JPET Fast Forward. Published on May 15, 2006 as DOI: 10.1124/jpet.105.097568
JPET Fast Forward. Published on May 15, 2006 as DOI: 10.1124/jpet.105.097568
This article has not been copyedited and formatted. The final version may differ from this version.

JPET #97568

# Discovery and Characterization of Triaminotriazine Aniline Amides as Highly Selective p38 Kinase Inhibitors

Tsung H. Lin, Axel Metzger, David J. Diller, Madhuri Desai,

Ian Henderson, Gulzar Ahmed, Earl F. Kimble, Elizabeth Quadros, and Maria L. Webb

Pharmacopeia Drug Discovery Inc., Princeton, NJ 08543

Downloaded from jpet.aspetjournals.org at ASPET Journals on March 20, 2018



Copyright 2006 by the American Society for Pharmacology and Experimental Therapeutics.

JPET Fast Forward. Published on May 15, 2006 as DOI: 10.1124/jpet.105.097568
This article has not been copyedited and formatted. The final version may differ from this version.

JPET #97568

# Discovery of Selective p38 Kinase Inhibitors

Corresponding author: Maria L. Webb,

Pharmacopeia Drug Discovery Inc.

PO Box 5350

Princeton, NJ 08543-5350

Phone: (609) 452-3622

Fax: (609) 655-4187

Email: Mwebb@pcop.com

Text pages: 32

Number of Tables: 1

Number of Figures: 6

Number of References: 29

Number of Words in Abstract: 243

Number of Words in Introduction: 539

Number of Words in Discussion: 969

Non-Standard Abbreviations: ECLiPS: Encoded Combinatorial Libraries on Polymeric Support; ERK: extracellular signal-regulated kinases; GST: glutathione-S-transferase; JNKs: c-Jun N-terminal kinases; LPS: lipopolysaccharide; MAP kinase: mitogen activated protein kinase; MKK: MAP kinase kinase; MTS: 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium TNFα: tumor necrosis factor α; TPA: O-tetradecanoyl-phorbol 13-acetate



Downloaded from jpet.aspetjournals.org at ASPET Journals on March 20, 2018

JPET Fast Forward. Published on May 15, 2006 as DOI: 10.1124/jpet.105.097568
This article has not been copyedited and formatted. The final version may differ from this version.



Recommended Section Assignment: Inflammation and Immunopharmacology

Downloaded from jpet.aspetjournals.org at ASPET Journals on March 20, 2018

JPET Fast Forward. Published on May 15, 2006 as DOI: 10.1124/jpet.105.097568
This article has not been copyedited and formatted. The final version may differ from this version.



## Abstract

The p38 MAP kinases are a family of serine/threonine protein kinases that play important roles in cellular responses to inflammation and external stress. Inhibitors of the p38 MAP kinase have shown promise for potential treatment of inflammatory disorders such as rheumatoid arthritis, acute coronary syndrome, psoriasis and Crohn's disease. We identified a novel class of p38 inhibitors via high throughput screening. PS200981, a representative compound identified from screening a collection of combinatorial libraries, amounting to 2.1 million compounds, inhibits p38α kinase and the LPS-induced increase in TNFα levels in THP-1 cell media with IC50 values 1 μM. The screening data revealed a preferred synthon, 3 – amino – 4 – methyl benzamide which is critical for the activity against p38. This synthon appeared almost exclusively in screening hits including PS200981, and slight variations of this synthon including 3-amino benzamide and 2-amino-4-methyl benzamide also contained in the library were inactive. PS200981 is equally potent against the α and β forms of p38 but did not inhibit p38γ and is > 25-fold selective versus a panel of other kinases. PS200981 inhibited the LPS-induced increase in TNFα levels when administered at 30 mg/kg to mice. Selectivity and *in vivo* activity of this class of p38 inhibitors was further demonstrated by PS166276, a highly structurally related but more potent and less cytotoxic inhibitor, in several intracellular signaling assays, and in LPS challenged mice. Overall, this novel class of p38 inhibitors is potent, active *in vitro* and *in vivo*, and is highly selective.

Downloaded from jpet.aspetjournals.org at ASPET Journals on March 20, 2018

JPET Fast Forward. Published on May 15, 2006 as DOI: 10.1124/jpet.105.097568
This article has not been copyedited and formatted. The final version may differ from this version.



## Introduction

The mammalian p38 mitogen-activated protein (MAP) kinases are central to transduce extracellular signals to the nucleus thereby enabling cells to respond to environmental insults. p38 kinases are activated by physical and chemical stresses including the pro-inflammatory cytokines tumor necrosis factor-α (TNFα) and interleukin 1, endotoxin, UV irradiation, heat, and osmotic shock (Pearson et al., 2001). Activation of p38 requires dual phosphorylation by upstream MAP kinase kinases (MKKs) 3 and 6 on a threonine and a tyrosine within a Thr-Gly-Tyr motif, characteristic of p38 kinases (Raingeaud et al., 1996). Once activated, p38 kinases phosphorylate and activate other kinases and transcription factors leading to increases or decreases in the expression of certain target genes, such as interleukin-1 and TNFα (Lee et al., 1994).

The up-regulation by activated p38α kinase of cytokine production, predominantly TNFα and interleukin-1, is a hallmark of inflammation associated with a wide variety of diseases such as rheumatoid arthritis, endotoxic shock, inflammatory bowel disease, multiple sclerosis, psoriasis, and others (Henry et al., 1999; Salituro et al., 1999). Several biological agents that sequester TNFα or inhibit the action of interleukin-1, including monoclonal antibody to TNFα (infliximab and Adalimumab) (Onrust and Lamb, 1998; Furst et al., 2003), soluble TNFα receptor-Fc fusion protein (Etanercept) (Jarvis and Faulds, 1999), or the interleukin-1 receptor antagonist (anakinra) (Cvetkovic and Keating, 2002) have been used to treat patients with chronic inflammatory diseases, such as rheumatoid arthritis, Crohn's disease and psoriasis. While these agents are efficacious, they are costly and limited by route of administration. Thus, a need exists for small

Downloaded from jpet.aspetjournals.org at ASPET Journals on March 20, 2018

JPET Fast Forward. Published on May 15, 2006 as DOI: 10.1124/jpet.105.097568
This article has not been copyedited and formatted. The final version may differ from this version.



molecule based therapeutics that can be administered orally and at lower cost to both patient and health care systems.

The challenge in identification of kinase inhibitors for use in chronic disease is the potential for off-target effects at other kinases that cause issues with the safety profile. This is due largely to the general conservation in the ATP binding site of kinases and the specific homology across related members of specific kinase sub-families. The MAP kinase family consists of three subfamilies that include the extracellular signal-regulated kinases (ERKs), the c-Jun N-terminal kinases (JNKs), and p38 kinases. Molecular cloning studies have led to the identification of four p38 isoforms: p38α, p38β, p38γ and p38δ. These isoforms differ in tissue distribution, substrate preference, and activation modes (Goedert et al., 1997; Wang et al., 1997). Among these four isoforms, p38α is the best characterized and perhaps the most physiologically relevant p38 isoform involved in inflammatory responses (Allen et al., 2000).

In the past few years, a number of small molecule p38α inhibitors have been shown to block the production of TNFα and interleukin-1 (Cirillo et al., 2002; Jackson and Bullington, 2002) and some of these have advanced to clinical studies (Kumar et al., 2003). Concerns about off-target effects, however, have been raised. For example, VX-745, an ATP-competitive p38 inhibitor, advanced to clinical trials but was withdrawn following issues with CNS toxicity and BIRB-796, an allosteric p38 inhibitor with a slow off-rate (Kumar et al., 2003), also advanced to clinical trials and was last reported in Phase III. In this paper, we report the discovery of a novel and highly selective class of

Downloaded from jpet.aspetjournals.org at ASPET Journals on March 20, 2018

JPET Fast Forward. Published on May 15, 2006 as DOI: 10.1124/jpet.105.097568
This article has not been copyedited and formatted. The final version may differ from this version.



p38α inhibitors that demonstrate a strong selection for the methylanilino amide synthon indicating a critical interaction with p38.

Downloaded from jpet.aspetjournals.org at ASPET Journals on March 20, 2018

JPET Fast Forward. Published on May 15, 2006 as DOI: 10.1124/jpet.105.097568
This article has not been copyedited and formatted. The final version may differ from this version.



## Methods

### Materials

Tissue culture reagents and plastic ware were purchased from BD Biosciences (San Jose, CA), reagents for PCR and cloning were from New England BioLabs (Beverly, MA), myelin basic protein was obtained from Sigma (St. Louis, MO). Kinases except p38 were provided by Upstate Biotechnology Inc (Charlottesville, VA). Antibodies were purchased from Cell Signaling Technology (Beverly, MA).

### Tissue culture

Human monocytic THP-1 cells were obtained from American Type Culture Collection (Manassas, VA), maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, and split twice a week. F7 cells were kindly provided by Dr. Hodaka Fujii, University of Tokyo (Hatakeyama, 1989). F7 cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum plus interleukin-2.

### Generation of p38 kinases

Human p38α cDNA was amplified from human liver Quick-Clone cDNA (BD Biosciences, San Jose, CA), and human p38β and p38γ cDNAs were amplified from human brain Quick-clone cDNA, using PCR technology. p38 cDNAs were subcloned in the prokaryotic expression vector pGEX which consisted of a glutathione-S-transferase (GST) sequence at the amino-terminal region (Amersham Biosciences, Piscataway, NJ). The expression vectors containing p38 α, β and γ were transformed into the BL21 (DE3) strain of *E. Coli.* Expression of GST-p38 fusion proteins was induced in the presence of



Downloaded from jpet.aspetjournals.org at ASPET Journals on March 20, 2018

JPET Fast Forward. Published on May 15, 2006 as DOI: 10.1124/jpet.105.097568
This article has not been copyedited and formatted. The final version may differ from this version.



isopropyl β-D-1-thiogalactopyranoside. GST-p38 fusion proteins were purified from bacterial pellets using affinity chromatography (Amersham Biosciences). p38 α, β and γ isozymes were activated using constitutively active MKK6.

p38 kinase assay

The p38 kinase screen was performed in a 96-well filtermat assay format with myelin basic protein as the substrate. p38 was preincubated with test compounds for 10 min. The reaction was initiated by adding substrate mix containing [γ-$^{33}$P]ATP and protein substrate. After a 45-min incubation, the reaction was terminated by adding EDTA (40 mM final), the protein substrate was then harvested onto filtermats, and [γ-$^{33}$P]ATP was removed using a Skatron Micro96 Cell Harvester (Molecular Devices, Sunnyvale CA). [γ-$^{33}$P] phosphorylated protein substrate was detected using a Microbeta scintillation counter (PerkinElmer, Wellesley, MA). The final concentrations of reagents in the reactions were 10 nM p38, 34 μg/ml myelin basic protein, 50 mM Tris pH 7.5, 10 mM $MgCl_2$, 50 mM NaCl, 1 mM DTT 1 μM ATP, 3 nM [γ-$^{33}$P]ATP, and 0.3% DMSO.

Kinase selectivity assays

Different assay formats were used in the evaluation of selectivity for p38α against a panel of non-p38 kinases. To ensure similar sensitivity across the different kinase assays, the ATP concentration was adjusted to equal or below Km for each kinase.

The filtermat protocol described in the **Methods** was used to determine selectivity against ERK1, ERK2, JNK1α1, JNK2α2, Mapkap K2, and protein kinase A. Myelin basic



Downloaded from jpet.aspetjournals.org at ASPET Journals on March 20, 2018

JPET Fast Forward. Published on May 15, 2006 as DOI: 10.1124/jpet.105.097568
This article has not been copyedited and formatted. The final version may differ from this version.



protein was used as the substrate for ERK1, ERK2 and Mapkap-K2; histone was used as the substrate for protein kinase A; and ATF-2 was used as the substrate for JNK1α1 and JNK2α2.

A time-resolved fluorescence assay was used to determine selectivity against Src, and ZAP-70. Briefly, the protein substrate, poly Glu-Tyr (25 μg/ml) for Src and cdb3 (10 μg/ml) for ZAP-70, was immobilized in black 384-well plates. Tyrosine kinases were preincubated with compounds for 10 min, and the reactions were initiated by adding ATP. The plates were incubated for 45 min at room temperature. The reaction mixes were removed, and the plates were washed twice with Tris buffered saline. Europium labeled anti-phosphotyrosine antibody (75 ng/ml) was added to the plates, and incubated for 1 h at room temperature. The plates were washed 5 times with Tris buffered saline. The signal from bound Europium labeled anti-phosphotyrosine antibody was measured using Victor (PerkinElmer) in the presence of enhancement solution.

A homogeneous time-resolved fluorescence assay was used to determine selectivity against Abl, and Tie2. Biotinylated Poly Glu-Ala-Tyr was used as the substrate. Kinases were preincubated with compounds for 10 min. The reaction was initiated by adding substrate solution containing biotinylated Poly Glu-Ala-Tyr and ATP. After incubation for 1h at room temperature, the reaction was terminated by adding stop/detection solution containing EDTA (3 mM final), streptavidin-Cy5 (1.5 μg/ml final) and Eu-PT66 (0.2 μg/ml final). The plate was read by Victor V (PerkinElmer) at both 615 nm and 665 nm. The signal is analyzed using the ratio of 665-nm to 615-nm fluorescence signal.

Downloaded from jpet.aspetjournals.org at ASPET Journals on March 20, 2018

JPET Fast Forward. Published on May 15, 2006 as DOI: 10.1124/jpet.105.097568
This article has not been copyedited and formatted. The final version may differ from this version.



Selectivity against PKBβ, RSK2 and CaMKIV was assessed using IMAP assays (Molecular Devices) according to the manufacturer's protocols.

Kinase assays for Aurora-A, BTK, CDK1/cyclinB, CHK1, CK1δ, CK2, CSK, EGFR, EphB4, FGFR3, Flt3, GSK3ß, Flt1, IGF-1R, IKKβ, KDR, Lck, Met, NEK2, PAK4, PDK1, PKCα, PKCθ, and ROCK-II were performed using the Kinase Profiler Assay Protocols from Upstate Biotechnology Inc (Charlottesville, VA) and validated with staurosporine.

Downloaded from jpet.aspetjournals.org at ASPET Journals on March 20, 2018

LPS-induced TNFα production in THP-1 cells

THP-1 cells were seeded in 96-well tissue culture plates. Test compounds or vehicle (1% DMSO final) were added to cells followed by addition of LPS (1 μg/ml final). Plates were incubated overnight at 37 $^{o}$C and 5% $CO_2$. TNFα in the medium was measured using a sandwich immunoassay. TNFα in the supernatant was immobilized by an anti-human TNF antibody (R&D, Minneapolis, MN, #MAB610) which was pre-coated in high-binding EIA plates. Immobilized TNFα was recognized by a biotinylated anti-human TNFα polyclonal antibody (R&D, #BAF210). Streptavidin conjugated to horseradish peroxidase was used in the ELISA, and the activity of peroxidase was quantified using a peroxide substrate kit (Pierce Biotechnology, Rockford, IL).

Cytotoxicity assay

Cytotoxicity was evaluated in THP-1 cells. THP-1 cells were harvested at log phase, re-suspended in fresh medium, and seeded in 96-well plates (30,000 cells/well).

JPET Fast Forward. Published on May 15, 2006 as DOI: 10.1124/jpet.105.097568
This article has not been copyedited and formatted. The final version may differ from this version.



Compounds were serially diluted and added to cells. After 48-h incubation cell viability was determined using the MTS reagent and assayed (Promega, Madison, WI) according to the manufacturer's instructions.

Mapkap-K2 immune complex kinase assay

THP-1 cells were treated as described above. Cells were lysed in RIPA buffer (50 mM Tris, pH 7.5, 1% NP-40, 0.1% sodium deoxycholate, 150 mM NaCl, 50 mM NaF, 1 mM sodium pyrophosphate, 1 mM sodium vanadate, 1 mM nitrophenylphosphate, 5 mM benzamidine, 0.2 μM calyculin A, 2 mM PMSF, 10 mg/ml aprotinin). Lysates were cleared by centrifugation (10,000 x g, 10 min) following incubation with antibody recognizing Mapkap-K2 for 1 h at 4 $^{o}$C, and protein G-Sepharose for an additional 2 h at 4 $^{o}$C. The precipitates were washed 3 times with cold wash buffer (0.25M Tris, pH 7.5, 0.1 M NaCl). The immune complexes were resuspended in 40 μl of kinase assay buffer containing 10 mM Tris, pH 7.5, 10 mM $MgCl_2$, 1 mM DTT, 10 μM ATP, 5 μCi [$\gamma$-$^{32}$P]ATP, and 2 μg hsp27, and incubated for 30 min at room temperature. Reactions were stopped by adding 40 μl of 2x sample buffer and boiling for 3 min. The samples were subjected to SDS-PAGE (12%), and the gels were dried. The dried gels were exposed to X-ray films.

Western blot analysis

Total cell lysates from equivalent cell numbers were separated using SDS-PAGE (10%) under reducing conditions. The proteins were transferred electrophoretically onto polyvinylidene fluoride membrane (Immobilon P, Millipore, Billerica, MA). The



Downloaded from jpet.aspetjournals.org at ASPET Journals on March 20, 2018

JPET Fast Forward. Published on May 15, 2006 as DOI: 10.1124/jpet.105.097568
This article has not been copyedited and formatted. The final version may differ from this version.



membranes were blocked with 1% BSA in PBS. The membranes were incubated first with primary antibody (1 μg/ml) in 1% BSA/PBS and then with secondary antibody conjugated with peroxidase in 5% non-fat dry milk/PBS. The immunocomplexes were detected using an enhanced chemiluminescence kit (Amersham Biosciences).

Inhibition of TNFα release in mice

Female BALB/c mice, weighing approximately 20 g, were used to evaluate PS200981 and PS166276 on TNFα release *in vivo*. PS200981 and PS166276 were dissolved in 5% ETOH, 5% Tween 80 and 90% water. Mice were dosed subcutaneously with PS200981, PS166276, PBS or vehicle 30 minutes prior to LPS challenge. LPS (0.2 ml of LPS suspended at 10 μg/ml in saline) was injected to mice intravenously. Blood samples were obtained 60 min after LPS injection and serum separated by centrifugation. Levels of TNFα were measured using an ELISA kit (R&D).

Synthesis of PS200981 and PS166276

The structures of PS200981 (3-(4-(1,4-diazepan-1-yl)-6-(((*1S,2R,5S*)-6,6-dimethylbicyclo[3.1.1]heptan-2-yl)methylamino)-1,3,5-triazin-2-ylamino)-4-methylbenzamide) and PS166276 ((*R*)-3-(4-(isobutyl(methyl)amino)-6-(pyrrolidin-3-ylamino)-1,3,5-triazin-2-ylamino)-4-methylbenzamide) are shown in Figure 1. A solution phase synthesis for this series of p38 inhibitors including PS200981 and PS166276 was described previously (Leftheris et al, 2004).



Downloaded from jpet.aspetjournals.org at ASPET Journals on March 20, 2018

JPET Fast Forward. Published on May 15, 2006 as DOI: 10.1124/jpet.105.097568
This article has not been copyedited and formatted. The final version may differ from this version.



## Results

Identification of p38α inhibitors

A total of 41 ECLiPS (Encoded Combinatorial Libraries on Polymeric Support) libraries, comprising 2.1 million compounds, were screened. Compounds were arrayed in plates at 10-20 compounds per well for the initial survey. Putative active libraries were then arrayed as a single compound per well at a 3-fold redundancy. The structures in the active wells were identified through a proprietary decoding process, followed by compound resynthesis to confirm activity (Diller and Hobbs, 2004).

A combinatorial library was found to contain numerous active compounds. This library contains ~180,000 members and was constructed through a linear, four combinatorial step synthesis (31 $R^1$ x 63 $R^2$ x 3 $R^3$ x 31 $R^4$) based on a triaminotriazine structure. The frequency of individual combinatorial synthons present within the hits is shown in Figure 2. Of 63 different $R^2$ synthons used in the construction of this combinatorial library, 63 out of 69 screening hits contains synthon $R^2$ 39, indicating an almost exclusive preference for synthon $R^2$ 39, which corresponds to a methylanilino amide. A somewhat broader preference is also evident within the $R^4$ components, while no preference is observed in the $R^1$ and $R^3$ components.

Inhibition of p38α and TNFα production by PS200981

PS200981, a triaminotriazine aniline amide compound that showed inhibition of p38α in the screen, was further tested in a panel of *in vitro* kinase and cell-based assays. Figure 3A shows the inhibition of p38α activity by PS200981 in a dose-dependent manner. The



Downloaded from jpet.aspetjournals.org at ASPET Journals on March 20, 2018

JPET Fast Forward. Published on May 15, 2006 as DOI: 10.1124/jpet.105.097568
This article has not been copyedited and formatted. The final version may differ from this version.



IC50 value was 1.0 μM in which the ATP concentration was 1 μM in the assay. Evaluation of the inhibition of TNFα production by PS200981 in cells was accomplished using LPS-induction of TNFα in human monocytic THP-1 cells. As seen in Figure 3B, PS200981 inhibited TNFα production with an IC50 value of 1.0 μM.

PS200981 is an ATP competitive inhibitor

Kinetic experiments were conducted to determine whether PS200981 was an ATP competitive inhibitor. The velocity (V) of p38α was determined in the presence of various concentrations of ATP in combination with different concentrations of PS200981. Figure 4 shows Lineweaver-Burke plots of the competition of PS200981 with ATP in p38α kinase activity. These data are consistent with a competitive mechanism of action for PS200981. The Ki of 1 μM is further consistent with the IC50 value of 1.0 μM assayed in the presence of 1 μM ATP. The value of Km[ATP] in this reaction is 27 μM, which is consistent with the results reported previously (Frantz, et al., 1998).

Kinase selectivity

The p38 family is one of three related kinase families, the others being ERK and JNK. Moreover, p38 kinase exists as four distinct isoforms (α, β, γ, and δ). Thus, a well-understood kinase selectivity profile is essential for use of p38 kinase inhibitors in chronic diseases. To this end, the kinase selectivity of the hit series was examined against a variety of recombinant kinases. PS200981 exhibits equipotent inhibitory activity against α and β isoforms, and no activity against the γ isoform (Table 1). It is of interest to note that several structurally diverse p38 kinase inhibitors, such as VX-745,

Downloaded from jpet.aspetjournals.org at ASPET Journals on March 20, 2018

JPET Fast Forward. Published on May 15, 2006 as DOI: 10.1124/jpet.105.097568
This article has not been copyedited and formatted. The final version may differ from this version.



SB203580, RWJ67657, BIRB-796, RPR200276A, inhibit the p38α and p38β isoforms but not the γ and δ isoforms (Pargellis, et al., 2002; Natarajan, et al., 2003; Kumar, et al., 1997; Wadsworth, et al., 1999; McLay, et al., 2001). As shown in Table 1, neither PS200981 or PS166276 inhibited other MAP kinase family members, such as ERK1, ERK2, JNK1α1, and JNK2α2. Furthermore, these compounds showed greater than 50-fold selectivity versus 31 other kinases including Mapkap K2, a down stream kinase of p38, while it only weakly inhibited Abl and Tie2.

In order to assess kinase selectivity at the cellular level, PS166276 (Figure 1), which has the same critical triaminotriazine aniline amide substitution at $R^2$, but has different $R^1$ and $R^3$ groups as compared with PS200981, was used. PS166276 has an IC50 of 28 nM at p38 kinase and 170 nM in the THP-1 TNFα assay (Figure 3). Similar to PS200981, PS166276 is also highly selective for p38α and β; it is greater than 1,000-fold selective versus a panel of 38 kinases (Table 1). Although PS200981 and PS166276 are structurally similar, both compounds exhibit different cytotoxicity. In THP-1 cells, PS200981 was cytotoxic at concentrations greater than 10 μM, while PS166276 showed no cytotoxicity up to 100 μM, the highest concentration used in the assay (data not shown). Because PS166276 is highly structurally related, 35-fold more potent, and 10-fold less cytotoxic than PS200981, it was more suitable for assessing kinase selectivity in intact cells. These assays monitored the effects of PS166276 on kinase-mediated signal transduction cascades, including the MAP kinase pathways (p38, ERK, JNK), IKK, and Jak/Stat pathways.

Downloaded from jpet.aspetjournals.org at ASPET Journals on March 20, 2018

JPET Fast Forward. Published on May 15, 2006 as DOI: 10.1124/jpet.105.097568
This article has not been copyedited and formatted. The final version may differ from this version.



The serine/threonine kinase Mapkap K2, a downstream kinase of p38, is phosphorylated and activated by p38 (Kotlyarov et al., 1999). Thus, inhibition of p38-mediated signal transduction was evaluated by examining the effects of compound treatment on Mapkap K2 activation by p38. THP-1 human monocytic cells were pretreated with PS166276 prior to challenge with LPS. Mapkap K2 was immunoprecipitated from cell lysates using an antibody. Kinase activity of Mapkap K2 in the immunocomplexes was determined using hsp27 as a substrate. LPS robustly increased Mapkap K2 activity, which was suppressed in a dose-dependent manner by PS166276 (Figure 5A). Higher compound concentrations (10 and 3 μM) completely abrogated Mapkap K2 activation, while at 1 and 0.3 μM, partial inhibition was observed. An IC50 value of 200 nM was estimated which is consistent with the potency observed in the LPS-induced TNF-α production assay (Figure 3). PS166276 did not inhibit Mapkap K2 kinase activity when added directly to immunoprecipitated MK2 (data not shown).

Effects on the ERK signal transduction pathway were examined by measuring ERK phosphorylation in phorbol ester-treated THP-1 cells. O-tetradecanoyl-phorbol 13-acetate (TPA) was used to stimulate ERK-1/ERK-2 via PKC, Raf and MKK1/2. The activation of ERKs is accompanied by phosphorylation of Thr202 and Tyr204, which was detected by immunoblot using an anti-phospho-ERK antibody. Significant activation was induced by TPA, which could be inhibited by the MKK1/2 inhibitor PD98059 (Figure 5D). PS166276 had no effect at concentrations of up to 30 μM, indicating that the compound did not inhibit upstream components, including protein kinase C, Raf and MKK1/2, in the TPA-induced ERK pathway.

Downloaded from jpet.aspetjournals.org at ASPET Journals on March 20, 2018

JPET Fast Forward. Published on May 15, 2006 as DOI: 10.1124/jpet.105.097568
This article has not been copyedited and formatted. The final version may differ from this version.



Similar to the p38 kinase pathway, JNK is activated by cellular stress via a signal transduction cascade that involves MKK4/7. Phosphorylation of JNK at Thr183 and Tyr185 results in catalytic activation. The substrates for JNK2 include transcription factors such as c-Jun and ATF-2. LPS treatment of THP-1 cells resulted in JNK activation, which was detected as phosphorylation of ATF-2 using an anti-phospho-ATF-2 antibody (Figure 5C). PS166276 had no effect on ATF-2 phosphorylation at concentrations of up to 30 μM, suggesting that the compound does not interfere with JNK signaling components.

The IKK pathway can be activated by endotoxin or pro-inflammatory cytokines. The IKK isozymes activate NF-kB-mediated signal transduction by phosphorylation of IκBα, which subsequently undergoes proteolytic degradation. Degradation of IκBα was monitored by immunoblot to determine the effects of PS166276 on the IKK pathway. As shown in Figure 5B, LPS promptly induced degradation of IκBα in THP-1 cells. No effect was observed at compound concentrations of up to 30 μM, indicating that the compound effects on TNF-α and the expression of other proinflammatory cytokines are not mediated through IKKβ inhibition. Similar results were observed in the TNFα-stimulated IκBα degradation in U937 cells (data not shown).

Similar analyses were applied to the Jak2 and Jak3 pathways using the mouse F7 pre-B lymphocyte cell line. These cells are dually-responsive to interleukin-2 (Jak1/3-dpendent) and interleukin-3 (Jak2-dependent). Following interleukin-2 or interleukin-3



Downloaded from jpet.aspetjournals.org at ASPET Journals on March 20, 2018

JPET Fast Forward. Published on May 15, 2006 as DOI: 10.1124/jpet.105.097568
This article has not been copyedited and formatted. The final version may differ from this version.



binding to its corresponding receptor, Jak2/3 kinases are activated by phosphorylation and are capable of catalyzing phosphoryl transfer to Stat5. The effects of PS166276 on Stat5 phosphorylation are shown in Figures 5E and 5F. PS166276 had no effect on the interleukin-2 and interleukin-3-induced Stat5 phosphorylation. Taken together, these results indicate that this series of compounds is highly selective for p38α versus other protein kinases.

PS200981 and PS166276 inhibit LPS-induced TNFα production in mice

Although PS200981 was identified directly from high throughput screening, its efficacy was demonstrated *in vivo*. Because PS200981 has low cellular permeability (data not shown), suggesting low oral absorption in animals, compounds were administered to mice subcutaneously. As seen in Figure 6A, PS200981, at 30 mg/kg, significantly inhibits LPS-induced TNFα production (45%) in comparison with the vehicle-treated group. PS166276, a 6-fold more potent compound as compared with PS200981 in the cellular assay, was also tested in the same model. Figure 6B demonstrates that PS166276 is more efficacious than PS200981 *in vivo*. PS166276, at 30 mg/kg, inhibits 81% of LPS-induced TNFα production versus the vehicle-treated group. No obvious toxicity was observed in PS200981 or PS166276 treated animals.

Downloaded from jpet.aspetjournals.org at ASPET Journals on March 20, 2018

JPET Fast Forward. Published on May 15, 2006 as DOI: 10.1124/jpet.105.097568
This article has not been copyedited and formatted. The final version may differ from this version.



## Discussion

Biological agents that act as TNFα sequestrants or inhibit the action of interleukin-1 have demonstrated efficacy as therapeutics in the treatment of chronic inflammatory diseases e.g. rheumatoid arthritis, and Crohn's disease. Although efficacious, the cost and route of administration of these biologicals are disadvantageous to patients and health care systems compared to a small molecule p38 inhibitor that can be administered orally if one can be identified and advanced. We report here the identification of a triaminotriazine aniline amide series of potent and selective p38 inhibitors from a collection of large combinatorial libraries. Forty-one combinatorial libraries averaging 50,000 members per library were screened. An active triaminotriazine library was identified. A strong preference for the aniline amide was observed from the screening data. This library has been screened at 25 other kinases and although subsets of this library have produced inhibitors of other kinases, the observed preference for the methylanilino amide is unique to p38α kinase. Other related substituents such as 3-amino benzamide and 2-amino-4-methyl benzamide were contained in the library but not found among the p38 inhibitors.

With one exception, all known p38α inhibitors bind to the ATP binding pocket and inhibit the kinase by directly competing with the binding of ATP. Uniquely, the p38 inhibitor, BIRB-796, binds to a kinase specificity pocket and to the ATP-binding site. This dual binding mode results in a conformational change in p38α and slow association kinetics of binding (Pargellis, et al., 2002). Kinetics studies have indicated that the current class of p38α inhibitors, like most other p38 inhibitors, competes with the binding of ATP.

Downloaded from jpet.aspetjournals.org at ASPET Journals on March 20, 2018

JPET Fast Forward. Published on May 15, 2006 as DOI: 10.1124/jpet.105.097568
This article has not been copyedited and formatted. The final version may differ from this version.



Over their entire kinase domains p38α and p38β have 83% sequence identity. Within the ATP binding site the two isoforms are nearly identical. P38α and β are identical within the main hydrophobic pocket including the key gate keeper residue for which they both have a Thr (Thr106 of p38α). It is noteworthy that p38γ, p38δ and most of the JNK and ERK kinases have a Met at the gate keeper position. The key gate keeper residue has been shown to be a critical determinant in kinase selectivity. This may explain the observation that all known p38α inhibitors, including our new series of p38α inhibitors, have limited selectivity between p38α and p38β, while showing good selectivity against p38γ and other protein kinases.

Mice null for p38α allele die during early embryonic development. The development arrest suggests that the different p38 isozymes do not perform redundant activities, at least during embryonic development. $p38\alpha^{-/-}$ embryonic stem cells fail to activate Mapkap K2 in response to chemical stress inducers, and generate minimal levels of interleukin-6 in response to interleukin-1, despite the fact that $p38\alpha^{-/-}$ embryonic stem cells express three other p38 kinases (Allen, et al., 2000). These results, together with pharmacological studies using specific p38α inhibitors (Adams et al., 2001), indicate that p38α is the key p38 kinase involved in the inflammatory response. Evidence from p38α inhibitors which have limited selectivity against p38β and which show no obvious adverse effects in animal tests and clinical trials suggests that some activity against p38β is also tolerated (Kumar et al., 2003).

Downloaded from jpet.aspetjournals.org at ASPET Journals on March 20, 2018

JPET Fast Forward. Published on May 15, 2006 as DOI: 10.1124/jpet.105.097568
This article has not been copyedited and formatted. The final version may differ from this version.



PS200981, a p38α inhibitor identified from high throughput screening, shows inhibition of p38α kinase and p38α-mediated cellular assays (Figure 3). This compound, and a highly related more potent analog PS166276, exhibit > 25-fold selectivity against a panel of non-p38 kinases (Table 1). Furthermore, PS200981 and PS166276 were tested *in vivo* and inhibited ~50 – 80 % of the LPS-induced TNFα production in mice (Figure 6A and 6B). These results indicate that the initial p38 hits, exemplified by PS200981, as well as early analogs such as PS166276, have suitable characteristics for further optimization and study.

PS166276, a highly related analogue of PS200981, was used to further assess selectivity by analyzing cellular events. PS166276 did not inhibit several distinct signal transduction pathways, which includes the signaling cascades that lead to phosphorylation of ERK1/2, ATF-2, Stat5, and degradation of IκBα. Each of these signaling cascades consists of multiple kinases and other signaling proteins that lead to the components that are analyzed by Western blotting (Figure 5). A total of at least 20 protein kinases are involved in these signaling pathways. Furthermore, PS166276 was not cytotoxic up to 100 μM indicating that the actions of PS166276 are not due to toxicity or pleiotropic effects.

In contrast to PS200981 and PS166276, members of the current triaminotriazine aniline amides, members of the original series of triaryl-imidazoles such as SB203580, inhibit JNK2 (data not shown), and have been shown to be inhibitors of a number of other kinases including JNK3, CSNK1E, GAK, RICK, and CK1δ (Fabian, et al, 2005; Godl, et



Downloaded from jpet.aspetjournals.org at ASPET Journals on March 20, 2018

JPET Fast Forward. Published on May 15, 2006 as DOI: 10.1124/jpet.105.097568
This article has not been copyedited and formatted. The final version may differ from this version.



al, 2003). It has also been observed that several known p38 inhibitors are more potent inhibitors of TNFα expression in human peripheral blood mononuclear cells than would otherwise be expected given their p38 kinase IC50 values (Diller, et al, 2005). This includes members of the triaryl-imidazole class of p38 inhibitors such as RWJ68354. Thus, there is reason to believe that the observed inhibition of TNFα expression is due to the inhibition of something other than their activity at p38. In contrast, the good correspondence between kinase, cellular, and in vivo data observed for the current compounds is consistent with limited off target effects, and is likely due to the strong preference for the methylanilino amide synthon in this series.

In conclusion, we have identified a novel class of p38α kinase inhibitors that block *in vitro* p38 kinase, p38 cell-based activity, *in vivo* activity, and which as exemplars of screening hits (PS200981) and early analogs (PS166276) are highly selective against other kinases. Moreover, p38 kinase shows a strong synthon preference for the methylanilino amide at $R^2$. This preferred methylanilino amide is a key component in the structure-activity of these compounds and is deemed critical for the selectivity profile observed to date versus other kinases.

Downloaded from jpet.aspetjournals.org at ASPET Journals on March 20, 2018

JPET Fast Forward. Published on May 15, 2006 as DOI: 10.1124/jpet.105.097568
This article has not been copyedited and formatted. The final version may differ from this version.



## Acknowledgements

We thank Ms. Vera Swensen for skilled preparation of the manuscript.

Downloaded from jpet.aspetjournals.org at ASPET Journals on March 20, 2018

JPET Fast Forward. Published on May 15, 2006 as DOI: 10.1124/jpet.105.097568
This article has not been copyedited and formatted. The final version may differ from this version.



## References

Adams JL, Badger AM, Kumar S and Lee JC (2001) p38 MAP kinase: molecular target for the inhibition of proinflammatory cytokines. *Prog Med Chem* **38**:1-60.

Allen M, Svensson L, Roach M, Hambor J, McNeish J and Gabel CA (2000) Deficiency of the stress kinase p38α results in embryonic lethality: characterization of the kinase dependence of stress responses of enzyme-deficient embryonic stem cells. *J Exp Med* **191**:859-869.

Cirillo PF, Pargellis C and Regan J (2002) The non-diaryl heterocycle classes of p38 MAP kinase inhibitors. *Curr Top Med Chem* **2**:1021-1035.

Cvetkovic RS and Keating G (2002) Anakinra. *BioDrugs* **16**:303-311.

Diller DJ and Hobbs DW (2004) Deriving knowledge through data mining high-throughput screening data. *J Med Chem* **47**:6373-6383.

Diller DJ, Lin TH and Metzger A (2005) The discovery of novel chemotypes of p38 inhibitors. *Curr Top Med Chem* **5**:953-965.

Fabian MA, Biggs WH, Treiber DK, Atteridge CE, Azimioara MD, Benedetti MG, Carter TA, Ciceri P, Edeen PT, Floyd M, Ford JM, Galvin M, Gerlach JL, Grotzfeld RM, Herrgard S, Insko DE, Insko MA, Lai AG, Lelias JM, Mehta SA, Milanov ZV, Velasco

Downloaded from jpet.aspetjournals.org at ASPET Journals on March 20, 2018

JPET Fast Forward. Published on May 15, 2006 as DOI: 10.1124/jpet.105.097568
This article has not been copyedited and formatted. The final version may differ from this version.



AM, Wodicka LM, Patel HK, Zarrinkar PP and Lockhart D J (2005) A small molecule-kinase interaction map for clinical kinase inhibitors. *Nat Biotechnol* **23**:329-336.

Frantz B, Klatt T, Pang M, Parsons J, Rolando A, Williams H, Tocci MJ, O'Keefe SJ and O'Neill EA (1998) The activation state of p38 mitogen-activated protein kinase determines the efficiency of ATP competition for pyridinylimidazole inhibitor binding. *Biochem* **37**:13846-13853.

Furst DE, Schiff MH, Fleischmann RM, Strand V, Birbara CA, Compagnone D, Fischkoff SA and Chartash EK (2003) Adalimumab, a fully human anti-tumor necrosis factor-a monoclonal antibody, and concomitant standard antirheumatic therapy for the treatment of rheumatoid arthritis: results of STAR (Safety Trial of Adalimumab in Rheumatoid arthritis*). J Rheumatol* **30**:2563-2571.

Godl K, Wissing J, Kurtenbach A, Habenberger P, Blencke S, Gutbrod H, Salassidis K, Stein-gerlach M, Missio A, Cotten M and Daub H (2003) An efficient proteomics method to identify the cellular targets of protein kinase inhibitors. *Proc Natl Acad Sci USA* **100**:15434-15439.

Goedert M, Cuenda A, Craxton M, Jakes R and Cohen P (1997) Activation of the novel stress-activated protein kinase SAPK4 by cytokines and cellular stresses is mediated by SKK3 (MKK6); comparison of its substrate specificity with that of other SAP kinases. *EMBO J* **16**:3563-3571.

Downloaded from jpet.aspetjournals.org at ASPET Journals on March 20, 2018

JPET Fast Forward. Published on May 15, 2006 as DOI: 10.1124/jpet.105.097568
This article has not been copyedited and formatted. The final version may differ from this version.



Hatakeyama M, Mori H, Doi T and Taniguchi T (1989) A restricted cytoplasmic region of IL-2 receptor beta chain is essential for growth signal transduction but not for ligand binding and internalization. *Cell* **59**:837-845.

Henry JR, Cavender DE and Wadsworth SA (1999) p38 mitogen-activated protein kinase as a target for drug discovery. *Drugs Future* **24**:1345-1354.

Jackson PF and Bullington JL (2002) Pyridinylimidazole based p38 MAP kinase inhibitors. *Curr Top Med Chem* **2**:1011-1020.

Jarvis B and Faulds D (1999) Etanercept: A review of its use in rheumatoid arthritis. *Drugs* **57**:945-966.

Kotlyarov A, Neininger A, Schubert C, Eckert R, Birchmeier C, Volk HD and Gaestel M (1999) MAPKAP kinase 2 is essential for LPS-induced TNF-a biosynthesis. *Nature Cell Biol* **1**:94-97.

Kumar S, Boehm J and Lee JC (2003) p38 MAP kinases: key signalling molecules as therapeutic targets for inflammatory diseases. *Nature Rev Drug Discov* **2**:717-726.

Kumar S, McDonnell PC, Gum RJ, Hand AT, Lee JC and Young PR (1997) Novel homologs of CSBP/p38 MAP kinase: activation, substrate specificity and sensitivity to inhibition by pyridinyl imidazoles. *Biochem Biophys Res Commun* **235**:533-538.

Downloaded from jpet.aspetjournals.org at ASPET Journals on March 20, 2018

JPET Fast Forward. Published on May 15, 2006 as DOI: 10.1124/jpet.105.097568
This article has not been copyedited and formatted. The final version may differ from this version.



Lee JC, Laydon JT, McDonnell PC, Gallagher TF, Kumar S, Green D, McNulty D, Blumenthal MJ, Heys JR and Landvatter SW (1994) A protein kinase involved in the regulation of inflammatory cytokine biosynthesis. *Nature* **372**:739-746.

Leftheris K, Ahmed G, Chan R, Dyckman AJ, Hussain Z, Ho K, Hynes J Jr, Letourneau J, Li W, Lin S, Lin TH, Metzger A, Moriarty KJ, Riviello C, Shimshock Y, Wen J, Wityak J, Wrobleski ST, Wu H, Wu J, Desai M, Gillooly KM, Loo D, McIntyre KW, Pitt S, Shen DR, Shuste DJr, Zhang R, Diller D, Doweyko A, Sack J, Baldwin J, Barrish J, Dodd J, Henderson I, Kanner S, Schieven GL and Webb M (2004) The discovery of orally active triaminotriazine aniline amides as inhibitors of p38 MAP kinase. *J Med Chem* **47**:6283-6291.

Downloaded from jpet.aspetjournals.org at ASPET Journals on March 20, 2018

JPET Fast Forward. Published on May 15, 2006 as DOI: 10.1124/jpet.105.097568
This article has not been copyedited and formatted. The final version may differ from this version.



McLay IM, Halley F, Souness JE, McKenna J, Benning V, Birrell M, Burton B, Belvisi M, Collis A, Constan A, Foster M, Hele D, Jayyosi Z, Kelley M, Maslen C, Miller G, Ouldelhkim MC, Page K, Phipps S, Pollock K, Porter B, Ratcliffe AJ, Redford EJ, Webber S, Slater B, Thybaud V and Wilsher N (2001) The discovery of RPR200765A, a p38 MAP kinase inhibitor displaying a good oral anti-arthritic efficacy. *Bioorg Med Chem* **9**:537-554.

Natarajan SR, Wisnoski DD, Singh SB, Stelmach JE, O'Neill EA, Schwartz CD, Thompson CM, Fitzgerald CE, O'Keefe SJ, Kumar S, Hop CECA, Zaller DM, Schmatz DM and Doherty JB (2003) p38 MAP kinase inhibitors. Part 1: design and development of a new class of potent and highly selective inhibitors based on 3,4-dihydropyrido[3,2-d]pyrimidone scaffold. *Bioorg Med Chem Lett* **13**:273-276.

Onrust SV and Lamb HM (1998) Infliximab: A review of its use in Crohn's disease and rheumatoid arthritis. *BioDrugs* **10**:397-422.

Pargellis C, Tong L, Churchill L, Cirillo PF, Gilmore T, Graham AG, Grob PM, Hickey ER, Moss N, Pav S and Regan J (2002) Inhibition of p38 MAP kinase by utilizing a novel allosteric binding site. *Nat Struct Biol* **9**:268-272.

Pearson G, Robinson F, Gibson TB, Xu BE, Karandikar M, Berman K and Cobb MH (2001) Mitogen-activated protein (MAP) kinase pathways: Regulation and physiological functions. *Endocr Rev* **22**:153-183.

Downloaded from jpet.aspetjournals.org at ASPET Journals on March 20, 2018

JPET Fast Forward. Published on May 15, 2006 as DOI: 10.1124/jpet.105.097568
This article has not been copyedited and formatted. The final version may differ from this version.



Raingeaud J, Whitmarsh AJ, Barrett T, Derijard B and Davis RJ (1996) MKK3- and MKK6-regulated gene expression is mediated by the p38 mitogen-activated protein kinase signal transduction pathway. *Mol Cell Biol* **16**:1247-1255.

Salituro FG, Germann UA, Wilson KP, Bemis GW, Fox T and Su MS-S (1999) Inhibitors of p38 MAP kinase: therapeutic intervention in cytokine-mediated diseases. *Curr Med Chem* **6**:807-823.

Wadsworth SA, Cavender DE, Beers SA, Lalan P, Schafer PH, Malloy EA, Wu W, Fahmy B, Olini GC, Davis JE, Pellegrino-Gensey JL, Wachter MP and Siekierka JJ (1999) RWJ 67657, a potent, orally active inhibitor of p38 mitogen-activated protein kinase. *J Pharm Exp Ther* **29**:680-687.

Wang XS, Diener K, Manthey CL, Wang SW, Rosenzweig B, Bray J, Delaney J, Cole CN, Chan-Hui PY, Mantlo N, Lichenstein HS, Zukowski M and Yao Z (1997) Molecular cloning and characterization of a novel p38 mitogen-activated protein kinase. *J Biol Chem* **272**:23668-23674.

Downloaded from jpet.aspetjournals.org at ASPET Journals on March 20, 2018

JPET Fast Forward. Published on May 15, 2006 as DOI: 10.1124/jpet.105.097568
This article has not been copyedited and formatted. The final version may differ from this version.



## Legends for Figures

**Fig. 1. Structures of PS200981 and PS166276.** The $R^1$, $R^2$, $R^3$ and $R^4$ positions are designated for the screening hit PS200981.

**Fig. 2. Synthon frequency plot of p38 active library.** The active library is constructed using a linear four combinatorial step synthesis (31 $R^1$ x 63 $R^2$ x 3 $R^3$ x 31 $R^4$). Each compound in this library contains four synthons. A total of 69 related hits, including PS200981, were identified from screening this particular library. The frequency of individual combinatorial synthons for the 69 hits was plotted.

**Fig. 3. Inhibition of p38α, and LPS-induced TNFα production in THP-1 cells by PS200981 and PS166276. A.** Various concentrations of PS200981 or PS166276 were included in the p38α kinase assay as described in **Methods**. Each compound dilution was tested in duplicate. The average IC50 values $\pm$ S.D are 1.0 $\pm$ 0.2 μM (n=11) and 0.028 $\pm$ 0.008 μM (n=8) for PS200981 and PS166276, respectively. **B.** PS200981 or PS166276 was added to THP-1 cells prior to LPS challenge. Cells were incubated for 18 h. TNFα in the medium was measured using an ELISA assay. Each compound dilution was tested in duplicate. The average IC50 values $\pm$ S.D are 1.0 $\pm$ 0.3 μM (n=5) and 0.17 + 0.08 μM (n=4) for PS200981 and PS166276, respectively.

**Fig. 4. Lineweaver-Burke plots of the competition of PS200981 with ATP in p38α kinase activity.** Different concentrations of PS200981 were added to p38α kinase assay



Downloaded from jpet.aspetjournals.org at ASPET Journals on March 20, 2018

JPET Fast Forward. Published on May 15, 2006 as DOI: 10.1124/jpet.105.097568
This article has not been copyedited and formatted. The final version may differ from this version.



in combination with various concentrations of ATP. The enzyme velocity (V) was calculated under each condition. The 1/V versus 1/[ATP] was plotted.

**Fig. 5. Selectivity of PS166276 in cell-based assays.**

THP-1 cells were pretreated with PS166276 for 30 min prior to the induction by LPS (1 μg/ml) for an additional 15 min. **A**. Cellular Mapkap K2 was immunoprecipitated, and its kinase was measured according to **Methods**. **B**. The degradation of IκBα was analyzed by anti-IκBα immunoblot. **C**. ATF-2 phosphorylation was visualized by anti-phospho-ATF-2 immunoblotting. **D**. THP-1 cells were treated with PS166276 or PD98059 for 15 min followed by incubating with TPA (10 ng/mg) for an additional 15 min. ERK phosphorylation was detected by immunoblot using an anti-phospho-ERK antibody. **E**&**F**. F7 cells were pretreated with PS166276 for 30 min. Cells were stimulated by interleukin-2 (100 ng/ml) or interleukin-3 (1 ng/ml). Stat5 phosphorylation was detected by anti-phospho-Stat5 immunoblot.

**Fig. 6. Inhibition of LPS-induced TNFα production in mice.**

Female Balb/c mice were pretreated with PBS, vehicle, PS200981 or PS166276 with dosages as indicated, by subcutaneous injection. Thirty minutes after pretreatment, the mice were challenged with LPS. Sixty minutes later, the mice were sacrificed, and blood samples were collected. Levels of TNFα in serum were detected by ELISA assay. **A**. Each group has three animals. * = $p < 0.1$ vs PBS and vehicle, Student's *t* test. **B**. Each group has eight animals. ** = $p < 0.001$ vs vehicle, Student's *t* test.

Downloaded from jpet.aspetjournals.org at ASPET Journals on March 20, 2018

JPET Fast Forward. Published on May 15, 2006 as DOI: 10.1124/jpet.105.097568
This article has not been copyedited and formatted. The final version may differ from this version.



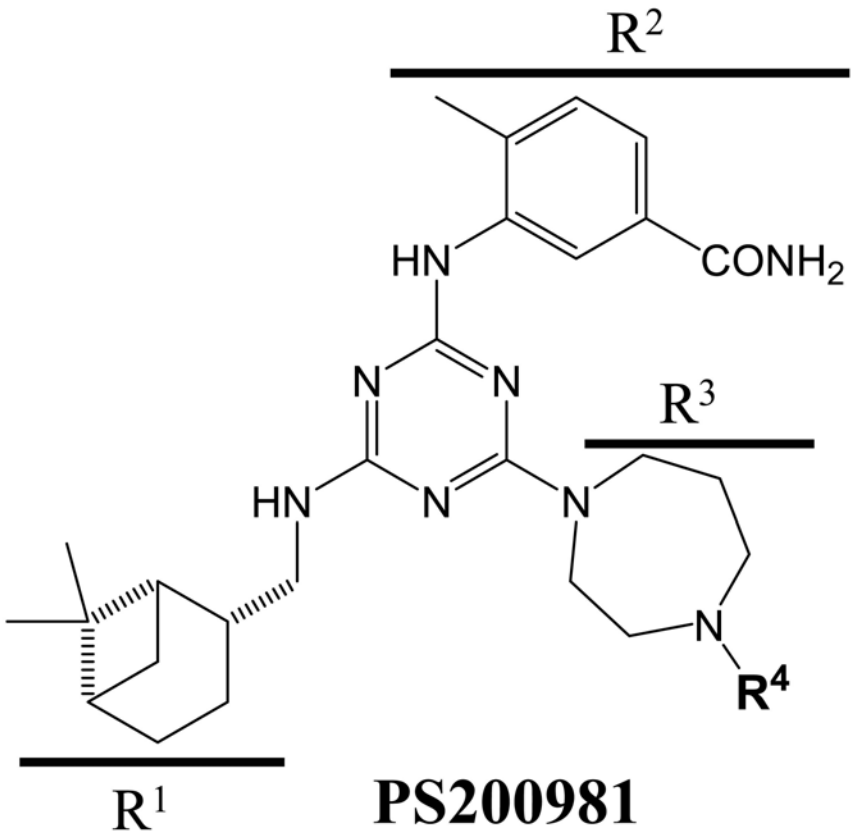
**Table 1. Activity of PS200981 and PS166276 at a panel of kinases.**

A panel of kinases was evaluated for activity of PS200981 and PS166276 at ≤ Km [ATP]. IC50 values are in μM.

| Kinase | PS200981 | PS166276 | Kinase | PS200981 | PS166276 |
|---|---|---|---|---|---|
| P38α | 1.0 | 0.028 | IGF-1R | >50 | >50 |
| P38β | 1.1 | 0.014 | IKKβ | >50 | >50 |
| P38γ | >100 | >100 | JNK1α1 | >50 | >50 |
| Abl | 25 | >50 | JNK2α2 | >50 | >50 |
| Aurora-A | >50 | >50 | KDR | >50 | >50 |
| BTK | >50 | >50 | Lck | >50 | >50 |
| CaMKIV | >50 | >50 | Mapkap K2 | >50 | >50 |
| CDK1/cyclinB | >50 | >50 | Met | >50 | >50 |
| CHK1 | >50 | >50 | NEK2 | >50 | >50 |
| CK1δ | >50 | >50 | PAK4 | >50 | >50 |
| CK2 | >50 | >50 | PDK1 | >50 | >50 |
| CSK | >50 | 32 | PKA | >50 | >50 |
| EGFR | >50 | >50 | PKBβ | >50 | >50 |
| EphB4 | >50 | >50 | PKCα | >50 | >50 |
| ERK1 | >50 | >50 | PKCθ | >50 | >50 |
| ERK2 | >50 | >50 | ROCK-II | >50 | >50 |
| FGFR3 | >50 | >50 | Rsk2 | >50 | >50 |
| Flt3 | >50 | >50 | Src | >50 | >50 |
| GSK3ß | >50 | >50 | Tie2 | 37 | >50 |
| Flt1 | >50 | >50 | Zap-70 | >50 | >50 |

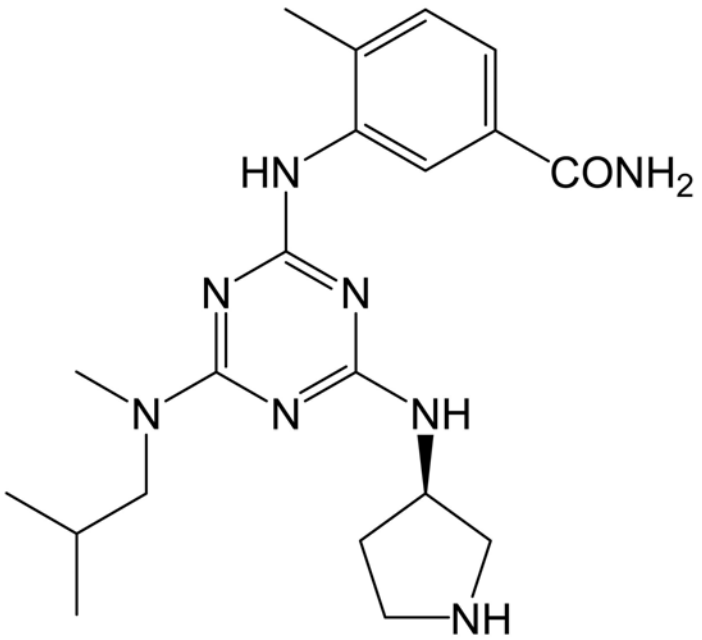
Downloaded from jpet.aspetjournals.org at ASPET Journals on March 20, 2018

# Figure 1




**PS200981**

**$R^4 = H$**

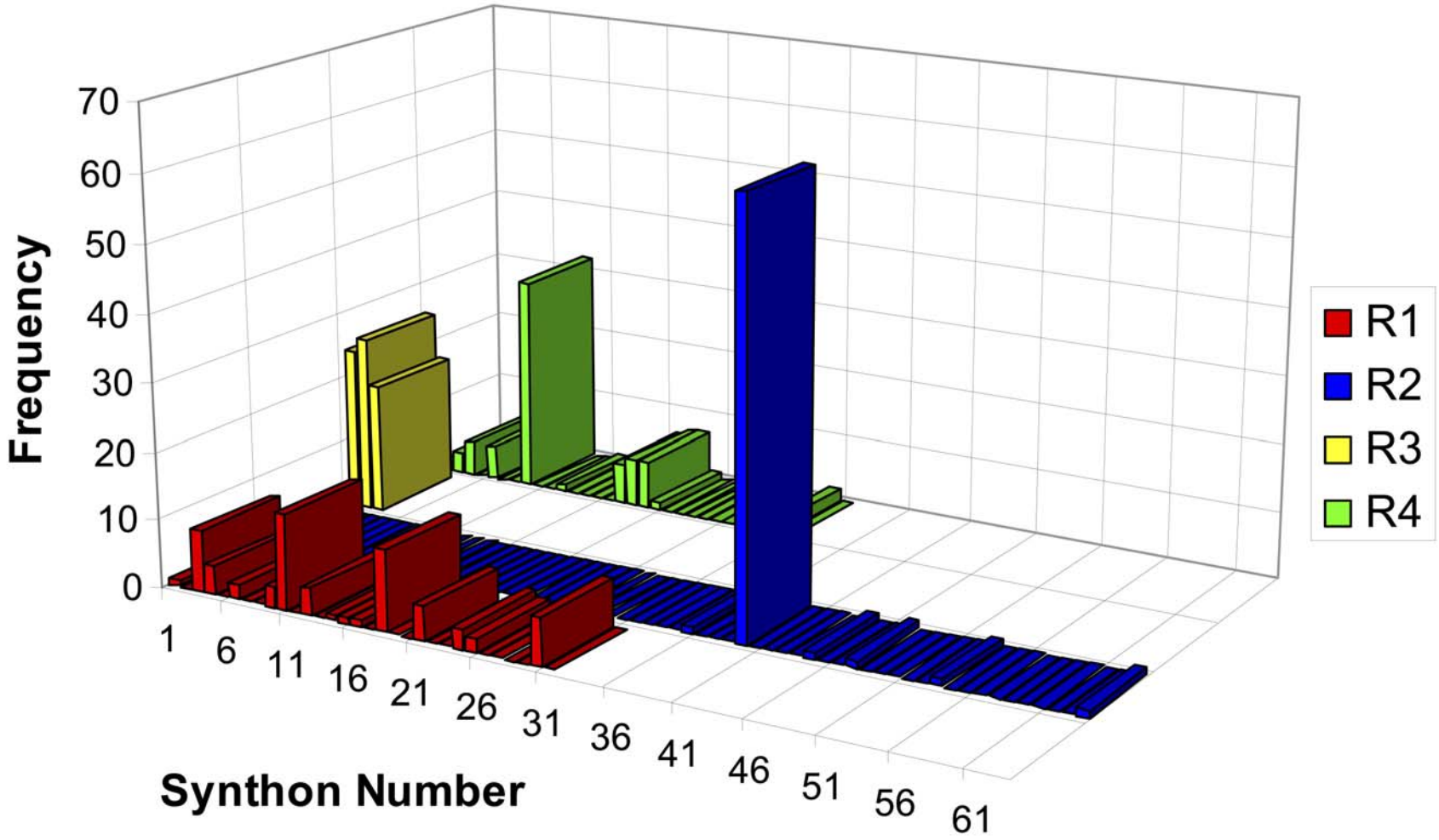



**PS166276**

JPET Fast Forward. Published on May 15, 2006 as DOI: 10.1124/jpet.105.097568
This article has not been copyedited and formatted. The final version may differ from this version.

Downloaded from jpet.aspetjournals.org at ASPET Journals on March 20, 2018

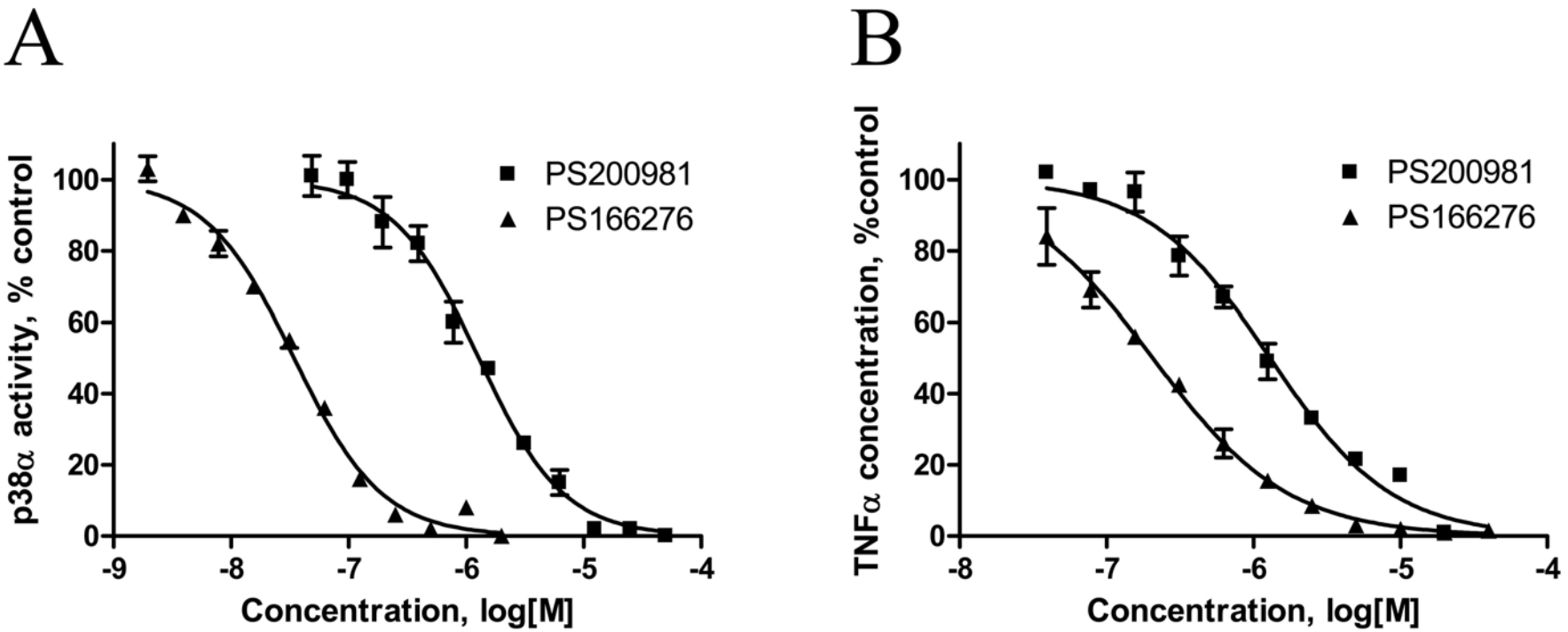
# Figure 2




JPET Fast Forward. Published on May 15, 2006 as DOI: 10.1124/jpet.105.097568
This article has not been copyedited and formatted. The final version may differ from this version.

Downloaded from jpet.aspetjournals.org at ASPET Journals on March 20, 2018

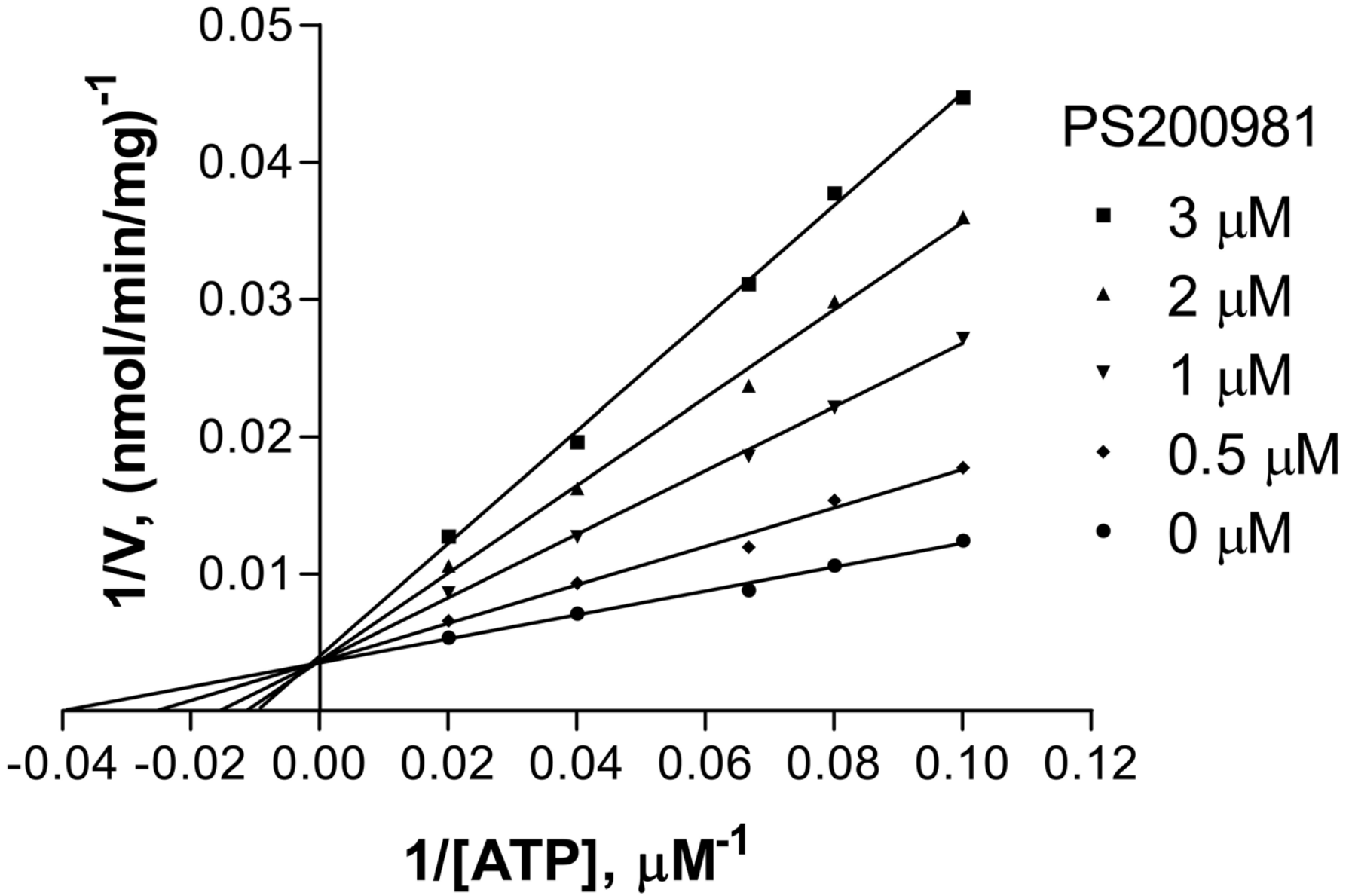
# Figure 3




JPET Fast Forward. Published on May 15, 2006 as DOI: 10.1124/jpet.105.097568
This article has not been copyedited and formatted. The final version may differ from this version.

Downloaded from jpet.aspetjournals.org at ASPET Journals on March 20, 2018

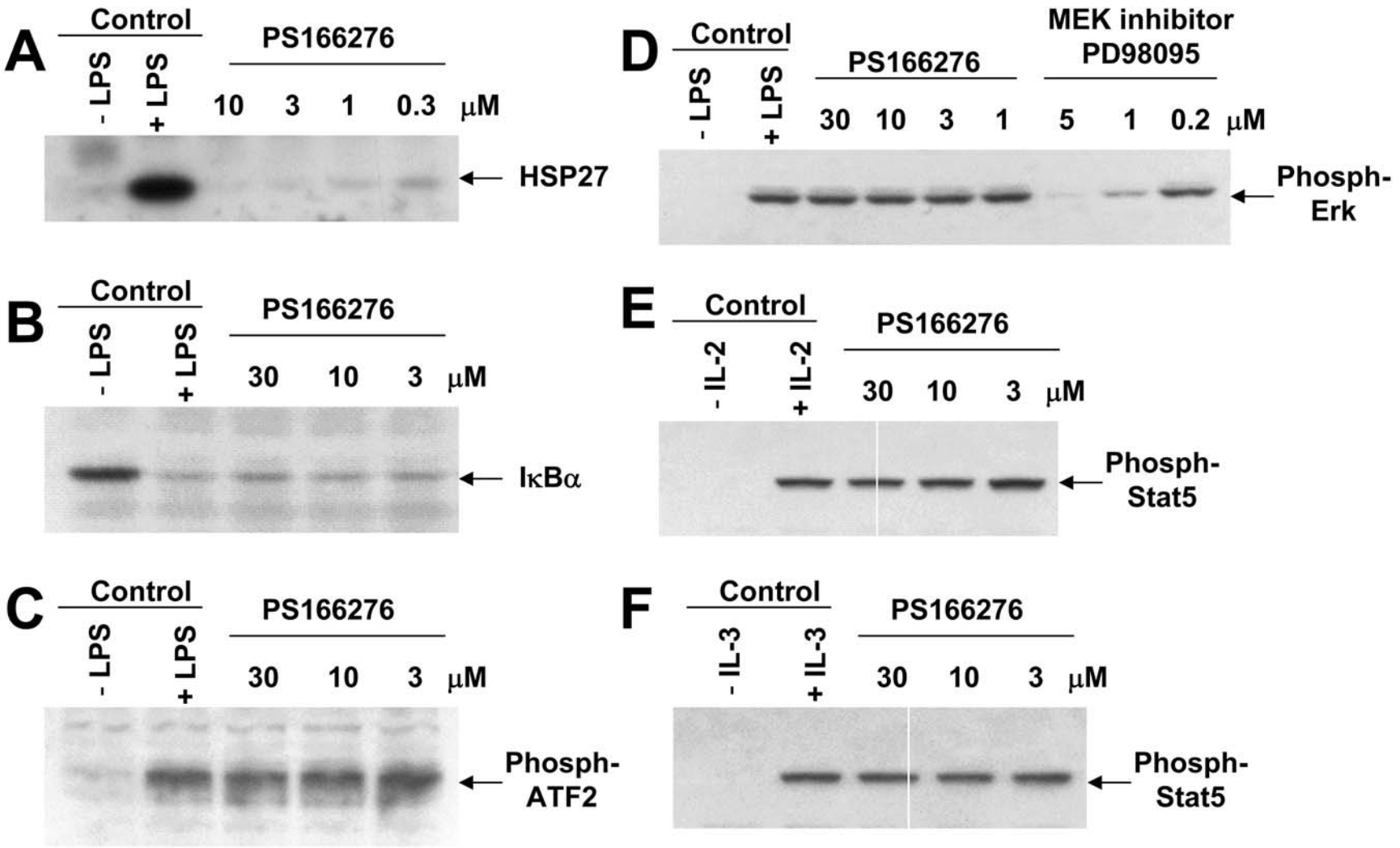
Figure 4




JPET Fast Forward. Published on May 15, 2006 as DOI: 10.1124/jpet.105.097568
This article has not been copyedited and formatted. The final version may differ from this version.

Downloaded from jpet.aspetjournals.org at ASPET Journals on March 20, 2018

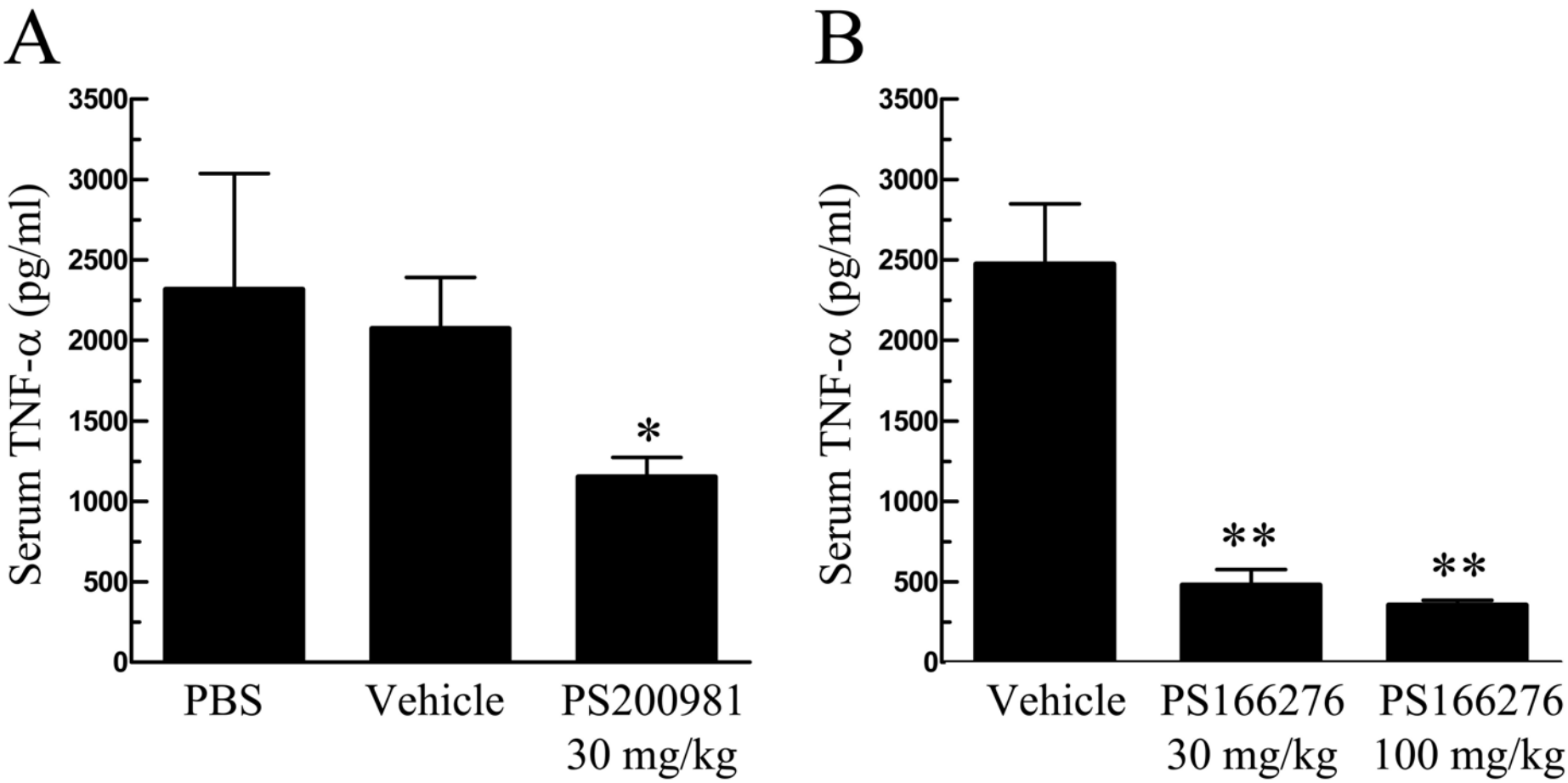
# Figure 5




JPET Fast Forward. Published on May 15, 2006 as DOI: 10.1124/jpet.105.097568
This article has not been copyedited and formatted. The final version may differ from this version.

Downloaded from jpet.aspetjournals.org at ASPET Journals on March 20, 2018

# Figure 6




JPET Fast Forward. Published on May 15, 2006 as DOI: 10.1124/jpet.105.097568
This article has not been copyedited and formatted. The final version may differ from this version.

Downloaded from jpet.aspetjournals.org at ASPET Journals on March 20, 2018