BIOCHEMICAL PHARMACOLOGY 77 (2009) 422–432

available at www.sciencedirect.com

ScienceDirect

journal homepage: www.elsevier.com/locate/biochempharm

Inhibition of pro-inflammatory cytokine production by the dual p38/JNK2 inhibitor BIRB796 correlates with the inhibition of p38 signaling

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ARTICLE INFO

Article history:
Received 5 September 2008
Accepted 28 October 2008

Keywords:
p38
Protein kinase inhibitor
T-cells
Neutrophils
Monocytes
High-content imaging

ABSTRACT

The characterization of the potent p38 inhibitor BIRB796 as a dual inhibitor of p38/Jun N-terminal kinases (JNK) mitogen-activated protein kinases (EC 2.7.11.24) has complicated the interpretation of its reported anti-inflammatory activity. To better understand the contribution of JNK2 inhibition to the anti-inflammatory activities of BIRB796, we explored the relationship between the effects of BIRB796 and analogues on cytokine production and on cellular p38 and JNK signaling. We determined the binding affinity for BIRB796 and structural analogues to p38α and JNK2 and characterized compound 2 as a p38 inhibitor that binds to p38α with an affinity equivalent to BIRB796 but does not bind to any of the JNK isoforms. High-content imaging enabled us to show that the inhibition of p38 signaling by BIRB796 and analogues correlates with the ability of these compounds to inhibit the lipopolysaccharide (LPS)-induced TNF-α production in THP-1 monocytes. This finding was extended to cytokine release by disease-relevant human primary cells: to the production of TNF-α by peripheral blood mononuclear cells, and of IL-8 by neutrophils. Furthermore, BIRB796 and compound 2 inhibited the production of TNF-α in THP-1 monocytes and the IL-12/IL-18-induced production of interferon-γ in human T-cells with similar potencies. In contrast, cellular JNK signaling in response to cytokines or stress stimuli was only weakly inhibited by BIRB796 and analogues and not affected by compound 2. In summary, our data suggest that p38 inhibition alone is sufficient to completely suppress cytokine production and that the added inhibition of JNK2 does not significantly contribute to the effects of BIRB796 on cytokine production.

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0006-2952/$ – see front matter
doi:10.1016/j.bcp.2008.10.032

1. Introduction

The mitogen-activated protein kinase (MAPK) p38α is activated by environmental stimuli, bacterial products and cytokines and plays a critical role in the production of pro-inflammatory cytokines such as TNF-α and IL-1β [1–3]. In T-cells, p38 is involved in the production of IFN-γ in T-helper type 1 (Th1) cells [4,5]. The mitogen-activated protein kinase-activated protein kinase-2 (MK2) has been shown to be the main target of p38 in the context of inflammatory cytokine production [2,6,7]. MK2 signaling is mediated through the phosphorylation of substrates such as the small heat-shock protein Hsp27 [8]. The Jun N-terminal kinases (JNK) comprising the products of three genes, JNK1, JNK2 and JNK3, have also been implicated in the control of pro-inflammatory cytokines, e.g. the production of TNF-α in stimulated macrophages and mast cells [9,10]. JNK signaling does not seem to be required for T-cell activation but contributes to the differentiation of $CD4^+$ T-helper cells to effector cells [11].

Elevated levels of cytokines have been associated with a variety of autoimmune disorders including rheumatoid arthritis, psoriasis, diabetes and inflammatory bowel disease [12,13]. Therefore, p38 signaling has been targeted for the development of orally active, small-molecule therapeutics for autoimmune disorders [14]. A family of ATP-competitive pyridinyl imidazole inhibitors exemplified by the compound SB203580 was critical for the identification and initial characterization of p38 signaling [15]. More recently, a diaryl urea class of highly potent p38 inhibitors including the former clinical candidate BIRB796 has been shown to block TNF-α production in cell culture at nanomolar concentrations and has demonstrated efficacy in animal models of endotoxin-stimulated TNF-α release and of collagen-induced arthritis [16]. BIRB796 also strongly inhibited the production of TNF-α in a model of human inflammation, lipopolysaccharide (LPS)-induced endotoxemia [17]. This compound class blocks p38 signaling by stabilizing a conformation of p38α (Phe-out) that is incompatible with ATP binding [16].

Initially, we reported significant inhibition of JNK2α2 by BIRB796 based on an IC_{50} determination of 98 nM [16]. This early work was performed using a weakly activated commercial preparation of JNK2α2 resulting in an underestimation of the true binding affinity. Subsequent work disclosed herein was directed toward determining the binding K_d for BIRB796 and related compounds to JNK2α2 using a solution competition assay. Meanwhile in a recent publication, BIRB796 was shown to display high-affinity binding to JNK2 (K_d = 5.6 nM) using a surface displacement system. Additionally, moderate affinity for JNK3 (K_d = 62 nM) and no significant binding to JNK1 were observed [18]. These findings raised the question whether inhibition of JNK2 might contribute to BIRB796-mediated cell and in vivo effects. SP600125, an inhibitor of JNK1, JNK2 and JNK3, had been shown to block the LPS-induced induction of TNF-α and IL1-β in human monocytes [19]. However, it remained unclear whether inhibition of JNK2 in the absence of JNK1 inhibition would be sufficient to exert significant effects on cytokine production. The lack of sufficiently JNK1-selective or JNK2-selective inhibitors has impeded the pharmacological analysis of the cellular effects of each of these JNK isoforms in the absence of effects on other JNK isoforms and other protein kinases.

It was recently reported that BIRB796 inhibits JNK signaling in response to osmotic shock with estimated IC_{50} values in the micromolar range [20]. However, the relevance of BIRB796 binding to JNK2 with regard to LPS- or cytokine-induced JNK signaling and to cytokine production was not addressed. For this reason, we have now systematically characterized the relationship between the effects of BIRB796 and analogues on p38 and JNK signaling and on cytokine production by relevant cell types. We have used fluorescence-based high-content imaging to show that the effects of BIRB796 analogues on p38 activation and p38 activity correlate closely with the inhibition of cytokine production. BIRB796 and its close analogue, compound 1, have only weak effects on cytokine-induced JNK signaling in a human monocytic cell line. Moreover, compound 2, a potent p38 inhibitor lacking activity against JNK1, 2 and 3 isoforms, is able to inhibit the production of TNF-α from human monocytic cells and of IFN-γ from human T-cells with potencies similar to those of BIRB796 and compound 1. We conclude that the cytokine inhibitory effects of BIRB796 are predominantly mediated by the inhibition of p38 and not of JNK signaling.

2. Materials and methods

2.1. Reagents used for the experiments

HeLa cells were obtained from ATCC (American Type Culture Collection, Manassas, VA). SB203580 was purchased from Novagen Calbiochem (San Diego, CA). SKF 86002 was a generous gift from Glaxo SmithKline Pharmaceuticals. All other p38 inhibitors were synthesized at Boehringer Ingelheim Pharmaceuticals (Ridgefield, CT). Lipopolysaccharide (LPS), anisomycin, TNF-α, leptomycin B, DEAE, HEPES, DMSO, deoxycholic acid, sodium orthovanadate, sodium fluoride, sodium pyrophosphate, β-mercaptoethanol, formaldehyde, and Triton X-100 were obtained from Sigma–Aldrich (St. Louis, MO). Unless otherwise specified, any and all other buffers and reagents were from Sigma–Aldrich (St. Louis, MO) and of reagent grade. All cell culture reagents (RPMI 1640, pen/strep, L-glut., neaa, phosphate-buffered saline (PBS), Na pyruvate, goat serum) were from Gibco Invitrogen (Grand Island, NY) except for heat-inactivated fetal bovine serum (FBS) which was from Hyclone (Logan, UT). Phospho-p38 MAPK (T180/Y182) and phospho-c-Jun (Ser63) rabbit polyclonal antibodies and phospho-JNK (Thr183/Tyr185) and Hsp27 mouse monoclonal antibodies were obtained from Cell Signaling Technology (Danvers, MA). Phospho-Hsp27 (Ser78) goat polyclonal antibody was from Santa Cruz Biotechnology (Santa Cruz, CA); Alexa Fluor 488 goat anti-rabbit, goat anti-mouse and donkey anti-goat IgG $F(ab')_2$ fragments were obtained from Invitrogen (Carlsbad, CA). Peroxidase goat anti-rabbit and donkey anti-goat IgG $F(ab')_2$ fragments were from Jackson ImmunoResearch Laboratories (Westgrove, PA). Nucleic acid stain Hoechst 33342 was from Cellomics (Pittsburgh, PA) and Draq 5 was from Biostatus (Shepshed, UK).

2.2. Determination of compound binding rate constants to p38α and JNK2α2

Recombinant p38α was expressed in *E. coli* using a B834(DE3)pLysS vector (Novagen EMD, Madison, WI) containing the cloned insert with an N-terminal $(His)_6$ tag. $(His)_6$-p38α expression was induced with IPTG (Invitrogen, Carlsbad, CA) and $(His)_6$-p38α was purified using a Nickel-NTA column (Qiagen, Valencia, CA). Final purification was achieved by ion exchange chromatography on a DEAE column. JNK2α2 was expressed in Sf21 insect cells using a modified pFASTBac1 vector (Invitrogen, Carlsbad, CA) with an N-terminal $(His)_6$ tag. Cells were grown in a stirred tank 20 l reactor and were infected on day 5. 72 h later, biomass was harvested and lysate was applied to a Nickel-NTA column (Qiagen, Valencia, CA), followed by ion exchange chromatography and affinity purification as described above for p38α. Enzyme concentration was determined by active site titration using SKF 86002 and the molecular weight of the purified protein was verified by LC/MS. Kinetic rate constants for the binding of the fluoroprobe, SKF 86002, to all proteins and for the binding of unlabeled inhibitors to these proteins were determined as described previously [16,21].

2.3. High-content imaging

HeLa cells were cultured in RPMI 1640 supplemented with 1% penicillin/streptomycin, 2 mM L-glutamine, non-essential amino acids, and 10% fetal bovine serum at 37 °C in humidified air/CO_2 (95%/5%). HeLa cells were plated in black 96 well-plates (Corning Costar, Corning, NY) at 5000 cells/well and allowed to adhere for 14–16 h at 37 °C in humidified air/CO_2 (95%/5%). Compounds were diluted from DMSO stocks into complete media and added to the cells yielding a final DMSO concentration of 0.05%. Cells were pre-incubated with compounds for 1 h at 37 °C prior to stimulation with anisomycin (5 μg/ml) for 30 min or TNF-α (0.5 μg/ml) for 15 min at 37 °C. Cells were fixed with 3.7% formaldehyde in PBS (15 min), and permeabilized with 0.5% Triton X-100. Cells were washed and blocked with 10% goat serum in PBS prior to incubation with phospho-specific antibodies for 30 min at room temperature. Cells were washed again, and bound antibody was visualized with Alexa Fluor 488 anti-rabbit and anti-goat secondary antibodies. Cell nuclei were labeled with Hoechst 33342 and cells were imaged on the ArrayScan II reader (Cellomics, Pittsburgh, PA). Fluorescence intensity was analyzed for 100 cells per well using Cellomics proprietary image analysis software (Nuclear translocation algorithm).

2.4. Isolation and stimulation of human neutrophils

Human neutrophils were separated as follows: 5 ml of heparinized human blood was layered over 4 ml of 1-Step Polymorphs (Accurate Chemical and Scientific, Westbury, NY) separation medium in a 15 ml centrifuge tube. Ten such tubes were prepared and centrifuged at 500 × *g* for 30 min at 20 °C. After centrifugation, neutrophils in the lower leukocyte band above the erythrocytes were harvested and diluted in one volume of 0.45% sodium chloride. Cells were centrifuged at 400 × *g* for 10 min at 20 °C, resuspended in PBS and centrifuged again similarly. Finally, cells were resuspended in RPMI 1640 with 10% FBS at 10^7 cells/ml.

Cells were incubated for 30 min at 37 °C, 5% CO_2 with various concentrations of BIRB796. The stock solution of BIRB796 was prepared in 100% DMSO and subsequent dilutions were made in RPMI 1640, 10% FBS. TNF-α was then added for a final concentration of 20 ng/ml and the incubation was continued for 5 min at 37 °C. Following the incubation, 400 μl of cold RPMI 1640, 10% FBS was added to each tube and tubes were centrifuged at 2000 rpm (Eppendorf, Model 5417R) for 10 min. The supernatant was aspirated and the cell pellets were resuspended in 60 μl of cold lysis buffer (10 mM Tris–HCl pH 7.5, 50 mM sodium chloride, 1% Triton X-100, 5 mM sodium fluoride, 1 mM sodium vanadate, 0.1% bovine serum albumin, 5 mM EDTA, protease inhibitor cocktail). The tubes were incubated for 30 min on ice. Lysates were centrifuged at 14,000 rpm (Eppendorf, Model 5417R) for 10 min at 4 °C. The supernatant was transferred to a new tube and an equal volume of 2× Tris–glycine SDS sample buffer (Invitrogen, Carlsbad, CA) containing 10% β-mercaptoethanol was added. Samples were boiled for 5 min at 95 °C and submitted to Western blotting.

2.5. IL-8 release assay

Human polymorph nuclear (PMN) cells (150 μl) were isolated as described above and were added to 25 μl of various concentrations of BIRB796 or medium in 96-well flat bottom plates (Corning Costar, Corning, NY). The stock solution of BIRB796 was prepared in 100% DMSO and subsequent dilutions were made in RPMI 1640, 10% fetal bovine serum. Cells were incubated for 40 min at 4 °C and TNF-α was then added to yield a final concentration of 20 ng/ml and the incubation was continued over night at 37 °C. The next day, the plates were centrifuged at 2000 rpm at room temperature in a Beckman GS-6R centrifuge for 10 min. The supernatants were collected and levels of IL-8 were measured using a human IL-8 immunoassay kit from R&D Systems (Minneapolis, MN) according to the manufacturer's instructions.

2.6. Isolation and stimulation of human peripheral blood mononuclear cell (PBMC)

Heparinized blood (25 ml) was diluted with 15 ml of PBS, layered onto 10 ml of Ficoll Paque Plus (GE Healthcare Life Sciences, Piscataway, NJ) and centrifuged at 500 × *g* (Beckman, Model GS-6R) for 30 min at room temperature. The peripheral blood mononuclear cells were collected and washed twice with PBS and resuspended at 3.33×10^6 cells/ml in RPMI 1640, 10% FBS.

The cells were incubated for 60 min at 37 °C, 5% CO_2 with various concentrations of BIRB796. The stock solution of BIRB796 was prepared in 100% DMSO and subsequent dilutions were made in RPMI 1640, 10% FBS. LPS was then added for a final concentration of 250 ng/ml and the incubation was continued for 20 min at 37 °C. Following the incubation, 400 μl of cold RPMI 1640, 10% FBS was added to each tube and tubes were centrifuged at 2000 rpm (Eppendorf, Model 5417R) for 10 min. The supernatant was aspirated and the cell pellets were resuspended in 60 μl of cold lysis buffer

(20 mM Tris–HCl pH 7.5, 120 mM sodium chloride, 1% Triton X-100, 20 mM sodium fluoride, 2 mM sodium orthovanadate, protease inhibitor cocktail). The tubes were incubated for 30 min on ice. Lysates were centrifuged at 14,000 rpm (Eppendorf, Model 5417R) for 10 min at 4 °C. The supernatant was transferred to a new tube and an equal volume of 2× Tris–glycine SDS sample buffer (Invitrogen, Carlsbad, CA) containing 10% β-mercaptoethanol was added. Samples were boiled for 5 min at 95 °C and subjected to Western blotting.

2.7. Cytokine release from human PBMC

Human PBMC were isolated as described above and added to various concentrations of BIRB796 or medium in 96-well flat bottom plates (Corning Costar, Corning, NY). Cells were incubated for 60 min at 37 °C, 5% CO_2. LPS was then added for a final concentration of 250 ng/ml and the incubation was continued over night (18–24 h). Supernatants were collected and frozen at −80 °C for future analysis. Levels of various cytokines (TNF-α, IL-1β, IL-6, IL-8, IL-10, and GM-CSF) in the supernatants were measured using ELISA kits from R&D Systems (Minneapolis, MN) according to the manufacturer's instructions.

2.8. Western blotting

Cell lysates (total protein 40 μg per lane) were electrophoresed on a Tris–HCl 15% linear gradient gel (Bio-Rad, Hercules, CA) at 200 V for 50 min and transferred onto polyvinylidene fluoride membranes (Bio-Rad, Hercules, CA). Membranes were blocked with 5% dry milk in Tris-buffered saline (TBS) and immunoblotted with antibodies against phosphorylated p38, MK2 or Hsp27 14–16 h at 4 °C. Membranes were washed 3× 5 min in TBS + 1% Tween-20 (Bio-Rad, Hercules, CA) prior to exposure to peroxidase conjugated anti-rabbit secondary antibody for 1 h at room temperature. After removal of secondary antibody, membranes were washed 3× 5 min in TBS + 1% Tween-20. Immuno-reactive proteins were detected using the ECL Western blot detection reagent kit according to the manufacturer's instructions (Amersham Biosciences, Piscataway, NJ).

2.9. Generation of inflammatory T-cells

Human peripheral blood was obtained via venipuncture from healthy volunteers. Mononuclear cells were isolated from peripheral blood by density centrifugation using CPT tubes (BD Biosciences, Franklin Lakes, NJ). Primary T-cell cultures were set up with the addition of phytohemagglutinin (PHA; Sigma–Aldrich, St. Louis, MO) at 10 μg/ml, and rIL-2 (R&D Systems, Minneapolis, MN) at 5 ng/ml to mononuclear cells suspended at 1×10^6 cells/ml in complete media (RPMI 1640, 10% FBS, 10 mM HEPES, 1 mM sodium pyruvate, 20 U/ml penicillin, 20 μg/ml streptomycin and 2 mM L-glutamine). Cultures were incubated for 48 h at 37 °C. Cells were then washed twice and resuspended in fresh media supplemented with rIL-2 and allowed to proliferate for an additional 12–14 days.

2.10. IFN-γ induction studies

Cultured 14–16 days T cells were washed twice with phosphate-buffered saline and resuspended at 1×10^6 cells/ml in complete media and added (100 μl/well) to 96-well plates (Corning Costar, Corning, NY) that were pre-coated for 1 h with anti-CD3 (Immunotech, France) at 1 μg/ml in PBS. For IL-12/IL-18-induced IFN-γ, cultured T-cells were treated with IL-12 and IL-18 (R&D Systems, Minneapolis, MN) at 10 ng/ml and 300 ng/ml, respectively, and added (100 μl/well) to uncoated wells of a 96-well plate (Corning Costar, Corning, NY). Compounds were diluted from DMSO stocks into complete media and added to the cells (100 μl/well) yielding a final DMSO concentration of 0.05%. Plates were incubated at 37 °C for 48 h with 5% carbon dioxide. After a brief centrifugation at ~100 × *g*, supernatants were removed, diluted and tested for the presence of IFN-γ using BenderMedsystems (Vienna, Austria) Instant ELISA kits according to the manufacturer's instructions.

2.11. Data analysis

Values in figures and tables are expressed as mean ± S.E. mean of *n* observations, unless otherwise noted. All treatments were compared with relevant vehicle control groups. All IC_{50} values were calculated using regression analysis following linearization of dose-response curves by logarithmic transformation. IC_{50} values for the inhibition of cytokine release from PMN and PBMC and for the inhibition of p38 and Hsp27 phosphorylation were generated by a four-parameter fit of fluorescence intensity data with the SAS statistical software system, Version 9.0 (SAS Institute, Cary, NC). IC_{50} values for compound inhibition of IFN-γ production were calculated with XLfit (idbs, Burlington, MA) Version 4.2 utilizing a four-parameter logistic model.

3. Results

3.1. Characterization of compound binding to p38 and JNK2

The binding affinity of BIRB796 and additional p38 inhibitors was determined using the paired progress curve method as described [16,21]. This method is particularly useful to determine rate constants for slowly associating and slowly dissociating systems otherwise requiring long pre-incubation periods to reach equilibrium. We applied this method to the determination of the binding affinity of compounds to p38α and JNK2α2. BIRB796 and its close analogue, compound 1, bound with high affinity to both p38α and JNK2α2 (Fig. 1 and Table 1). However, the affinity to p38α was about 30-fold higher than the affinity to JNK2α2 (Table 1). Compound 1 inhibited JNK3 approximately 100-fold weaker than JNK2 and showed no significant inhibition of JNK1 (IC_{50} determinations using kinase activity assays—data not shown). Compound 2 (structurally distinct from BIRB796 but also binding to the Phe-out conformation of p38) bound to p38α with an affinity equivalent to BIRB796 but did not show any affinity for JNK2α2 (Fig. 1 and Table 1), JNK1 or JNK3 (data not shown). Consistent with our previous publication, all three in-house p38 inhibitors demonstrated both slow association and slow dissociation rate constants in contrast with the archetypal ATP-site binder (Phe-in binder) SKF 86002.

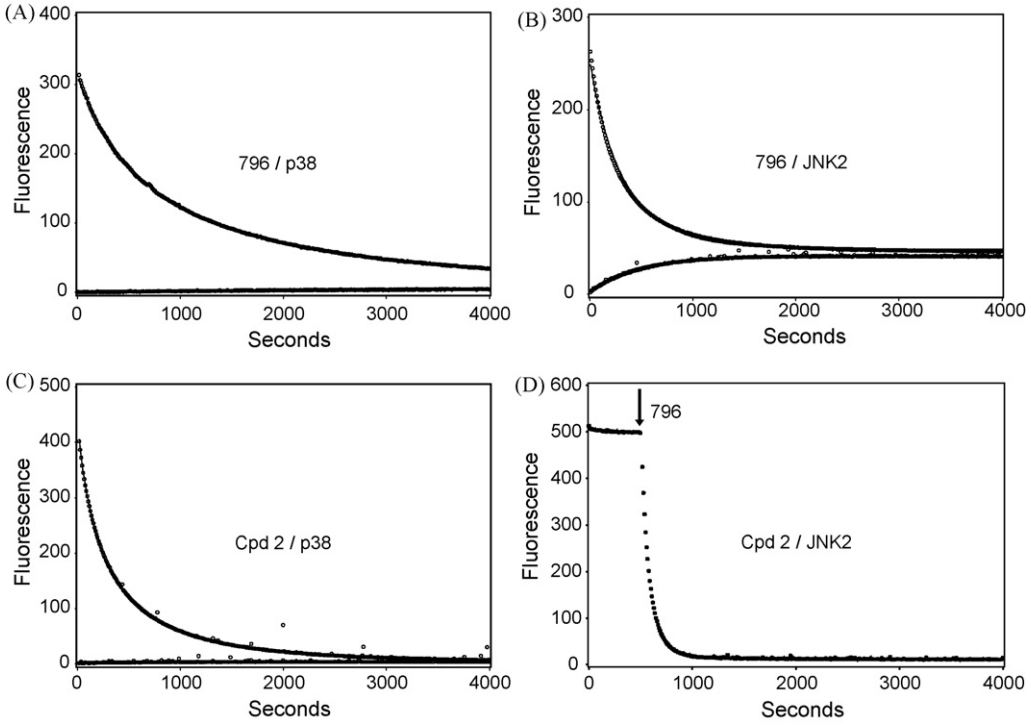

Fig. 1 – Competitive binding between SKF 86002 and various p38 inhibitors. Rate constants for the binding of the fluoroprobe, SKF 86002, to both p38α and JNK2α2, were determined using stopped flow association binding data as reported previously [16]. Raw data (circles) was fitted to an expression by nonlinear regression analysis (solid line) yielding both the association and dissociation rate constants for competitor binding (see Section 2). (A) [p38α] = 1.74×10^{-7} M, [BIRB796] = 2.90×10^{-7} M, and [SKF 86002] = 1.50×10^{-6} M. (B) [JNK2α2] = 1.30×10^{-7} M, [BIRB796] = 4.03×10^{-7} M, and [SKF 86002] = 1.00×10^{-6} M. (C) [p38α] = 1.70×10^{-7} M, [compound 2] = 2.08×10^{-7} M, and [SKF 86002] = 1.00×10^{-6} M. (D) [JNK2α2] = 1.30×10^{-7} M, [compound 2] = 4.00×10^{-7} M, and [SKF 86002] = 1.00×10^{-6} M. Note that no displacement of SKF 86002 was evident following addition of compound 2 to JNK2α2 indicating negligible binding affinity. The addition of BIRB796 (400 nM) at 500 s (indicated by arrow) led to the rapid displacement of SKF 86002.

3.2. *Correlation of p38 inhibition with inhibition of cytokine release*

We have previously reported that BIRB796 and analogues potently inhibit the production of pro-inflammatory cytokines [16,22,23]. BIRB796 prevents the activation of p38α leading to decreased levels of its phosphorylated form [20,24]. In-house experiments indicate that BIRB796 does not inhibit the upstream activator of p38 (MKK6) but simply titrates available p38 protein (data not shown).

In order to investigate the dose-dependent effects of BIRB796, its analogues and compound 2 on cellular p38 signaling, we performed immunofluorescence-based assays for p38 and Hsp27 phosphorylation on the Cellomics ArrayScan II automated imaging platform (Cellomics, Pittsburgh, PA). BIRB796, compound 1 and compound 2 potently inhibited the phosphorylation of p38 and Hsp27 in this assay yielding IC_{50} values between 7 and 20 nM (Fig. 2A and B and Table 2). These values were also comparable to the IC_{50} values for the inhibition of TNF-α production in THP-1 human monocytic cells (Table 2). Similar IC_{50} values were also obtained in murine and human cells stimulated with TNF-α or LPS (data not shown). In comparison, the p38 inhibitor SB203580 displayed a lower potency than BIRB796

Table 1 – Molecular inhibition profiles of selected p38 inhibitors binding to p38α and JNK2α2. All standard errors of the fitted parameters, k_{on} and k_{off}, are less than 4%. K_d is calculated from the formula: $K_d = k_{off}/k_{on}$ (no inhib.: no inhibition).

Code	p38α			JNK2		
	k_{on} ($M^{-1}\,s^{-1}$)	k_{off} ($M^{-1}\,s^{-1}$)	K_d (M)	k_{on} ($M^{-1}\,s^{-1}$)	k_{off} ($M^{-1}\,s^{-1}$)	K_d (M)
SKF 86002	$4.3 \times 10^{+7}$	7.7×10^{0}	1.8×10^{-7}	$6.0 \times 10^{+7}$	5.0×10^{0}	8.3×10^{-8}
BIRB796	$4.6 \times 10^{+4}$	7.5×10^{-6}	1.6×10^{-10}	$8.2 \times 10^{+4}$	3.8×10^{-4}	4.6×10^{-9}
Cpd 1	$2.6 \times 10^{+4}$	1.2×10^{-5}	4.6×10^{-10}	$8.7 \times 10^{+4}$	1.3×10^{-4}	1.5×10^{-9}
Cpd 2	$1.3 \times 10^{+5}$	8.7×10^{-6}	6.7×10^{-11}	No inhib.	No inhib.	No inhib.

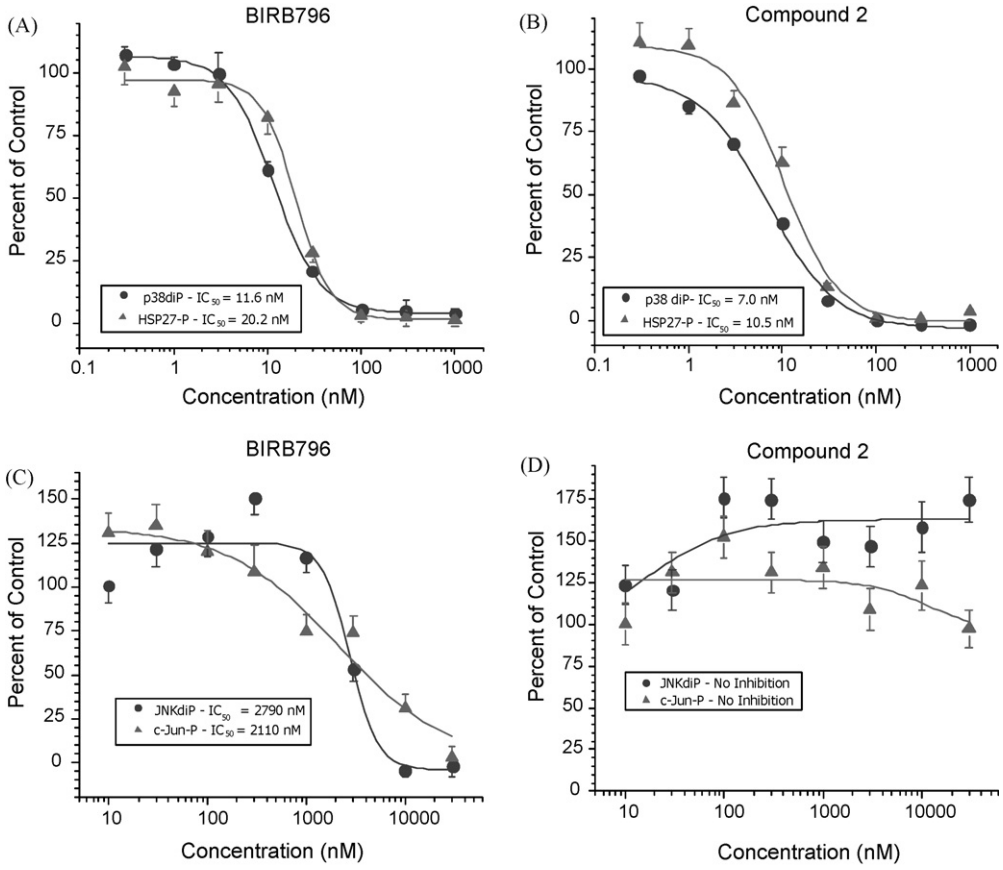

Fig. 2 – BIRB796 and compound 2 potently inhibit p38 signaling but have weak or no inhibitory effects on JNK signaling. (A and B) HeLa cells were cultured in 96-well plates over night and stimulated with anisomycin (5 μg/ml) for 30 min prior to fixation and permeabilization. In separate wells, cells were pretreated with BIRB796 (A) or compound 2 (B) to yield final compound concentrations between 0.2 and 1000 nM for 1 h prior to stimulation. Immunofluorescence staining was performed with an antibody directed against phosphorylated p38α/β (●) or phosphorylated Hsp27 (▲) in combination with secondary anti-rabbit and anti-goat IgG antibodies conjugated to Alexa Fluor 488. Fluorescence staining was detected via imaging with the Cellomics Arrayscan II automated imaging platform. Phosphorylation levels were analyzed using the Cellomics Nuclear Translocation algorithm and were normalized to stimulated levels in vehicle-treated control cells. Data fitting for the generation of IC_{50} values was performed using SAS 9.0 and Origin software. (C and D) HeLa cells were cultured in 96-well plates over night and stimulated with TNF-α (0.5 μg/ml) for 15 min prior to fixation and permeabilization. In separate wells, cells were pretreated with BIRB796 (C) or compound 2 (D) to yield final compound concentrations between 10 and 30,000 nM for 1 h prior to stimulation. Immunofluorescence staining was performed with antibodies directed against phosphorylated JNK (●) or phosphorylated c-Jun (▲) in combination with secondary anti-mouse or anti-rabbit IgG antibodies conjugated to Alexa Fluor 488. Fluorescence staining was imaged and analyzed as described for (A) and (B).

for the inhibition of p38 signaling and of cytokine release (Hsp27-P: $IC_{50} = 218 \pm 78$ nM; TNF-α: $IC_{50} = 76 \pm 19$ nM). In order to confirm the close relationship between the inhibition of cellular p38 signaling and TNF-α release for BIRB796, we expanded our analysis to include fifty BIRB796 analogues with varying levels of p38 potency in molecular assays [23]. In this analysis, we observed a linear correlation for the inhibition of p38 signaling and TNF-α release supporting the p38-dependency of compound effects on cytokine release (Fig. 3B and C). In addition, we also found a linear correlation between the inhibition of p38 and Hsp27 phosphorylation consistent with the dual inhibition of p38 activation and activity by BIRB796 that had been described earlier (Fig. 3A) [20,24].

3.3. *Weak effects on cellular JNK signaling*

Western blotting experiments have demonstrated that BIRB796 inhibits the phosphorylation of JNK and its substrate c-Jun in stress-activated cells with estimated IC_{50} values in the micromolar range [20]. In order to further evaluate the effects of BIRB796 on JNK signaling, we used immunofluorescence-based assays for JNK and c-Jun phosphorylation that allowed us to quantitate the effects of BIRB796 and its analogues using automated fluorescence microscopy (as described above). We found that BIRB796 and compound 1 inhibited cellular JNK signaling in response to TNF-α at least 100 times less potently than p38 signaling and cytokine release (Fig. 2 and Table 2). Compound 2, a potent p38 inhibitor lacking activity against

Table 2 – Effects of BIRB796, compound 1 and compound 2 on cellular p38 and JNK signaling. IC_{50} values (nM) are compared for the inhibition of p38 (p38diP) and Hsp27 (Hsp27-P) phosphorylation in HeLa cells stimulated with anisomycin (5 μg/ml), for the inhibition of JNK (JNKdiP) and c-Jun (c-Jun-P) phosphorylation in HeLa cells stimulated with TNF-α (0.5 μg/ml) and for the inhibition of TNF-α release in THP-1 cells (TNF-α). Numbers in parentheses are the standard deviation ($n = 2$).

	BIRB796	Compound 1	Compound 2
p38diP	11.6 (0.4)	6.6 (0.2)	7.0 (0.8)
Hsp27-P	20.2 (1.6)	13.7 (1.2)	10.5 (1.8)
JNKdiP	2790 (590)	3190 (1196)	No inhibition
c-Jun-P	2110 (740)	5950 (1800)	No inhibition
TNF-α	18 (3)	21 (6)	40 (25)

JNK2 in molecular assays (Fig. 1 and Table 1) did not inhibit cellular JNK signaling (Fig. 2D and Table 2). However, this compound potently inhibited TNF-α release with an IC_{50} value comparable to the IC_{50} values for the inhibition of p38 signaling by BIRB796 and compound 1 (Table 2). In summary, p38 inhibition alone is sufficient to completely suppress cytokine production and the less potent inhibition of JNK2 appears to have no added impact on cytokine production.

To evaluate the pan-JNK inhibitor SP600125 as an additional tool for our analysis, we tested the effect of this compound on cellular JNK signaling and cytokine production. SP600125 inhibited c-Jun phosphorylation and TNF-α release with estimated IC_{50} values of 12 and 20 μM, respectively. Our findings are consistent with the weak effect of SP600125 on cytokine production that has been reported previously [19]. Thus, high concentrations of this compound are required to achieve full inhibition of cellular JNK signaling. Cellular effects obtained at these concentrations may be mediated by the inhibition of protein kinases other than JNK and the utility of SP600125 as a tool to investigate JNK signaling is therefore limited.

3.4. *Inhibition of p38 signaling and cytokine release in human primary cells*

We also investigated the effect of BIRB796 on p38 signaling and cytokine release in human primary cells. BIRB796 potently inhibited the production of IL-8 in TNF-α stimulated neutrophils and the release of TNF-α in LPS-stimulated PBMC. The IC_{50} values obtained in these assays were similar to the IC_{50} values for the inhibition of p38 signaling (Fig. 4). Moreover, the observed potency was also comparable to the potency in other cell types described above (Fig. 2 and Table 2). In an expanded analysis, BIRB796 also potently inhibited the production of additional cytokines in LPS-stimulated human PBMC including IL-1β, IL-6, IL-8, IL-10 and GM-CSF (Table 3). In contrast, BIRB796 displayed only a very weak inhibition of cellular JNK signaling in these cells (Table 3).

It had been reported that p38 signaling is critically involved in the production of IFN-γ in murine T-cells [21]. Therefore, we examined the effects of BIRB796 and other compounds on cultured human inflammatory T-cells. BIRB796, compound 1 and compound 2 potently inhibited the production of IFN-γ in response to stimulation with IL-12/IL-18 but had no effect on

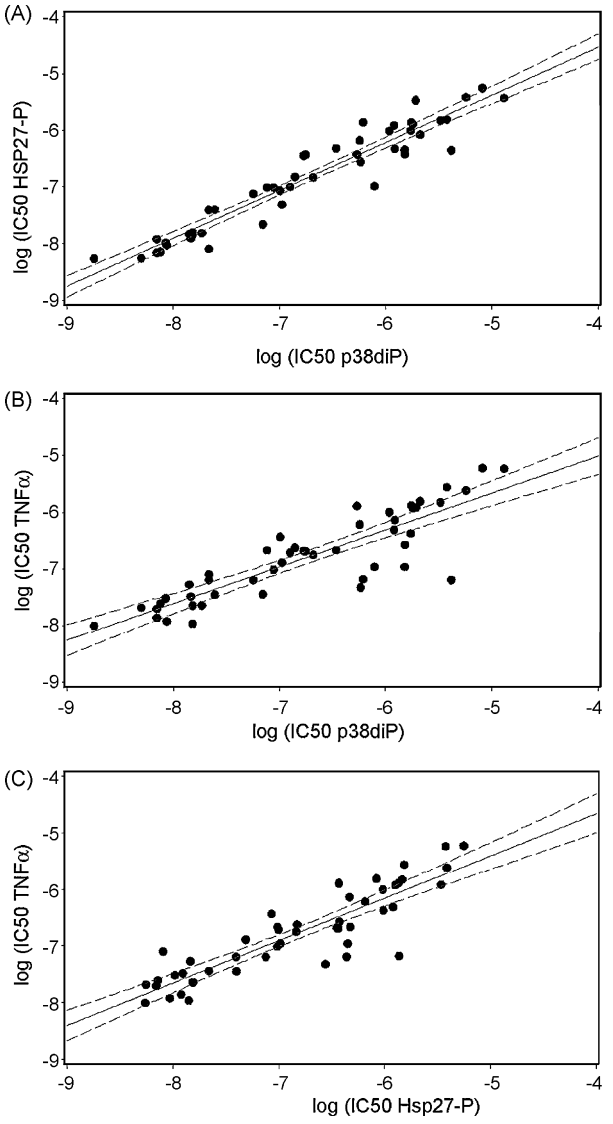

Fig. 3 – IC_{50} values for the inhibition of p38 and Hsp27 phosphorylation correlate with IC_{50} values for the inhibition of TNF-α production. The correlation between the IC_{50} values obtained for the inhibition of p38 phosphorylation (IC50_p38diP) and Hsp27 phosphorylation (IC50_HSP27-P) in HeLa cells and TNF-α release by THP-1 cells (IC50_TNFα) was analyzed for a set of 50 BIRB p38 inhibitors using SAS 9.0 software. The LPS-induced release of TNF-α by THP-1 cells was determined as described previously [16,22]. (A) Slope = 0.89 (%CV: 3.59); intercept = −0.75 (%CV: 29.89); adjusted $R^2 = 0.94$. (B) Slope = 0.76 (%CV: 4.92); intercept = −1.61 (%CV: 16.24); adjusted $R^2 = 0.90$. (C) Slope = 0.82 (%CV: 5.40); intercept = −1.20 (%CV: 25.69); adjusted $R^2 = 0.88$.

the production of IFN-γ elicited by T-cell receptor (TCR) stimulation (Fig. 5). In contrast, cyclosporine A did not affect the cytokine-induced response but completely blocked the TCR-induced IFN-γ production. These findings suggest that in both human and murine T-cells the cytokine induced but not the TCR-induced production of IFN-γ is p38-dependent [5]. The

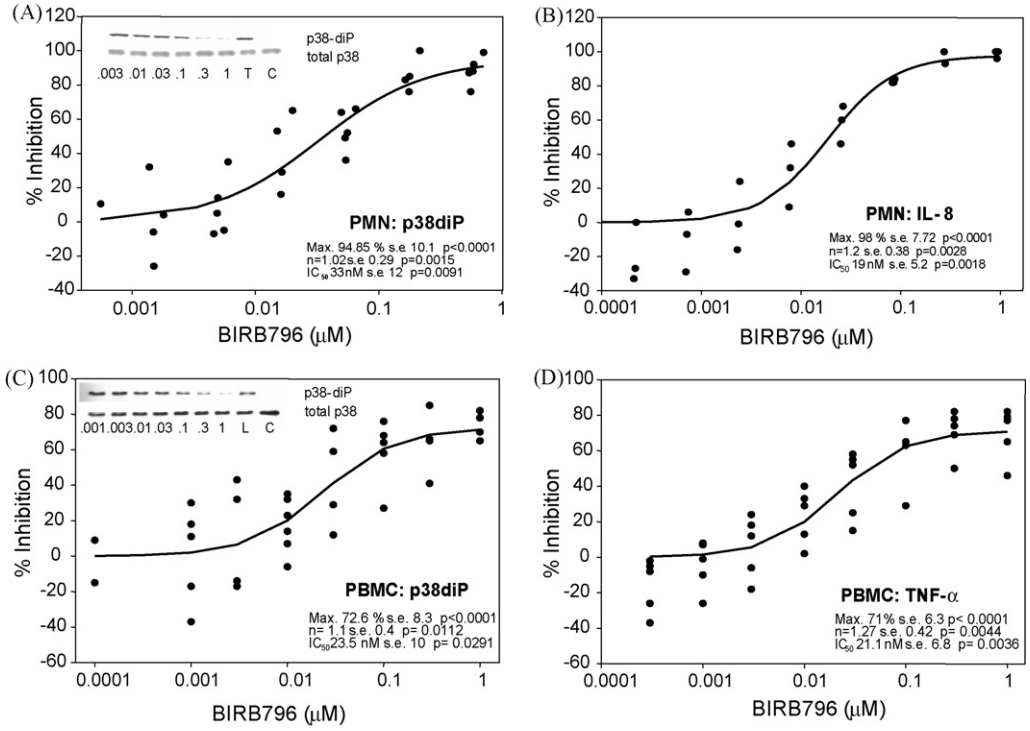

Fig. 4 – Effect of BIRB796 on p38 MAPK phosphorylation and cytokine release in human neutrophils and PBMC. Phosphorylation of p38 (A) and IL-8 release (B) in TNF-α stimulated human neutrophils. p38 MAPK phosphorylation and IL-8 release were measured in five different human donors for unstimulated cells (C), TNF-α stimulated cells (T) and cells treated with different concentrations of BIRB796 (0.0003–1 μM) prior to stimulation. The data was pooled and analyzed using non-linear regression analysis yielding the following parameters: max.: maximum level of inhibition; n: Hill coefficient or slope at IC_{50}; S.E.: standard error; *p*: probability or confidence level. All S.E. and *p* values were within acceptable limits ($p < 0.05$). The minimum level of inhibition was set to zero. Effect of BIRB796 on p38 MAPK phosphorylation (C) and TNF-α release (D) in LPS stimulated PBMC. p38 MAPK phosphorylation and TNF-α release were measured for six different human donors in unstimulated cells (C), LPS stimulated cells (L) and cells treated with different concentrations of BIRB796 (0.0001–1 μM) prior to stimulation. The data was pooled and analyzed as described above.

potency of compound 2 against p38 was comparable to the potency of BIRB796 and compound 1 suggesting that the inhibitory activity of BIRB796 and compound 1 towards JNK2 does not significantly contribute to the inhibitory effect on IFN-γ production. In addition, IC_{50} values for the inhibition of IFN-γ production were similar to IC_{50} values obtained in other human primary cells and cell lines (Tables 2–4).

Table 3 – Inhibition of the production of different cytokines by BIRB796 in LPS-stimulated human PBMC. The effects of BIRB796 on the production of different cytokines and on c-Jun phosphorylation were examined as described under Section 2. IC_{50} values (nM) are mean ± standard error of three experiments.

	BIRB796 IC_{50} (nM)	S.E.	Max. inh.
TNF-α	7.6	1	90
IL-1β	4.4	1	94
IL-6	18.4	6	70
IL-8	8.7	3	67
IL-10	13.9	3	90
GM-CSF	3.8	1	92
c-Jun-P	19,000	5000	56

4. Discussion

In this study, we examined the contribution of p38 and JNK2 inhibition by BIRB796 to the anti-inflammatory effects of this compound in various cellular models. Using quantitative analysis of protein phosphorylation levels, we show that the inhibition of LPS-elicited cytokine production by BIRB796 and its analogues correlates closely with the ability of these compounds to inhibit p38 activation and p38 activity. In contrast, these inhibitors have only very weak effects on cellular JNK signaling. Furthermore, a potent p38 inhibitor that binds to the Phe-out conformation of p38 similarly to BIRB796 but lacks activity against all JNK isoforms (compound 2) inhibits TNF-α release in human THP-1 monocytes and the production of IFN-γ in human T-cells with potencies comparable to BIRB796. We conclude that the effects of BIRB796 on cytokine production are predominantly mediated via the inhibition of the p38 signaling pathway.

The critical role of the p38 pathway in the production of pro-inflammatory cytokines has triggered efforts to develop small molecule, orally available p38 inhibitors as potential therapeutics for autoimmune diseases. One of the first available p38

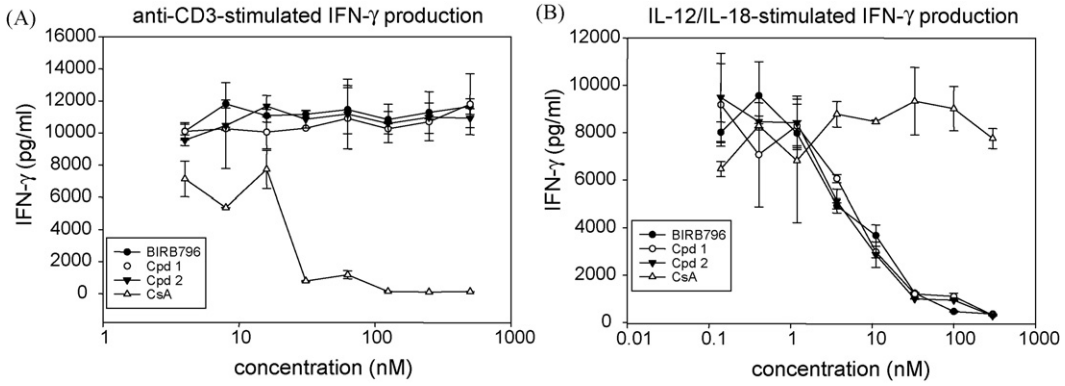

Fig. 5 – IFN-γ production in human inflammatory T cells induced by anti-CD3 or IL-12/IL-18 stimulation. Representative experiment ($n = 2$) demonstrating the induction of IFN-γ in human T-cells stimulated with anti-CD3 (A) or IL-12/IL-18 (B) for 48 h in the presence of BIRB796, compound 1, compound 2 or cyclosporine A (CsA).

inhibitors, the pyridinyl imidazole compound SB203580, has been used extensively to characterize the role of p38 in cellular and animal models: SB203580 has been shown to inhibit the production of TNF-α in human monocytes, to decrease LPS-induced TNF-α production in mice and rats, and to reduce inflammation in several animal models of arthritis [15,25].

However, studies have shown that most protein kinase inhibitors lack selectivity [18,26]. Both SB203580 and BIRB796 have been shown to bind tightly to JNK2. This finding raises the question whether the inhibition of JNK2 may contribute to the observed anti-inflammatory activities of these inhibitors. Published data indicate that JNK1 may be more critical than JNK2 for pro-inflammatory responses [27,28]. Unfortunately, the lack of inhibitors with sufficient selectivity for JNK1 or JNK2 has hindered the pharmacological analysis of this question. To investigate the effect of overall JNK inhibition, we tested the pan-JNK inhibitor SP600125 which displayed only a very weak inhibition of cellular JNK signaling and cytokine release in our hands. This finding is consistent with published information on the weak effect of this inhibitor on cytokine release [19].

Prior to this work, it remained unclear whether the tight binding of BIRB796 to JNK2 would translate into potent inhibition of JNK signaling in cells. As most cells co-express JNK1 and JNK2, JNK1, which is not affected by BIRB796, may be able to compensate for the inhibition of JNK2 in cells. So far, this issue has only been studied for the inhibition of JNK signaling in HEK293 cells following osmotic shock [20]: IC_{50} values for the inhibitory activity of BIRB796 were estimated based on the densitometric analysis of phospho-JNK and phospho-c-Jun signals from immunoblots and found to be in the micromolar range. They are therefore significantly higher than the IC_{50} values for the inhibition of cytokine production by BIRB796 disclosed in this and in previous publications. However, the relevance of this finding for the LPS or cytokine-mediated stimulation of p38/JNK signaling and for cytokine production was not addressed.

Our study is the first to provide a quantitative assessment of the inhibition of JNK signaling by BIRB796 and selected analogues in cells. We demonstrate that BIRB796 affects JNK signaling at least 100 times less potently than p38 signaling (Fig. 2 and Table 2). This difference may be due to the 30-fold difference in binding affinity BIRB796 displays for p38α and JNK2 or may be mediated by the compensatory action of JNK1 in cells. In order to further study the relationship between the inhibition of the p38 pathway and cytokine production, we investigated the correlation between compound effects on cellular p38 activation and activity and on TNF-α production in the human monocytic cell line THP-1. Using BIRB796 analogues with a wide range of potencies for p38 inhibition, we found a linear relationship between the inhibition of p38 signaling and of cellular TNF-α production (Fig. 3). This result indicates that p38 inhibition is predominantly responsible for the cellular activity observed in the TNF-α release assay. As expected, we also observed a correlation between compound inhibitory effects on the phosphorylation and activation of p38 and on the phosphorylation of Hsp27, an indicator of p38 activity (Fig. 3A). Furthermore, we show that the correlation between the inhibition of p38 signaling and cytokine production also applies to human primary cells. BIRB796 inhibits cytokine production in PBMCs and PMNs at concentrations that are significantly lower than those needed to affect cellular JNK signaling (Fig. 4). The p38-biased pharmacology is likely due to the higher affinity of BIRB796 for p38 over JNK2 and to the stronger inhibition of cellular p38 signaling compared to

Table 4 – Effects of BIRB796, compound 1 and compound 2 on IFN-γ production in human T-cells. The effects of compounds on IFN-γ production were examined as described for Fig. 5. Values expressed are IC_{50} determinations (nM) where the number in parentheses is the standard deviation ($n = 2$).

Treatment	BIRB796	Compound 1	Compound 2	CsA
Anti-CD3	No inhibition	No inhibition	No inhibition	4.6 (1.6)
IL-12/IL-18	5.0 (1.8)	7.6 (1.6)	4.0 (1.2)	No inhibition

JNK signaling (Fig. 2 and Table 2). Additional support for the p38-dependency of BIRB796 effects is provided by compound 2, a p38 inhibitor with equivalent p38 potency to BIRB796 but lacking inhibitory activity against all JNK isoforms: this compound inhibits the production of TNF-α in human monocytic cells and of IFN-γ production in T-cells with similar potency to BIRB796 (Fig. 5).

The ability of small molecule p38 inhibitors to inhibit cytokine production in a p38-dependent manner is also supported by studies with another p38 inhibitor unrelated to BIRB796. The p38 inhibitor VX-745, a former clinical candidate for the treatment of rheumatoid arthritis, displayed potent inhibition of cytokine production in human PBMCs and in human whole blood [29]. This compound possesses a protein kinase selectivity profile different from BIRB796 and does not show significant binding to JNK isoforms [18]. The shared activity and potency of VX-745 and BIRB796 with regard to the inhibition of cytokine production support the p38-dependency of these effects.

In T-cells, p38 plays a role in the production of IFN-γ by murine Th1 cells but seems not to be required for the production of IL-4 and IL-5 by Th2 cells [21]. Interestingly, we find no effect of BIRB796 and analogues on anti-CD3-induced IFN-γ production by human T-cells. In contrast, the IL-12/IL-18 mediated activation of p38 in human T-cells is fully inhibited by BIRB796 and compound 2 with IC_{50} values similar to those we obtained for BIRB796 with human PBMCs and PMNs (Figs. 4 and 5). Our finding for human T-cells is consistent with recent data from the murine system describing the selective requirement of p38α for IL-12/IL-18 induced but not for TCR-induced IFN-γ production [5]. Another potent p38 inhibitor, RWJ 67657, was also found to be inactive with regard to the TCR-mediated production of IFN-γ in human PBMCs [30].

In summary, we have demonstrated that the tight binding of BIRB796 to JNK2 does not translate into a potent inhibition of JNK signaling in cells and that a BIRB796 analogue lacking activity against JNK2 shows cellular potency for the inhibition of cytokine production similar to BIRB796. These findings together with the correlation between the inhibition of p38 signaling and cytokine production by numerous BIRB796 analogues lead us to conclude that the cellular effects of BIRB796 on cytokine production are due to the inhibition of the primary therapeutic target, p38 MAPK.

Acknowledgements

We are indebted to Teresa Roma and Elda Gautschi for experimental assistance with the purification of p38 and JNK2 recombinant proteins. We would like to thank Neil Moss for helpful comments on the manuscript.

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