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Pharmacokinetics and Pharmacodynamics of DPC 333, a Potent and Selective Inhibitor of Tumor Necrosis Factor-α Converting Enzyme in Rodents, Dogs, Chimpanzees and Humans

Mingxin Qian[1], Stephen A. Bai[2], Bernice Brogdon[3], Jing-Tao Wu[4], Rui-Qin Liu, Maryanne B. Covington[5], Kris Vaddi[5], Robert C Newton[5], Michael J Fossler[6], C. Edwin Garner[7], Yuzhong Deng, Thomas Maduskuie[5], James Trzaskos, James J.-W. Duan, Carl P. Decicco and David D. Christ[8]

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Corresponding author:
Mingxin Qian, Dr.
Pharmacokinetics, Pharmacodynamics and Bioanalytical Sciences,
Genentech Inc.
One DNA Way, South San Francisco, CA 94080, USA
Phone: 650-467-3087
Fax: 650-225-7592
Email: mqian@gene.com

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A list of abbreviations:

AUC: area under the plasma concentration-time curve; CIA: collagen antibody-induced arthritis; CLs: systemic clearance; C_{max}: maximum observed concentration achieved following oral dosing; DA: Dark Agouti; F: extravascular or oral bioavailability; IC_{50}: concentration producing 50% of maximal inhibition; iv: intravenous; ip: intraperitoneal; LC/MS/MS: liquid chromatographic tandem mass spectrometry; LPS: lipopolysaccharide; po: oral; PK: pharmacokinetics; PD: pharmacodynamics; RA: rheumatoid arthritis; SD: Sprague-Dawley; TACE: TNF-α converting enzyme; T_{max}: time

to reach maximum observed concentration; TNF-α: tumor necrosis factor-α; $t_{1/2}$: terminal half-life; Vss: steady-state volume of distribution; WBA: whole blood assay.

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ABSTRACT

DPC 333 is a potent and selective inhibitor of tumor necrosis factor (TNF)-α converting enzyme (TACE). It significantly inhibits lipopolysaccharide (LPS) induced soluble TNF-α production in blood from rodents, chimpanzee and human, with IC_{50} values ranging from 17 to100 nM. In rodent models of endotoxemia, DPC 333 dose-dependently inhibited the production of TNF-α, with an oral ED_{50} ranging from 1.1 to 6.1 mg/kg. Oral dosing of DPC 333 at 5.5 mg/kg daily for 2 weeks in a rat collagen antibody-induced arthritis (CIA) model suppressed the maximal response by approximately 50%. DPC 333 was distributed widely to tissues including the synovium, the site of action for anti-arthritic drugs. Pharmacokinetic and pharmacodynamic studies in chimpanzee revealed a systemic clearance of 0.4L/h/kg, a Vss of 0.6L/kg, an oral bioavailability of 17% and an *ex vivo* IC_{50} for the suppression of TNF-α production of 55 nM (n=1). In a Phase I clinical trial with male volunteers following single escalating doses of oral DPC 333, the terminal half-life was between 3-6 h and the *ex vivo* IC_{50} for suppressing TNF-α production was 113 nM. Measurement of the suppression of TNF-α production ex vivo may serve as a good biomarker in evaluating the therapeutic efficacy of TACE inhibitors. Overall, the

pharmacological profiles of DPC 333 support the notion that suppression of TNF-α with TACE inhibitors like DPC 333 may provide a novel approach in the treatment of various inflammatory diseases including RA, via control of excessive TNF-α production.

INTRODUCTION

Tumor necrosis factor alpha (TNF-α) is a ubiquitous, multifunctional cytokine produced primarily by activated monocytes, macrophages and T-cells, and plays a key role in fighting infection, eradicating tumors, and mediating the acute and chronic inflammatory effects of the immune system. Excessive production of TNF-α, however, has been directly implicated in a wide variety of diseases that differ considerably in their etiology and clinical manifestations. These include rheumatoid arthritis (RA), psoriasis, Crohn's disease, ankylosing spondylitis, refractory asthma (Erzurum, 2006) as well as cachexia and septic shock. Over the past decade, the important contributory role that TNF-α plays in these diverse diseases has led to the discovery of a number of agents antagonizing the effects of TNF-α that are effective therapies for RA, psoriasis, Crohn's disease and ankylosing spondylitis (Olsen and Stein, 2004). The most notable of these are the biologics: etanercept (Enbrel®), infliximab (Remicade®) and aldalimumab (Humira®).

There are, however, significant limitations in the use of these biologic therapies, notably the need for parenteral administration, the high cost of treatment, and the potential development of immunogenicity that may render them ineffective (Finckh et al., 2006). Moreover, some patients still have poorly or incompletely controlled disease with these therapies (Olsen and Stein, 2004), with non-responsive rates ranged from 20% to 50%, depending on the disease stage (Feldmann and Steinman, 2005). An orally administered, selective small molecule inhibitor of TNF-α production with a shorter half-life would provide an alternative and significant therapeutic advantage over the biologics from the standpoint of ease of administration, reduced cost of therapy, patient compliance, and the ability to more precisely control TNF-α levels and duration of TNF-α blockade.

Human TNF-α is synthesized as a biologically active 26 kDa membrane bound precursor that is cleaved by a cell membrane associated metalloprotease known as TNF-α converting enzyme (TACE) to form a 17 kDa soluble TNF-α (McGeehan et al., 1994, Black et al., 1997, Killar et al.,1999). Soluble TNF-α exerts its biological effects by binding to cell surface receptors TNFR1 and TNFR2, and ultimately triggering a cascade of cellular activation producing inflammatory mediators like IL-6, matrix degrading proteases, active oxygen species that produce tissue damage (Feldmann et al.,1996,

Kaufman and Choi, 1999). Inhibition of TACE and therefore the subsequent release of the soluble cytokine offer a potentially novel mechanism to modulate the activity of TNF-α. Numerous studies have suggested that small molecule TACE inhibitors under development may be as effective as other anti-TNF-α therapies in the treatment of inflammatory diseases (Newton et al., 1999, 2001; Conway et al., 2001; Beck et al., 2002; Zhang et al., 2004). DPC 333 (Figure 1) selectively inhibits TACE both *in vitro* and *in vivo* and is orally available (Duan et al., 2001; Vaddi et al., 2001; Wu. et al., 2002). In the current paper we report in detail the pharmacokinetics and pharmacodynamics of DPC 333 in mice, rats, dogs and chimpanzees as well as in a first-in-human clinical phase I trial in healthy volunteers.

METHODS AND MATERIALS

Chemicals

DPC 333, (2R)-2-((3R)-3-amino-3{4-[2-methyl-4-quinolinyl) methoxy] phenyl}-2oxopyrrolidinyl)-N-hydroxy-4-methylpentanamide), and the internal standards used in the analysis of DPC 333 were synthesized and characterized at DuPont Pharmaceutical Co.(later Bristol Myers Squibb), Wilmington, DE. Medical grade soluble TNF-R2: IgG fusion protein (etanercept,) was obtained from Immunex Corporation (now Amgen, Seattle, WA). Anti-murine TNF-α antibody, formed in hamsters, and ELISA kits to measure TNF-α in rodent plasma samples were obtained from Genzyme Corporation (Cambridge, MA). Bovine type II collagen, collagen monoclonal antibodies and incomplete Freund's adjuvant were obtained from Chondrex LLC (Seattle, WA). Lipopolysaccharide (derived from *E. coli*) and dinitrofluorobenzene (DNFB) were obtained from Sigma Chemical Co (St. Louis, MO). Alzet miniosmotic pumps were obtained from the Durect Corporation (Cupertino, CA). HPLC-grade solvents used for

LC/MS analysis and all other chemicals were obtained from commercial sources and were of analytical grade or higher.

Animals

Balb/c mice (18-20 g) and female CD rats (150-200 g) were obtained from Taconic Farms (Germantown, NY). Male and female Sprague-Dawley (SD), Dark Agouti (DA) and Lewis rats weighting approximately 225-350 g were obtained from Charles River Laboratories (Wilmington, MA). Male beagle dogs (11 ± 2 kg) were obtained from Marshall Farms (North Rose, N.Y). All animals were housed in temperature-control rooms with appropriate light/dark cycles and exercise regimens. The animals were fasted before dosing but were given access to water *ad lib*. Food was provided to the rats and dogs 4 h after dosing. Some SD rats had jugular vein cannulas placed for sample collection. All animal studies were conducted according to protocols reviewed and approved by the Institutional Animal Care and Use Committee. The *in vivo* chimpanzee (Pan Troglodytes) studies were conducted according to protocols approved by the Animal Care and Use Committee at the New Iberia Research Center (New Iberia, Louisiana). Four male

adolescent chimpanzees, weighing between 59.0 to 78.6 kg were used in these studies. A veterinarian observed each animal daily for sign of illness or distress during the study. Clinical and laboratory data were also monitored during the course of the study.

***In Vitro* Studies**

Whole Blood TNF Assay: Blood was drawn from mice, rats, dogs, chimpanzees and human volunteers into heparinized tubes (Becton Dickinson, Franklin Lakes, NJ) and a 225 μL aliquot of blood was pipetted directly into BioRad (Hercules, CA) polypropylene tubes. Test compounds, including DPC 333, were added at varying concentrations and preincubated with the blood cells for 10 minutes. LPS (Calbiochem, La Jolla, CA) was added to achieve a final concentration of 100 ng/mL and the blood incubated for 5 hours at 37°C. A 750 μL aliquot of AIM-V serum free medium (Gibco-BRL, Frederick MD) was added and the cell pellets were centrifuged at 1500 RPM for 15 minutes. A 500 μL aliquot of supernatant was collected and assayed for TNF-α using a standard sandwich ELISA assay. Blood collected from animals dosed with DPC 333 was also used to determine the amount of TNF-α generated after LPS stimulation. The degree of inhibition

in post DPC 333 addition or administration was calculated in relation to the pretreatment values.

Serum Protein Binding: The *in vitro* protein binding of DPC 333 in mouse, rat, dog, chimpanzee and human serum, as well as with purified human serum albumin and α-1-acid glycoprotein (AAG) were determined by ultrafiltration using Centrifree micropartition devices with a molecular weight cutoff of >30,000 Daltons (Millipore, Billerica, MA). DPC 333 was added to serum and purified protein samples in triplicate to yield final concentrations of 1 µM. Human serum albumin and α-1-acid glycoprotein solutions were prepared in de-ionized water at physiological concentrations of 40 mg/mL and 0.5 mg/mL, respectively. Following incubation at 37°C for 1 h, the devices were centrifuged at 1700 g for 10 min at 37°C. Aliquots of the serum ultrafiltrate were extracted on a C_8 solid phase extraction column and analyzed by LC/MS/MS. The unbound fraction (f_u) s was calculated as the concentration of DPC 333 in the serum or purified protein ultrafiltrate divided by the nominal concentration in the serum or purified protein reservoir. Values are reported as means ± S.D. for three determinations.

***In Vivo* Studies**

Dose administration and sample collection: All doses were administered as solutions in either isotonic saline (iv, ip and oral) or in 1% Tween 80 in 0.5% Methylcellulose (oral). Blood samples were collected in EDTA vacutainers and the plasma was obtained by centrifugation. Urine samples, when collected, were obtained *via* a collection pan placed under the cage that drained into a vessel maintained at 4^{o}C by wet ice. All biological samples were frozen immediately after collection at -20°C until they were analyzed. Specifics of each study are detailed below.

Single Dose Pharmacokinetic Studies in Mice and Rats: DPC 333 was administered to Balb/c mice, SD or DA rats intravenously, orally or intraperitoneally at doses ranging from 3.4 mg/kg to 68 mg/kg. Blood samples (0.5 ml) were collected from mice in a composite study design (3 mice pooled per time point) and from individual rats (n=3 for each dose route) at predose and at specific times up to 24 h post dose.

Synovial Pharmacokinetic Study in Rats: The pharmacokinetic profile of DPC 333 in synovial fluid was determined using female Lewis rats (~200g, n=18), a strain used as the rodent model for collagen induced arthritis. The rats were given single oral dose of 51

mg/kg of DPC 333 in 1% Tween 80 and 0.5% Methocellulose. Three animals were sacrificed at each time point; predose, 1, 2, 4, 8 and 24 h post dose. Blood samples (0.5 ml) were collected by cardiac puncture. Synovial fluid samples were collected under surgical microscopy according to the following procedure. The patellar ligament of the stifle knee joint was cut and the synovial cavity was incised and opened. Forty microliters of normal saline containing a structurally similar internal standard TA034 (0.5 μM) was injected into the joint space of the knee, then aspirated back to get approximate 40 μl synovial fluid sample after rinsing the space carefully and thoroughly. The collection process was completed within 5 minutes to minimize diffusion and drug loss. The use of the internal standard allowed a more precise estimate of the actual volume of synovial fluid collected, which was used for calculating the final concentrations of DPC 333 in each sample. Plasma concentrations of DPC 333 were determined as described previously.

Mouse and Rat Models of Endotoxemia: Male Balb/c mice (18-20 g) or female CD rats were randomly assigned to groups of 5-10 animals/group. All groups except the negative control group received *E. coli* derived LPS ip (10 μg/mouse or 50 μg/rat). For the dose-response studies, vehicle (saline) or different doses of DPC 333, ranging from 0.2 mg/kg to 68 mg/kg, were administered po or ip along with LPS. The mice or rats were sacrificed

1 hr after dosing, and blood was obtained by cardiac puncture and placed in EDTA for the determination of plasma TNF-α concentrations. Results were expressed as mean TNF-α concentration for all the animals in the group or as percent inhibition compared to the vehicle treated group.

Collagen-Induced Arthritis Rat Model: Animals were treated as described by Holmdahl et al. (1989). Briefly, DA rats were immunized by two intra-dermal injections at the base of the tail on day 0 and again on Day 7 with bovine type II collagen emulsified in incomplete Freund's adjuvant. Five days later all rats were given a single ip injection of LPS (50 μg/rat). Signs of arthritis such as erythema and swelling of the hind paws are typically observed between 5 and 10 days after the LPS injection. Rats that developed the disease (5-7/group) were randomly assigned to 4 treatment groups: vehicle control, DPC 333 treatment twice daily with oral doses of 5.5 or 10.5 mg/kg, and treatment with Etanercept at 2.5 mg/rat twice a week by intraperitoneal administration. Paw swelling and the clinical signs were monitored 3 times a week and clinical scores were assigned based on a scale of 0-3, with 3 being most severe. Results were expressed as total arthritic score per rat from all rats in the group. To determine the percent suppression of disease, the areas under the curve (AUC) of the clinical score from the different treatment groups were compared. The

AUCs of the clinical score were calculated as previously described (Bendele et al., 2000). To determine a DPC 333 concentration-effect relationship of DPC 333 in the rat CIA model, blood was collected from a cannulated jugular vein and at different time-points after the final dose and analyzed for DPC 333.

Single Dose Pharmacokinetics in Dogs: DPC 333 HCl salt was administered to male beagles (n=3 for each dose route) as single intravenous and oral doses of 5 mg/kg. Blood samples (~2 ml) were collected by jugular venipuncture into EDTA containing tubes at predose and 2, 5, 10, 15, 30 min, and 1, 1.5, 2, 4, 6, 8, 10, 12, and 16 h post dose.

Single Dose Pharmacokinetics and Pharmacodynamics of DPC 333 in Chimpanzees: The single oral dose pharmacokinetics and pharmacodynamics, which was assessed as the percent inhibition from predose levels of ex vivo LPS-stimulated TNF-α production of DPC 333 were determined in three male chimpanzees. The chimpanzees were given DPC 333 as a single dose of 2.7 mg/kg (as free base) formulated in aqueous Tang orange drink. Blood samples were collected predose and at 1, 2, 3, 6, 9, 12, 24, and 36 h after DPC 333 administration. Blood samples were stimulated with LPS at 37°C for 5 h to produce TNF-α, which was measured by ELISA as described previously, and the percent inhibition from

the predose sample was calculated. Plasma concentrations of DPC 333 were determined by LC/MS/MS. Plasma concentration-effect data were only available for one of the chimpanzees because ketamine was used to sedate the other two animals to facilitate the collection of blood samples. Ketamine has been shown to potentially inhibit the production of TNF-α (Takenaka *et al*., 1994). In a separate cassette dose PK study using one male chimpanzee, the intravenous pharmacokinetics of DPC 333 was determined along with five other structurally similar compounds following a 30 min infusion of 0.5 mg/kg each. Blood samples were obtained predose and at 0.08, 0.25, 0.5, 1, 2, 3, 4, 6, 10, 12, 18, 24, 36, and 48 h after starting the infusion. Urine samples were also collected from 0-6, 6-12, 12-24, and 24-48 h postdose. All plasma and urine samples were kept frozen at -20°C until analysis.

Single Dose Pharmacokinetic and Pharmacodynamic Study in Humans: The safety, tolerability, pharmacokinetics and ex vivo TNF-α concentrations following single oral doses of DPC 333 (15, 25, 40, 80, 120, 225, 345, and 530 mg) were determined in a double-blinded, randomized, placebo-controlled (6 active: 2 placebo) Phase 1 study in healthy subjects. The study was reviewed and approved by an authorized Institutional Review Board and all subjects provided informed consent prior to participation. Among

the subjected evaluated, 90.0% (72/80) of subjects were male and 10.0% (8/80) were female; the majority of subjects were Caucasians (96.3%, 77/80). The mean age, height, weight, and BMI were 32.0 years of age, 175.7 cm, 77.5 kg, and 25.0 kg/m2, respectively. Safety assessments include: adverse experiences (AEs), clinical laboratory tests (including hematology, serum chemistry, and urinalysis), vital sign measurements, electrocardiograms (ECGs), physical examinations, and tolerability. DPC 333 was administrated after an overnight fast as either an oral solution (dosages of 15 and 25 mg) or as a suspension (dosages ≥40 mg). Blood samples for the determination of plasma DPC 333 concentrations were drawn from indwelling catheters or by direct venipuncture into Vacutainer® tubes containing EDTA prior to dosing and at 0.25, 0.5, 1, 1.5, 2, 2.5, 3, 4, 6, 8, 10, 12, 18, 24, 36, and 48 h following DPC 333 administration. Blood samples for the determination of DPC 333 concentrations were centrifuged for 10 minutes at 1500 g at approximately 4 °C and plasma was stored at -20 C or below until analysis. Whole blood samples (5 mL) were collected into heparinized Vacutainer® tubes at pre-dose, and at 2, 4, 6, and 8 h after dose administrations for the determination of TNF-α concentrations in plasma using the ELISA Quantikine ® TNF-α kit (R&D Systems, MN, USA). The

validated assay range using 200 μL of cell-free supernatant was 15.6 to 1000 pg/mL. Inter-assay precision and accuracy ranged from 1.6-8.3% and 93.4 -106.9%, respectively.

Analytical methods

Analysis of DPC 333 by LC/MS/MS: DPC 333 concentrations in all biological samples were determined by a liquid chromatography/tandem mass spectrometry (LC/MS/MS) based method. Briefly, an aliquot of plasma or serum (0.2-0.5 mL) was mixed with a structural analog internal standard (TA034) or a deuterium-labeled isotope of DPC 333, TA769-d_5 , and loaded onto preconditioned Isolute C_8 cartridges. The cartridges were washed with two 1 mL aliquots of water, dried and then eluted with 1 mL of 2% ammonium hydroxide in methanol. The eluent was evaporated to dryness under nitrogen at 40°C and the residue was reconstituted with 0.10-0.15 mL of acetonitrile: aqueous formic acid (0.1%) (5:95, v/v). Urine samples were diluted with acetonitrile: aqueous formic acid (0.1%) (5:95, v/v) containing the internal standard and analyzed directly. The chromatographic separation of DPC 333 and the internal standard TA034 was accomplished on a reversed-phase column (Metachem AQ C_{18} column, 2.1 x 50 mm, 5 μm), using a gradient elution at a flow rate of 0.3 mL/min. The gradient solvents used

were 0.1% formic acid in water and 0.1% formic acid in acetonitrile for mobile phases A and B, respectively. The initial solvent composition was 7% solvent B, which was increased to 60% in 3.5 min and maintained at 60% for 1 minute. The content of solvent B was then decreased to 7% in 0.5 min and maintained at 7% for 3 min. The total run time was 8 min. DPC 333 was detected using a Micromass Quattro LC/MS/MS with positive electrospray interface in the multiple reaction monitoring mode (MRM). The parent/daughter ion transitions monitored were m/z 477 →157 for DPC 333 and 466 →276 for TA034. The linear range for the standard curves in plasma was 1 to 1000 nM, and the lower limit of quantification was 1 nM. A similar validated LC/MS/MS method was used to determine DPC 333 concentrations in human plasma. The validated assay concentration range for DPC 333 using 200 µL of plasma was 1.05 to 2098 nM. Inter-assay precision and accuracy ranged from 4.56-5.88% and 0.86-8.53%, respectively.

Pharmacokinetic and Pharmacodynamic Analysis: The plasma concentration-time data were analyzed by noncompartmental analysis using WinNonlin® Professional Version 3.0 (Pharsight Co. Mountain View, CA) and the pharmacokinetic parameters CLs, Vss and t1/2 were calculated using standard formulae (Gibaldi and Perrier, 1982). T_{max} and C_{max} were taken from the observed data. Bioavailability (F) was estimated in species where

DPC 333 was given intravenously by the dose-normalized AUC ratio data of DPC 333 after intravenous and extravascular administration. Individual pharmacokinetic parameters were generated for each animal and human subject and the data for each pharmacokinetic parameter are reported as the mean ± S.D. The relationship between the inhibition of LPS-stimulated TNF-α production and the concentration of DPC 333 in the chimpanzee was analyzed using a two compartment model and a sigmoid Emax model. The individual PK/PD parameters were estimated by nonlinear regression analysis using WinNonlin.

A preliminary pharmacokinetic/pharmacodynamic (PK/PD) model was also developed for the clinical phase I data using the observed concentrations of TNF-α and DPC 333. The actual TNF-α concentrations were used in this model (rather than percent inhibition from baseline) to avoid potential transformation errors. The model was then used to generate percent inhibition curves after the fitting procedure. The pharmacodynamic model used in this analysis was:

$$TNF(t) = TNF_0\left(1 - \frac{C(t)^{\gamma}}{IC_{50}^{\gamma} + C(t)^{\gamma}}\right) \qquad (1)$$

where TNF-(t) is the concentration of TNF-α at time t, TNF-$_0$ is the baseline (pre-dose) TNF-α concentration, C(t) is the plasma concentration of DPC 333 at time t, IC_{50} is the

concentration of DPC 333 which causes a 50% inhibition of TNF-α, and γ is a shape factor which describes the slope of the concentration-response curve.

For interspecies scaling of the pharmacokinetics, the mean values of CLs and Vss obtained from mouse, rat, dog and chimpanzee as well as from cynomolgus monkey (unpublished data) were plotted against mean body weight (kg) on a log-log scale. The following allometric equations were used for the analysis:

$$CLs = aBW^{x} \quad (2)$$

and

$$Vss = bBW^{y} \quad (3)$$

where *a* and *b* are the allometric coefficients, BW the body weight, and *x* and *y* the allometric exponents for CLs and Vss, respectively. The allometric equations obtained were used to estimate human pharmacokinetic parameters CLs and Vss. A 70-kg body and an oral bioavailability of 17% (derived from the chimpanzee study) were assumed in the human study. Human plasma *AUC* was estimated using the following equation:

$$AUC = FDose / CLs \quad (4)$$

RESULTS

Anti-TNF-α Activity of DPC 333 in blood

The in vitro IC_{50} values for the inhibition of TNF-α production were 39, 30, 17 and 100 nM using blood from mice, Lewis rats, SD rats and humans, respectively and complete inhibition of TNF-α production was observed at 190, 300, 134 and 1860 nM in these respective species.

Serum Protein Binding of DPC 333

Protein binding results of DPC 333 are summarized in Table 1. The binding of DPC 333 to purified human albumin and α-1-acid glycoprotein, the two major drug binding proteins in blood, was less extensive than binding in serum, 18.6 and 18.1% respectively at 1μM.

Pharmacokinetics of DPC 333 in Mice and Rats

Following an iv bolus dose in mice and rats, DPC 333 plasma concentrations declined rapidly, with an average terminal half-life of approximately 1 h (Table 2). The mean systemic plasma clearance was 6.2 and 2.7 L/h/kg and the mean Vss was 1.5 and 0.6

L/kg in mice and rats, respectively. After oral or intraperitoneal dosing, DPC 333 was rapidly absorbed, with maximal plasma concentrations (C_{max}) observed within 30 minutes post-dose. The apparent oral bioavailability was 11% and 17% in mice and SD rats, respectively, however the dose studied in the mouse was high, 68 mg/kg. The bioavailability after intraperitoneal dosing was high in the two species, 98% and 78%, respectively.

Synovial pharmacokinetics of DPC 333 following a single oral dose to rats

The plasma and synovial fluid concentration- time profiles of DPC 333 after a single oral dose of 60 mg/kg to Lewis rats are shown in Figure 2. Mean peak plasma and synovial concentrations of 13.489 and 2.970 µM, respectively were observed one hour after oral dosing. Concentrations in plasma and synovium declined in parallel to 0.394 and 0.267µM four hours postdose and were still measurable at 24 h. The estimated half life of DPC 333 was approximately 4 hours. DPC 333 exposure, measured as the AUC, in the synovium fluid was 26.4% of the plasma exposure.

Suppression of Plasma TNF-α Levels by DPC 333 in Rodent Models of Endotoxemia

The effect of increasing oral and ip doses of DPC 333 on the TNF-α response following LPS challenge in a mouse model of endotoxemia is shown in Figure 3A. The suppression of TNF-α production was dose-dependent with a calculated ED_{50} of 6.1 and 1.9 mg/kg following oral and ip administration, respectively. Similar experiments using rats revealed an ED_{50} of 1.1 mg/kg after ip administration (Figure 3B).

Efficacy and PK-PD Relationship of DPC 333 in the Rat Model of Collagen-Induced Arthritis

The effect of DPC 333 on the average clinical score in the rat collagen-induced arthritis model is shown in Figure 4. At doses of 5.5 mg/kg and 10.5 mg/kg given twice daily, DPC 333 markedly decreased the severity of the arthritis, with a mean reduction of the clinical score of 56% and 65%, respectively (Table 3). The magnitude of this reduction was indistinguishable from that produced by Etanercept, the positive control.

To determine the PK-PD relationship of DPC 333 in this rat model of arthritis, plasma concentration-time data on Day 14 of the experiment were obtained following the 5.5 or 10 mg/kg doses of DPC 333. As shown in Table 3, TNF-α production was reduced by

56% relative to control animals when the plasma AUC was 358 nM•h and the C_{max} was 444 nM. Suppression of the clinical score by the 10 mg/kg dose was similar, 65%.

Pharmacokinetics of DPC 333 in dogs

Pharmacokinetic parameters of DPC 333 in dogs are summarized in Table 2. Systemic plasma clearance was high, 1.8 L/h/kg, a value equivalent to hepatic blood flow in this species. The volume of distribution at steady state was 2.4 L/kg, approximately 4 times the total body water in the dog (0.6L/kg), suggesting extensive tissue distribution. The terminal half-life of DPC 333 was 3.9 h. Following oral dosing, DPC 333 was rapidly absorbed in dog, with mean maximal plasma concentration of 740 nM occurring approximately 45 min postdose. The mean oral bioavailability of a 5 mg/kg dose of DPC 333 was 15%.

Pharmacokinetics and pharmacodynamics of DPC 333 in Chimpanzees

Plasma concentrations of DPC 333 declined polyexponentially after termination of an iv infusion of 0.5 mg/kg. The calculated systemic plasma clearance was 0.4 L/h/kg, the volume of distribution at steady-state and terminal half-life was 0.6 L/kg and 1.8 h, respectively (Table 2). The fraction of DPC 333 excreted in urine unchanged was 1.6% of

total dose, with most appearing within the first 24h following dose. The small percent of the dose recovered in the urine suggests that the renal clearance plays a minor role in the elimination of DPC 333 in the chimpanzee. The apparent oral bioavailability was low, averaged 15% from 3 chimpanzees. The mean peak plasma concentration (C_{max}) was 589 nM, occurred approximately 2 h post dose.

Figure 5 shows the relationship between plasma concentrations of DPC 333 and the percent inhibition of ex-vivo LPS stimulated TNF-α production in whole blood from the one chimpanzee dosed orally who was not sedated with ketamine. The inhibition of TNF-α production was dependent on the plasma concentrations of DPC 333 in this conscious chimpanzee. The inhibition of TNF-α production by DPC 333 was maximal from 1 to 3 hours when total plasma concentrations were greater than 336 nM and was sustained over approximately 6-7 hours. Negligible inhibition was observed 9 hours postdose when the plasma concentrations were at or below 3 nM. The whole blood assay IC_{50} of 55.1 nM was estimated and maximal suppression was seen at concentrations greater than 92.5 nM using a sigmodial Emax model with a sigmoidicity factor of 3.54. In the other two chimpanzees sedated with ketamine for blood collection, no clear relationship between DPC 333 concentrations and % TNF-α suppression was observed. This is consistent with the potent suppressive effect of ketamine on LPS induced TNF-α

production *in vitro* and *in vivo* (Takenaka *et al*., 1994). Thus, the two animals were excluded from the PD analysis.

Single Oral Dose Pharmacokinetics and pharmacodynamics of DPC 333 in Humans

Overall, DPC 333 was generally safe and well tolerated. A total of 32 (48.5%) subjects who received DPC 333 in the study had an AE compared with 8 (40.0%) placebo-treated subjects. The most commonly observed AE was taste disturbance (28.3%, 17/60 DPC 333 vs. 20.0%, 4/20 placebo). For all laboratory parameters, vital sign measurements, and ECG parameters, no dose- or gender-related mean trends were apparent.

The mean plasma concentration - time profiles from single escalating oral doses of DPC 333 in healthy male volunteers are shown in Figure 6, while a summary of the pharmacokinetics results is shown in Table 4. DPC 333 was rapidly absorbed after oral dosing, with T_{max} values ranging at 0.25 to 1 hour post-dose. The mean half-life for each dose cohort ranged between 2 and 6 hours. The C_{max} and AUC values increased in a dose proportional manner from15 to 225 mg, however, doses larger than 225 mg produced greater-than-proportional increases in both C_{max} and AUC.

The compiled pharmacodynamic data from all healthy male subjects given DPC 333 is illustrated in Fig 7. The degree of inhibition for the LPS-stimulated TNF-α release

closely followed DPC 333 plasma concentrations. Total inhibition of TNF-α release was seen 2 hours following the 225 mg dose. Near-maximal inhibition of TNF-α release was achieved over the entire 8 hour sampling period at the 345 mg dose. Pharmacodynamic fitting of these data using a sigmoid Emax model (Figure 7) resulted in a calculated IC_{50} value (total drug concentration) of 113 nM (95% CI of 95-131 nM), a value indistinguishable from that obtained from *in vitro* studies conducted with human whole blood (100 nM).

Interspecies Scaling

Using the mean PK data from mice, rats, dogs, monkeys (unpublished data) and chimpanzees and a simple allometric approach, human CLs and Vss were estimated to be 0.48 L/kg/h and 0.72 L/kg, with a correlation coefficient (r^2) of 0.96 and 0.95 and an allometric exponent of 0.69 and 0.95, respectively (Fig 8).

DISCUSSION

Rheumatoid arthritis (RA), a devastating disease, afflicts approximately one percent of the general population and three times more women than men (Alamanos and Drosos, 2005). The pro-inflammatory cytokine TNF-α has been shown to play a pivotal role in RA and many other serious diseases such as Crohn's disease and psoriasis. Although substantial progress has been made in the treatment of these diseases with the use of anti- TNF-α biological agents, including Etanercept, Remicade and Humira, there still remains a sizable percentage (30~40%) of patients who are unresponsive to all currently used and approved medications (de Vries and Tak, 2005). Therefore, the discovery of new compounds that can be administered orally and whose mechanism of action differs from that of the biological TNF-α inhibitors would significantly add to the armamentarium available for treating RA and other immunological disorders. One molecule that fits such a profile is DPC 333, a potent, selective and orally available compound that inhibits TACE and decreases circulating TNF-α levels (Duan et al., 2003).

The plasma clearance of DPC 333 in rodents and in dogs is high, approximately the hepatic blood flow in these species, and is consistent with the low oral bioavailability (<20%) seen in these species. The clearance of DPC 333 in the chimpanzee, however, is approximately one-third of the apparent hepatic blood flow in humans. It's reasonable to make this comparison between chimpanzee and human because of the similarities in body size and allometric relationships between size and organ perfusion. However, these differences in clearances between the chimpanzee and the other animal species did not translate into a greater oral bioavailability for DPC 333. The oral bioavailability of DPC 333, given as either a solution or as a suspension, in all of the preclinical species tested ranged from 11-17%. It is unknown whether the lower than expected oral bioavailability in the chimpanzee is due to gastrointestinal tract metabolism, pharmaceutical issues with the formulation that was used, or the effects of the anesthesia used to collect blood samples from the chimpanzees. DPC 333 is rapidly absorbed in all species after oral dosing in solution, with T_{max} values of less than 2 hours, and in some cases less than 30 minutes.

DPC 333 is moderately bound to serum proteins in all species tested, although there are notable species variations in the binding. The free fraction is 2-3-times greater in rodents versus dog, chimpanzee and human. The reason for this difference is not

clear. The unbound fraction was the same using purified human albumin and α-1-acid glycoprotein, approximately 18-19%, and was greater than the free fraction determined with human serum, 7%. These data suggest that other proteins in serum, perhaps a globulin fraction, also bind DPC 333. Because α-1-acid glycoprotein levels can increase in rheumatoid arthritis (Belpaire and Bogaert, 1990), the significance of DPC 333 bound to α-1-acid glycoprotein should be determined in sera from these patients.

Pharmacokinetic data for the chimpanzee was used to predict the oral human exposure in the single ascending dose "first in human" trial with DPC 333, although there are some notable limitations of the chimpanzee data, such as the low number of animals used and the small dose range studied. Wong et al. (2004) and Riska et al. (1999) have shown that the pharmacokinetics of a wide range of xenobiotics in chimpanzees closely mimic those seen in humans. While the mechanisms responsible for systemic clearance of DPC 333 are unknown, the excellent correlation of the projected CLs and Vdss from the allometry suggests that the biochemical differences in chimpanzee CYP isozymes (Wong et al., 2004) may not be important and that the chimpanzee seems to be an appropriate model. Furthermore, our human exposure estimate utilizing a simple weight derived allometric scaling also supports the use of the chimpanzee as a suitable model for the human pharmacokinetics of DPC 333 (Fig. 8-9).

There is close agreement between the predicated and actual systemic exposure after oral doses studied in the Phase I clinical trial, especially at the lower doses. This validates the rationale and importance of using allometric scaling or the chimpanzee model to estimate starting clinical dose of DPC 333, one of most daunting challenges during the translational phase from preclinical to the first in human trial. At higher doses the predicted exposure underestimated the actual human data, as seen in Figure 9. This is most likely due to the fact that the predicted data assume linear kinetics in humans, which may not be the case, since the AUC and C_{max} values increase modestly in a greater than dose proportional manner, approximately two-fold. This implies that with an increase in dose in human volunteers either the clearance of DPC 333 fell or the absorption of the compound increased. Any change in clearance is difficult to detect without IV doses for humans. The limited data available does not rule out the possibility that the actual clearance in humans may be greater and hence oral bioavailability more susceptible to saturation of presystemic extraction. Another possibility for the greater than dose proportional increase in AUC with oral doses may be in part due to saturation of efflux pump/transporter throughout the intestinal tract at the higher oral doses. Recently, Luo et al. (2007) have reported that DPC 333 may be a

substrate for pgp and that glucuronide conjugates of DPC 333 may inhibit the Mrp-2-mediated transport of methotrexate.

The rodent models of inflammation represent simple yet versatile systems to assess the *in vivo* activity of TNF-α inhibitors (Williams et al., 1992 and Michie et al., 1998). Following the injection of mice or rats with bacterial endotoxin, systemic TNF-α levels are routinely elevated 100-fold over pretreatment levels within 1 to 2 hours and remain elevated above baseline values for an additional 4 hours (Bemelmans et al., 1993). Similar concentration-time-profiles for TNF-α levels have been shown in humans after a LPS-challenge (van der Poll et al., 1997; Dekkers et al., 1999). Following single escalating po and ip doses of DPC 333 to LPS-treated mice, the ED_{50} values of DPC 333 to lower TNF-α level are 6.1 and 1.9 mg/kg, respectively. These results demonstrate that DPC 333 can effectively reduce induced TNF-α levels in vivo. The ~3-fold difference between the ED_{50} values derived from po and ip routes of administration in mice is most likely due to differences in the systemic availability between the two routes of administration. The apparent bioavailability of DPC 333 was between 11 to 17% after oral administration, while after ip doses it ranged from 78 to 98%. Bioavailability from the mouse experiments must be interpreted cautiously however, since the oral dose was high (68 mg/kg) and the linearity of the pharmacokinetics is unknown.

The anti-inflammatory potential of DPC 333 was also assessed in the collagen-induced arthritis (CIA) rat. This animal model is well characterized and has a number of pathological similarities to human rheumatoid arthritis (Anthony and Haqqi, 1999). Like human rheumatoid arthritis, synovitis and erosions of cartilage and bone are hallmarks of the CIA rat model. In patients with RA, Deleuran et al. (1992) found that up-regulated TNF-α and TNF-α-receptor (TNF-α-R) expression are present in the synovium and particularly in the cartilage-pannus junction. The target site of anti-arthritic drugs, such as DPC 333, is mainly in the joint space. DPC 333 appears to distribute well into the synovial space in the rat as the exposure in the synovial fluid is approximately 26% of that in plasma following oral administration. More importantly, DPC 333 treatment of CIA rats dramatically reduces the clinical signs of arthritis to a similar extent to that seen in the rats treated with the positive control etanercept (Enbrel®).

Despite a clear response to the LPS challenge that produce large amounts of TNF-α from animal species and a highly consistent TNF-α inhibition profile by DPC 333 in our study using the rat CIA model (Table 3), there seems to be lack of direct correlation between DPC 333 doses (or plasma concentrations) and clinical scores (% suppression of disease). The reasons for the lack of a dose-response relationship remain unclear, but may be in part because of the fact that the lower dose and resulting plasma

concentrations had already produced the maximal effect (Emax), i.e. 50-65% decrease from control. In a number of clinical trials, a similar no-response rate (20~50%) was found with anti-TNF therapeutics (Olsen and Stein, 2004, Feldmann and Steinman, 2005). The lack of response to anti-TNF-α treatment has been associated with genetic variation in the production and effector pathways of its targets (Ulfgren, et al., 2000, de Vries and Tak, 2005). Because of the complexity of the downstream cytokine cascade and signal transduction triggered by elevated TNF-α, it is reasonable to use the rat CIA model only to demonstrate the proof of concept in efficacy, rather than to predict appropriate dose regimens of TNF inhibitors for treatment of human rheumatic arthritis.

The ability of DPC 333 to inhibit ex vivo LPS-stimulated TNF-α production/release from whole blood was assessed as a biomarker for anti TNF- α activity. A preliminary experiment using one unanesthetized chimpanzee demonstrated that the pharmacodynamic response (% inhibition of TNF-α) could be fitted to an Emax pharmacodynamic model, with an apparent IC50 of 55 nM. Similarly, the apparent IC_{50} in humans is 113 nM, calculated using this pharmacodynamic model to fit the more extensive ex vivo human data. This value is almost identical to the in vitro IC_{50} value obtained with human blood (100 nM). The similarity between the in vitro and ex vivo

human plasma IC_{50} suggests that contributions from any potential active metabolites to inhibit TNF-α production or release are negligible. Consistently, in preliminary in vitro studies on the biotransformation of DPC 333, no metabolites retaining significant pharmacological activity have been identified (data not presented). A close examination of the in vivo pharmacodynamic relationship in humans between inhibition of soluble TNF-α in vivo and plasma concentrations of DPC 333 reveals that the peak concentrations (C_{max}) achieved in most subjects at the lowest dose (15 mg) are generally greater than the IC_{50} values measured in vitro for soluble TNF-α release. Concentrations approaching or exceeding the IC_{50} for inhibition of TNF-α production (113 nM) are easily reached and last approximate 4 h post dose after single doses of 25 to 40 mg. The results show a dose-dependent maximum inhibition of TNF-α release with greater than 90% inhibition at the 225 mg dose, along with a C_{max} of 3139 nM and an AUC of 3000 nM•h. These values are quite similar to those observed in the LPS mouse infusion study. Measurement of TNF-α level using the WBA as described in these studies may serve as a good biomarker in evaluating the therapeutic efficacy of TACE inhibitors.

Because TNF-α is a key cytokine that acts by inducing several other pro-inflammatory cytokines, chemokines, adhesion molecules and matrix degrading enzymes

(Feldmann et al., 1996), diminishing TNF-α level for a short duration with DPC 333 may be sufficient to provide long lasting anti-inflammatory effects. Alternatively, with a relatively short half-life (<6 h) compared with the anti-TNF-α biologics (>4 days to 2 weeks), DPC 333 may offer the ability to more precisely control regulation of TNF-α levels, as well as the duration of TNF-α blockade, allowing for timely discontinuation in the event of intercurrent illness. This could minimize the risk of infection and other adverse events seen with some of the anti-TNF-α biologics with long half-lives (Wallis et al., 1998; Feldmann and Steinman, 2005).

In summary, DPC 333 is a potent inhibitor of TACE and shows a dose (concentration) dependent reduction of LPC-induced TNF-α in blood in vitro, in vivo and ex vivo from mouse, rat, chimpanzee and human. Pharmacokinetic and pharmacodynamic characteristics of DPC 333 support the concept that TACE inhibitors may provide a novel approach in treatment of various diseases including RA for which a precise control of excessive soluble TNF-α production is deemed desirable.

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Footnote

Current affiliation: [1]Genentech, Inc., South San Francisco, CA; [2]Endo Pharmaceuticals, Chadds Ford, PA; [3]Adolor Corporation, Exton, PA; [4]MillenniumPharmaceuticals, Inc., Cambridge, MA; [5]Incyte Corporation, Newark, DE; [6]GlaxoSmithKline, King of Prussia, PA; [7]AstraZeneca Plc, Waltham, MA; [8]SNC Partners LLC, Newark, DE .

Legends for Figures

Figure 1. Chemical structure of DPC 333.

Figure 2. Plasma and synovial concentration-time profiles of DPC 333 following a single oral dose of 60 mg/kg to Lewis rats.

Figure 3. Suppression of TNF-α Production in Mouse and Rat Models of Endotoxemia by DPC 333. Dose-dependent suppression of TNF-α production in the mouse (A) and rat (B) endotoxemia model following po (dotted line) and ip (solid line) administration of DPC 333.

Figure 4. Efficacy of DPC 333 and Etanercept in a Rat Model of Collagen-Induced Arthritis. Results are expressed as group mean (± S.D.) of clinical score/rat.

Figure 5. Time course of DPC 333 plasma concentrations (●) and the effect on *ex vivo* inhibition of TNF-α by DPC 333 (○) after a single oral administration of 2.7 mg/kg to an alert Chimpanzee.

Figure 6. Plasma concentrations-time profiles of DPC 333 following a single oral dose to human volunteers in the Phase I clinical trial

Figure 7. Ex Vivo Percent Inhibition of TNF-α Production as a Function of DPC 333 Plasma Concentrations Following a Single Oral Dose to Human Volunteers in the Phase I clinical trial.

Figure 8. Interspecies allometric scaling for systemic clearance (Cls) and volume of distribution at steady state (Vss) .The regression line was determined by the least-squares analysis of the five animal data, excluding the projected human data.

Figure 9. Comparison of predicted and actual systemic exposure to DPC 333 in various human doses.

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Table 1. Species Dependent Protein Binding of 1 µM DPC 333

species	**matrix**	concentrations µM	**unbound** %
Mouse	serum	1000	6.0 ± 0.1
Rat	serum	1000	4.6 ± 1.2
Dog	serum	1000	19.9 ± 2.8
Chimpanzee	serum	1000	14.0 ± 0.7
Human	serum	1000	6.7 ± 0.7
	HSA	1000	18.6 ± 0.8
	α1-Acid Glycoprotein	1000	18.1 ± 0.8

Table 2. Pharmacokinetic parameters of DPC 333 in different preclinical animal species

SPECIES	IV				PO				IP			
	dose	CLs	Vss	T½	dose	C_{max}	Tmax	F	dose	C_{max}	Tmax	F
	mg/kg	l/h/kg	l/kg	h	mg/kg	nM	h	%	mg/kg	nM	h	%
Mouse	11	6.2	1.5	1	68	13811	0.3	11	11	16038	0.1	98
SD rat	3.4	2.7±0.3	0.6±0.1	0.9±0.9	6.8	2496±799	0.1±0.0	17±3	6.8	9666±1010	0.1±0.1	78±12
Beagle dog	5	1.8±0.4	2.4±0.4	3.9±0.7	5	740±287	0.7±0.3	15±5	NA	NA	NA	NA
Chimpanzee	0.3	0.4	0.6	1.8	2.7	589±22	2.0±1.0	15±4	NA	NA	NA	NA

Table 3. PK-PD Relationship of DPC 333 in the Rat Model of Established Arthritis

Test Compound	Dose Regimen	DPC 333 AUC (nM•h)	DPC 333 C_{max} (nM)	% Suppression of clinical score from positive control
DPC 333	5.5 mg/kg, bid, po	358	444	56
DPC 333	10 mg/kg, bid,po	1398	2130	65
Etanercept	2.5 mg/rat, twice a week, ip	NA	NA	54

Table 4. Pharmacokinetic and pharmacodynamic parameters of DPC 333 following a single oral administration to healthy human volunteers

Parameter	15 mg (n=6)	25 mg (n=6)	40 mg (n=6)	80 mg (n=12)	120 mg (n=6)	225 mg (n=6)	345 mg (n=6)	530 mg (n=6)
AUC (nM x h)	341.9 ± 86.3	675.9 ± 223.1	1179.0 ± 244.6	3202.2 ± 957.1	4773.6 ± 1436.5	7888.4 ± 1507.8	23598.4 ± 5220.5	30762.2 ± 8718.5
Cmax (nM	133.6 ± 45.1	303.2 ± 132.0	505.0 ± 148.8	1420.3 ± 450.5	1941.5 ± 404.7	3139.5 ± 510.8	11001.7 ± 4415.5	13761.0 ± 4310.8
Tmax (h) [a]	0.75 (0.5-1.0)	0.5 (0.5-1.0)	0.5 (0.25-1.0)	0.5 (0.5-1.0)	0.5 (0.5-1.0)	0.75 (0.5-1.0)	0.75 (0.25-1.0)	0.5 (0.5-1.0)
T1/2 (h) [b]	2.18 ± 0.21	2.86 ± 0.86	3.41 ± 0.67	4.05 ± 0.51	4.89 ± 0.99	5.53 ± 0.88	6.39 ± 0.73	6.04 ± 0.58
C_{12h} (nM)	2.09 ± 0.79	4.41 ± 2.08	6.36 ± 1.03	20.4 ± 6.60	30.26 ± 12.74	49.03 ± 9.37	152.8 ± 32.2	167.3 ± 43.9
% inhibition of TNFα release	39.4 ± 16.0	51.8 ± 15.8	58.1 ± 25.8	82.2 ± 10.1	81.6 ± 7.3	91.0 ± 3.7	96.9 ± 1.2	96.1 ± 0.02
Range	(25.5-58.5)	(30.9-67.7)	(27.1-92.1)	(57.1-100)	(76.6-93.4)	(86.4-94.8)	(95.6-98.5)	(93.9-98.2)
Median Tmax (h)	2	2	2	2	2	2	2	2

[a] Median (Range)

[b] harmonic mean ± pseudo-standard deviation

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Figure 1.

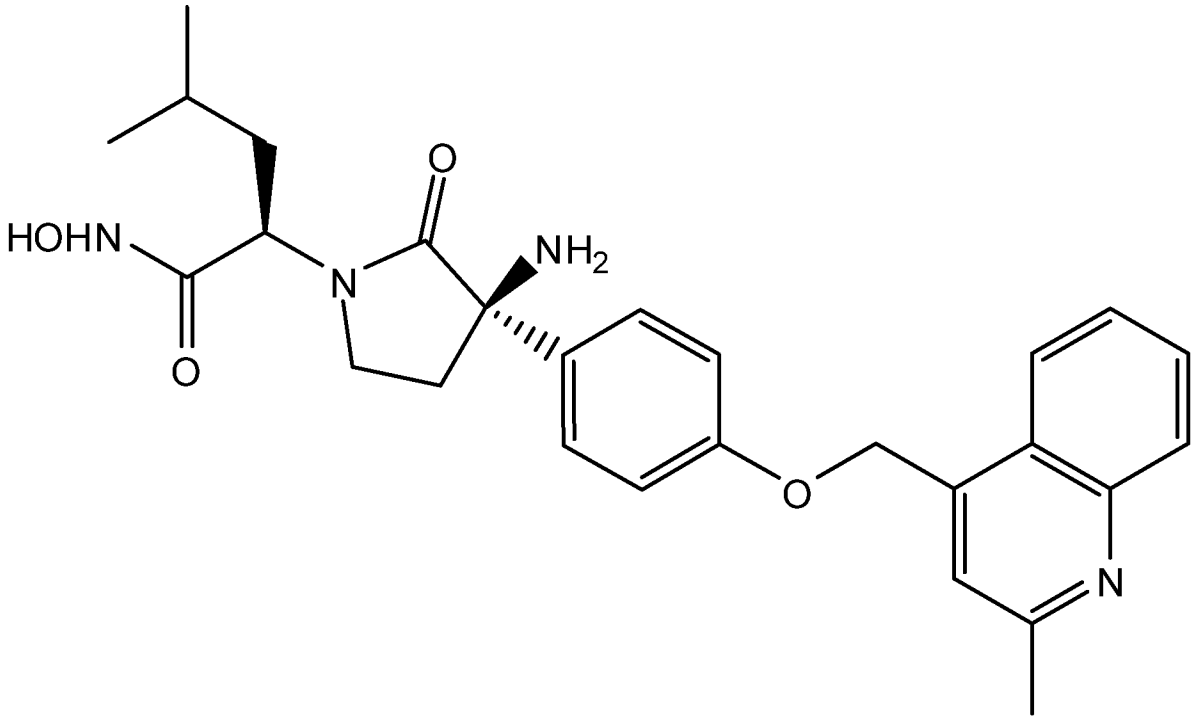

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Figure 2.

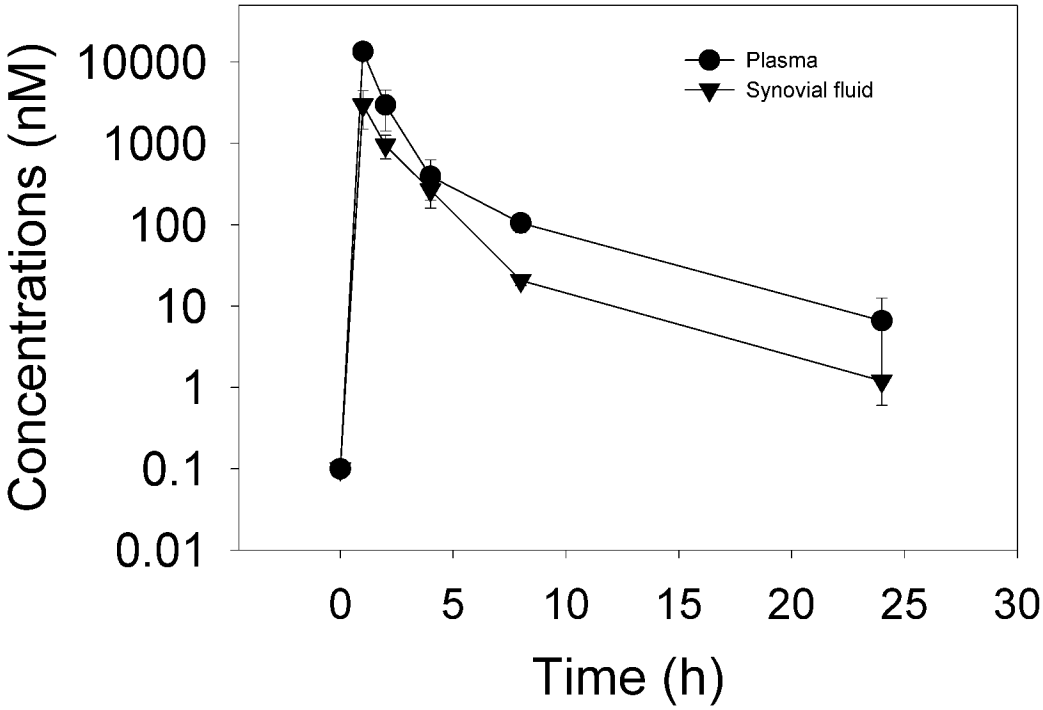

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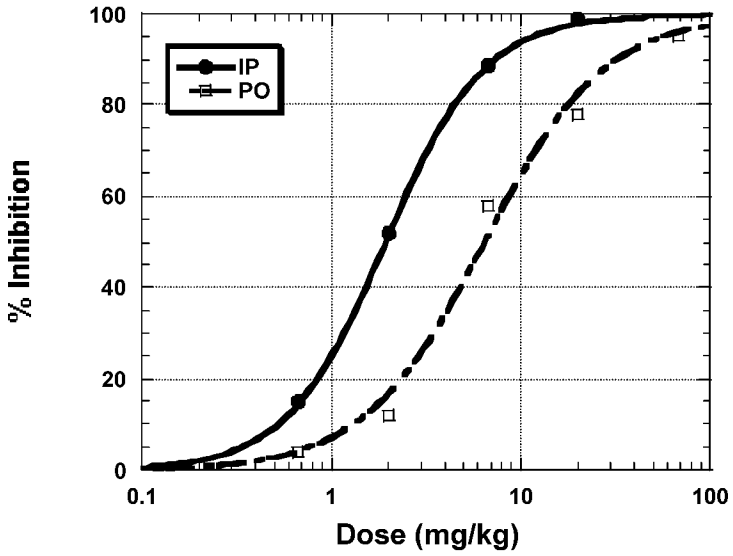
Figure 3.

A.



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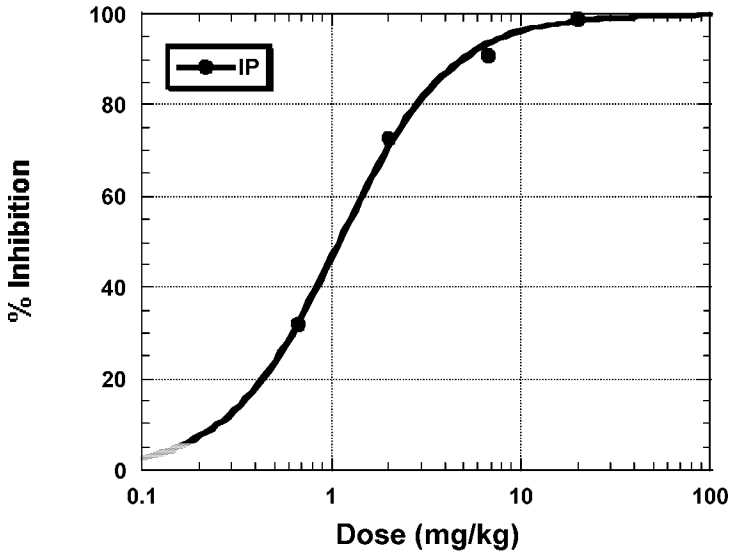

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Figure 4.

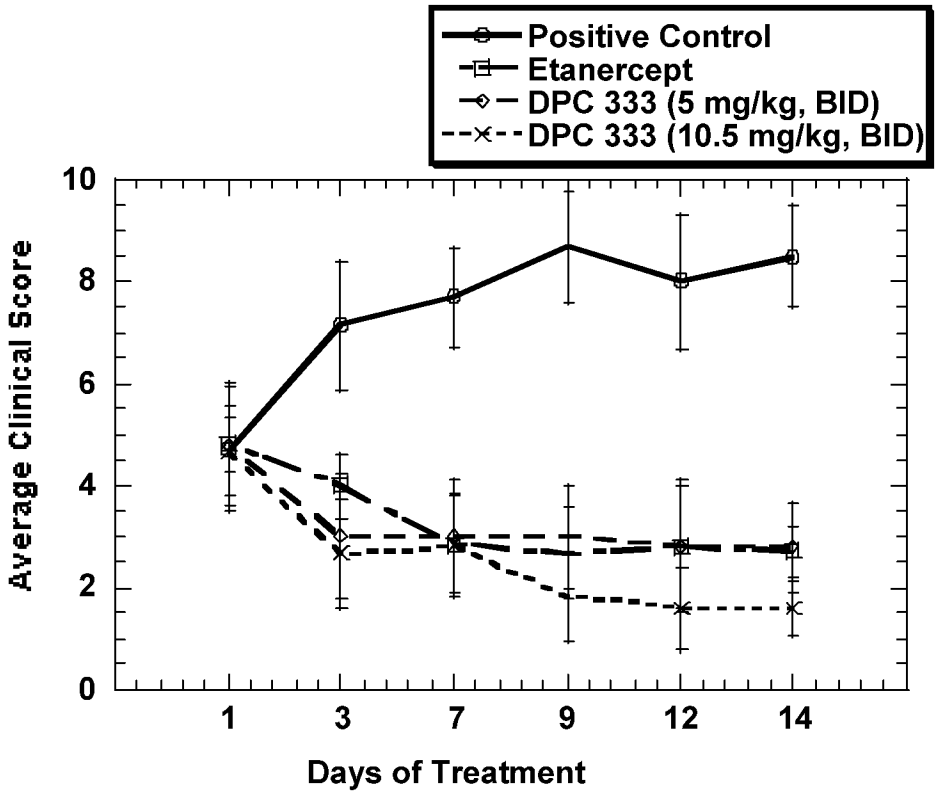

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Figure 5

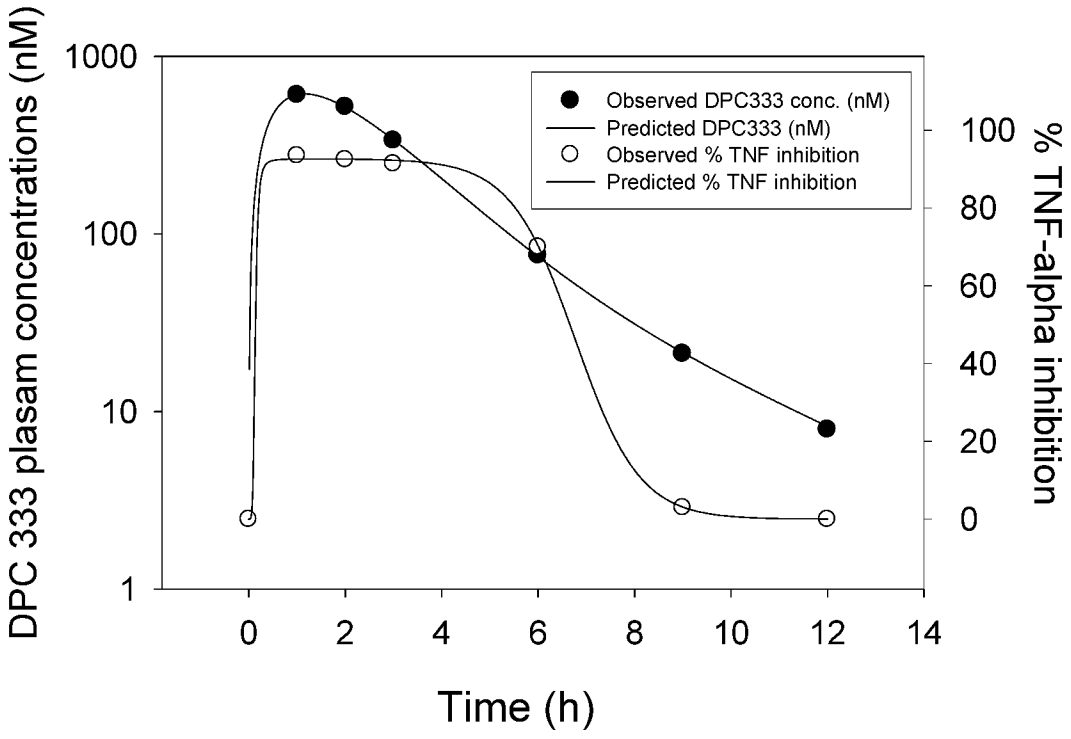

Figure 6

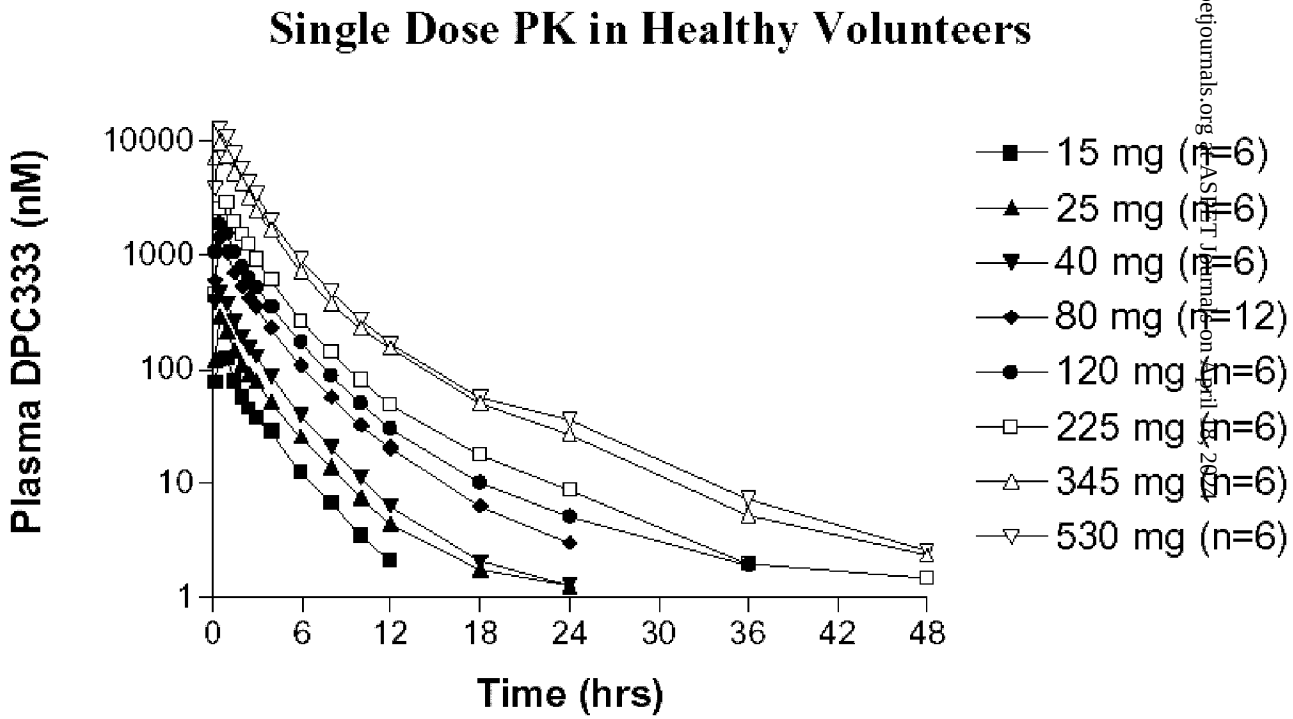

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Figure 7.

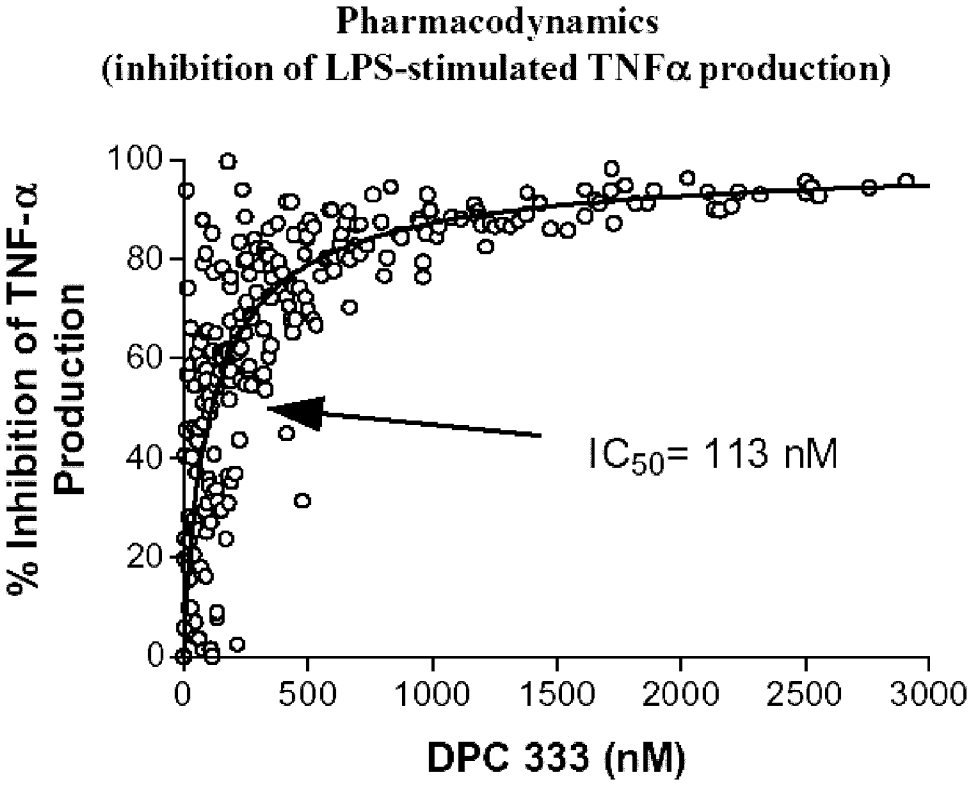

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Figure 8

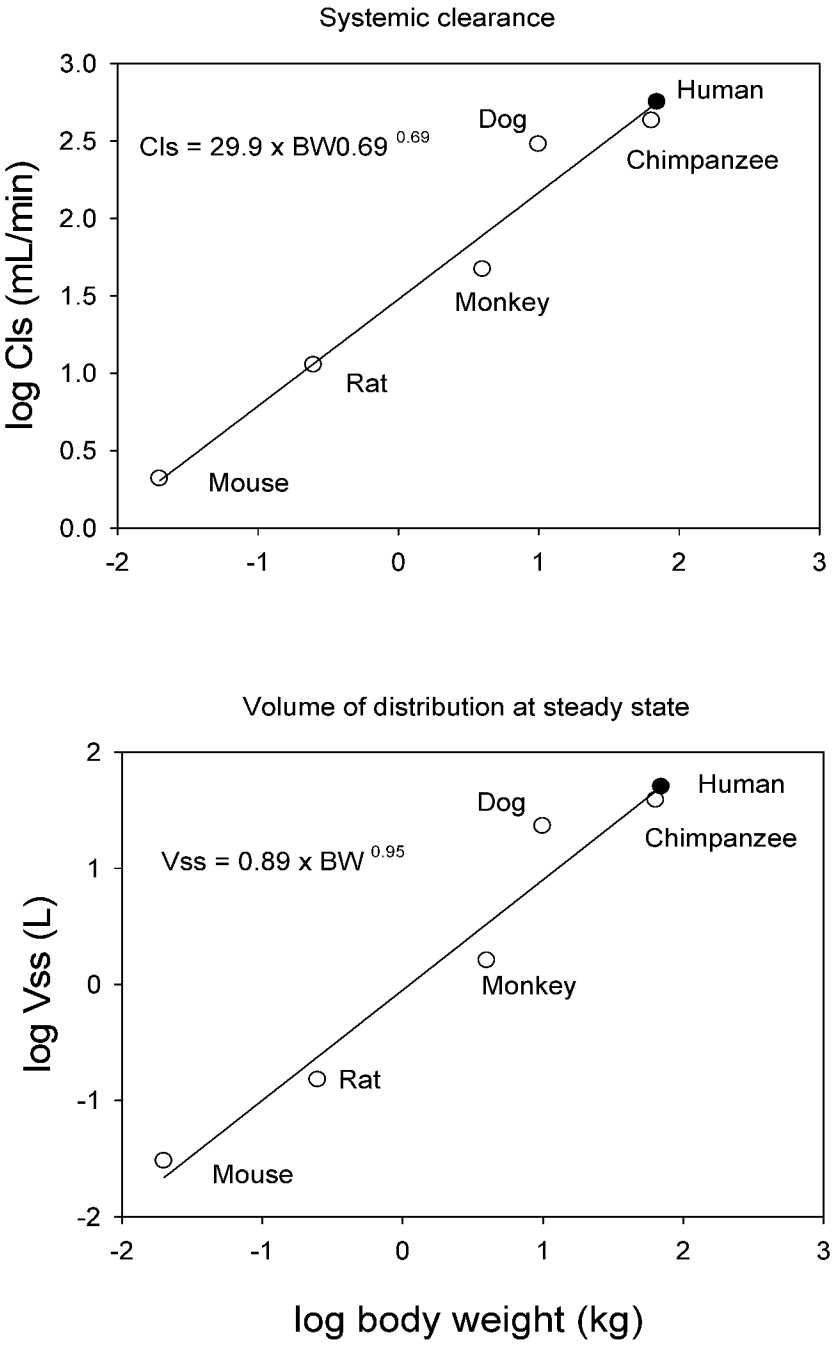

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Figure 9