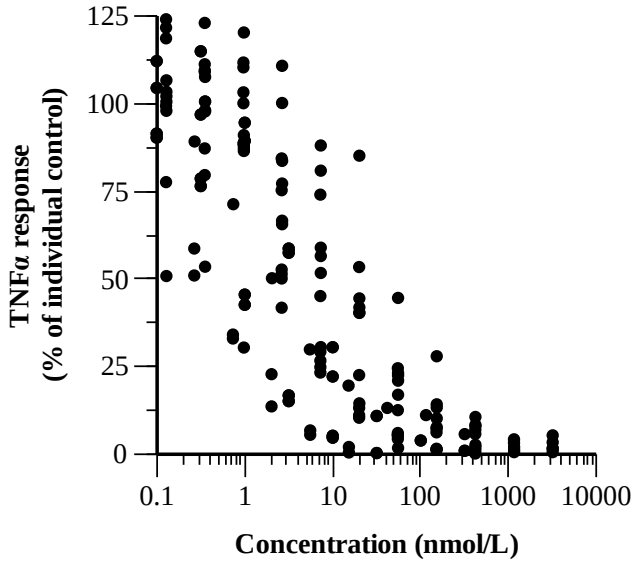

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Figure 3

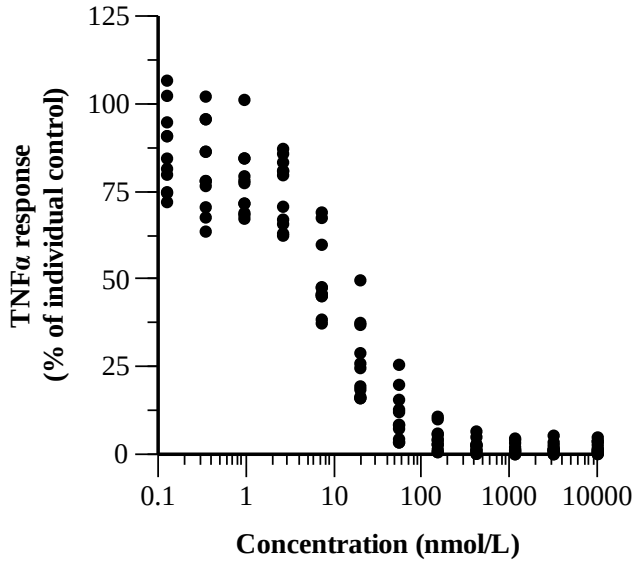

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Figure 4

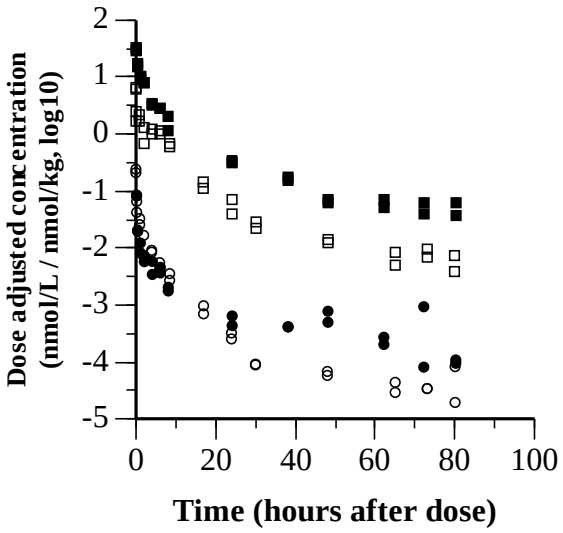

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Figure 5

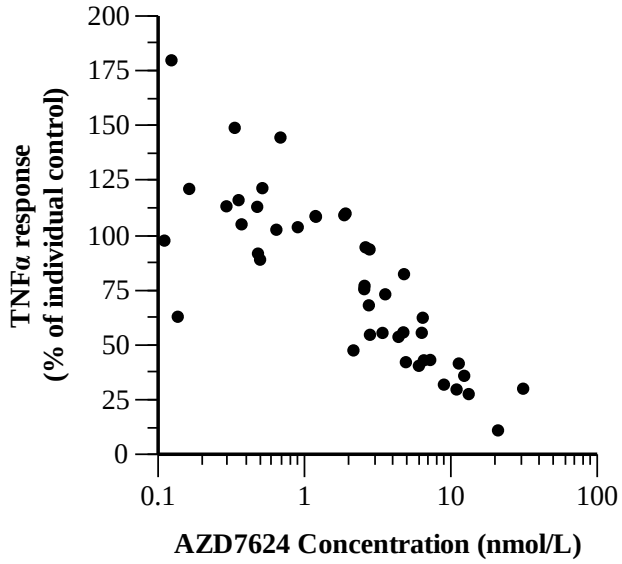

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Figure 6