EXTENDED REPORT

Differential influence of p38 mitogen activated protein kinase (MAPK) inhibition on acute phase protein synthesis in human hepatoma cell lines

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Background: Inhibition of intracellular signal transduction is considered to be an interesting target for treatment in inflammation. p38 MAPK inhibitors, especially, have been developed and are now in phase II clinical trials for rheumatoid arthritis (RA).

Objective: To investigate the influence of p38 MAPK inhibition on acute phase protein (APP) production, which is dependent on both JAK/STAT and p38 MAPK pathways.

Methods: The effects of p38 MAPK inhibition on APP production and mRNA expression in four human hepatoma cell lines was investigated, after stimulation with interleukin (IL)6 and/or IL1 β or tumour necrosis factor α .

Results: Two out of four cell lines produced C reactive protein (CRP), especially after combined IL6 and IL1 β stimulation. CRP production was significantly inhibited by the p38 MAPK specific inhibitor RWJ 67657 at 1 μ mol/l, which is pharmacologically relevant. Fibrinogen production was also inhibited at 1 μ mol/l in all cell lines. Serum amyloid A (SAA) was produced in all four lines. In contrast with CRP, SAA production was not inhibited by RWJ 67657 at 1 μ mol/l.

Conclusion: Production and mRNA expression of CRP and fibrinogen, but not SAA production and mRNA expression, were significantly inhibited by p38 MAPK specific inhibitor in hepatoma cell lines. For p38 MAPK inhibitor treatment in RA SAA might be a better marker of disease activity than CRP and fibrinogen, because SAA is not directly affected by p38 MAPK inhibition.

Rheumatoid arthritis (RA) is a chronic inflammatory disease, which leads to the destruction of cartilage and bone in the joints. Cytokines like interleukin (IL) 1 and tumour necrosis factor (TNF) α play an essential part in the pathogenesis of RA.¹ The liver is an important target of systemic inflammatory mediators, leading to the production of many components for defence, and of the acute phase plasma proteins (APPs).^{2,3} Measurement of the APP C reactive protein (CRP) is useful in managing disease, because the concentration reflects the inflammatory status of a patient. In RA, serial measurements of CRP are of prognostic value.⁴

IL6 is recognised as the principal regulator of most APP genes (the so-called type 2 or IL6-like APPs), including the three chains of fibrinogen, haptoglobin, and the protease inhibitors α_1 antichymotrypsin, α_1 antitrypsin, and α_2 macroglobulin. Type 1 APPs are regulated by the IL1-like cytokines and include α_1 acid glycoprotein, complement C3, serum amyloid A (SAA), and CRP. The IL6-like cytokines bind to plasma membrane receptor complexes containing the common signal transducing receptor chain gp130 (glycoprotein 130). Signal transduction involves the activation of JAK (Janus kinase) tyrosine kinase family members, leading to the activation of transcription factors of the STAT (signal transducers and activators of transcription) family.⁵ The importance of the JAK/STAT pathway in RA has not been completely established yet, but STAT-1 and STAT-3 seem to have both protective and pathogenic properties,⁶ with a regulating role for the suppressors of cytokine signalling (SOCS).⁷

Dimerisation of IL6-type cytokine receptor not only activates the JAK/STAT pathway but may also stimulate the mitogen activated protein kinase (MAPK) cascade through activation of Ras, a GTP-binding protein.⁵ Simultaneous

activation of the JAK/STAT and MAPK pathways in a rat hepatoma cell line has also been described for IL22, an IL10 related cytokine.⁸ A role for one of the MAPK homologues, p38 MAPK, in IL6 induced functions has been established in different studies.^{9–10} Zauberman *et al* reported that p38 MAPK activity was required for biological functions, including acute phase protein production from HepG2 hepatoma and proliferation of B9 hybridoma cells, an IL6 dependent cell line.¹⁰

p38 MAPK has a dominant role in signal transduction pathways in inflammatory diseases, and in recent years several specific inhibitors have been developed (for review see Kumar *et al*).¹¹ The p38 MAPK inhibitor RWJ 67657 has been shown to significantly inhibit the release of TNF α not only from lipopolysaccharide treated human peripheral blood mononuclear cells¹² but also from macrophages.¹³ Because p38 MAPK inhibitors are now in a phase II clinical trial for RA,¹⁴ it is important to know whether these inhibitors have a direct effect on the production of acute phase proteins, due to cross talk between the JAK/STAT and p38 MAPK cascades. This will elucidate whether these APPs are a valuable marker of disease activity during treatment with p38 MAPK inhibitors.

In this study we investigated the effect of p38 MAPK inhibition on IL6, IL1 β , and TNF α induced acute phase protein production in four different hepatoma cell lines, both

Abbreviations: ANOVA, analysis of variance; APP, acute phase protein; CRP, C reactive protein; DMEM, Dulbecco's modified Eagle's medium; ELISA, enzyme linked immunosorbent assay; FCS, fetal calf serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; JAK, Janus kinase; IL, interleukin; MAPK, mitogen activated protein kinase; RA, rheumatoid arthritis; RT-PCR, reverse transcriptase-polymerase chain reaction; SAA, serum amyloid A; STAT, signal transducers and activators of transcription; TNF, tumour necrosis factor

Figure 1 Western blot of p38 MAPK, phospho-p38 MAPK, phospho-MAPKAPK2, phospho-STAT-3, and STAT-3 after stimulation with (combinations of) cytokines, with or without 1 hour's pretreatment with RWJ 67657 at 1 μ mol/l. Hep3B cells were stimulated with 10 ng/ml IL1 β , TNF α , 50 ng/ml IL6 or combinations of these in DMEM with 1% FCS and 0.1 μ mol/l dexamethasone for 30 minutes. Phosphorylation was measured by western blot using specific antibodies against p38 MAPK, phospho-p38 MAPK, phospho-MAPKAPK2, STAT3, and phospho-STAT3.

at the level of mRNA expression and at the level of protein production. The effects on CRP, SAA, complement factor 3 (C3), and fibrinogen were studied. For mRNA analysis we studied the SAA-1 gene. Fibrinogen was studied by analysing both fibrinogen- β and fibrinogen- γ genes. Fibrinogen- β chain synthesis is considered to be rate limiting for assembly and secretion of mature fibrinogen. The promoter regions of both β and γ genes share the IL6 responsive element, but the γ gene lacks the C/EBP response element.¹⁵

MATERIALS AND METHODS

Reagents

RWJ 67657 was provided by Johnson and Johnson (RW Johnson Pharmaceutical Research Institute, Raritan, NJ, USA). The human hepatocellular carcinoma cell lines HepG2 and Hep3B, and the hepatoma cell line PLC/PRF/5 were purchased from the American Type Culture Collection (Manassas, VA, USA). The hepatoma cell line HuH7 was a kind gift from Dr R Kleemann (TNO, Leiden, The Netherlands). Recombinant human IL1 β , IL6, and TNF α were from R&D Systems (Minneapolis, MN, USA). Fetal calf serum (FCS) and DMEM (Dulbecco's modified Eagle's medium) were obtained from Biowhittaker (Verviers, Belgium).

Specific antibodies to p38 MAPK, phospho-p38 MAPK, phospho-MAPKAPK2, STAT-3, and phospho-STAT3 were purchased from Cell Signaling Technologies (Beverly, MA, USA) and detecting antibody peroxidase-swine-antirabbit IgG was from DAKO (Glostrup, Denmark). Antibodies for CRP and fibrinogen enzyme linked immunosorbent assay (ELISA) were obtained from DAKO, capture antibodies for C3 ELISA were from Calbiochem (San Diego, CA, USA), and

detecting antibodies were from ICN/Biomedicals (Irvine, CA, USA). Antibodies and ELISA for SAA were developed in our laboratory.¹⁶

All reagents for RNA isolation and reverse transcriptase reaction were purchased from Invitrogen, Life Technologies (Gaithersburg, MD, USA). Reagents for real time reverse transcriptase-polymerase chain reaction (RT-PCR) were obtained from Applied Biosystems (Foster City, CA, USA).

Culture of hepatoma cell lines

Cell lines were maintained in DMEM supplemented with 10% FCS and gentamycin in a humidified atmosphere of 5% CO₂/95% air. Hep3B and PLC/PRF/5 were passaged twice a week in a 1:3 ratio, HuH7 in a 1:10 ratio. HepG2 was passaged weekly in a 1:3 ratio. For experiments, the hepatoma cells lines were grown to confluence in 12-well (1 ml) or 24-well (0.5 ml) tissue culture plates (Corning, Schiphol, The Netherlands). Activation of the cells with 50 ng/ml IL6 and/or 10 ng/ml IL1 β or 10 ng/ml TNF α was performed in DMEM with 1% FCS and 0.1 μ mol/l dexamethasone.

Activation of p38 MAPK and STAT-3

Phosphorylation of MAPKAPK2, p38 MAPK, and STAT-3 in hepatoma cell lines was analysed by western blotting. Confluent cells were stimulated with (combinations of) IL6, IL1 β , or TNF α for 30 minutes in DMEM with 1% FCS and 0.1 μ mol/l dexamethasone with or without pretreatment with RWJ 67657 at 1 μ mol/l. Cell extracts were prepared and semidry blotting was performed as described previously.¹³ Blots were stripped with Restore Western Blot

Figure 2 Effect of RWJ 67657 pretreatment on CRP (A) and SAA (B) production in hepatoma cell lines after stimulation with (combinations of) cytokines. Cells were unstimulated or stimulated with IL1 β , IL6, TNF α , IL1 β + IL6, or TNF α + IL6 for 48 hours, with or without pretreatment with RWJ 67657 at 0.01, 0.1, 1, and 10 μ mol/l. CRP and SAA levels were measured in cell supernatants by ELISA and expressed in ng/ml. The mean and SEM is shown. ($p < 0.05$, $p < 0.001$, one way ANOVA with Dunn's multiple comparison test, tested against the stimulated control).

Stripping Buffer (Pierce, Rockford, IL, USA) after the first detection for second or third immunodetection.

Production of acute phase proteins by hepatoma cell lines

Cells were stimulated with IL6, IL1 β , and TNF α alone or in combination during 48 hours. The effect of p38 MAPK inhibition was investigated by preincubation for 1 hour with a concentration range of RWJ 67657 (0, 0.01, 0.1, 1, and 10 μ mol/l, diluted from stock solution of 10 mmol/l in dimethylsulphoxide). Production of acute phase proteins was determined in the culture supernatants by ELISAs. The detection limits of the assays were 0.1 ng/ml and 40 ng/ml for CRP and fibrinogen respectively. The detection limit for the C3 assay was 80 ng/ml.

The SAA ELISA was performed as described previously.¹⁶ In brief, a capture monoclonal antibody (Reu.86.5, which reacts

with all subtypes of SAA) was coated (1:1000), followed by incubation with diluted supernatants or standards. Detection was with a peroxidase conjugated monoclonal antibody (Reu.86.1, specific for SAA-1), followed by the substrate reaction. The detection limit of the assay was 2 ng/ml.

mRNA analysis of acute phase proteins

Hepatoma cells (PLC/PRF/5 and Hep3B) were grown to confluence in 12-well plates and stimulated for 24 hours as described above. One hour's pretreatment with RWJ 67657 at 0, 0.1, 1 and 10 μ mol/l was done for all stimulations. Total RNA was isolated from the cells with TRIzol reagent, according to the manufacturer's instructions (Invitrogen, Life Technologies). DNase treatment (Ambion, Huntingdon, Cambridgeshire, UK) was performed and, subsequently, cDNA was synthesised from 2.0 μ g of total RNA using M-MLV Reverse Transcriptase and oligo (dT)₁₄₋₁₈ (Invitrogen,

Figure 3 Effect of RWJ 67657 pretreatment on mRNA expression of CRP, SAA-1, fibrinogen- β , fibrinogen- γ , and complement C3 in Hep3B cells after stimulation with (combinations of) cytokines. Cells were stimulated with IL1 β , IL6, IL1 β + IL6, TNF α , and TNF α + IL6 for 24 hours with or without pretreatment with RWJ 67657 at 0.1, 1, and 10 $\mu\text{mol/l}$. mRNA expression was determined with real time RT-PCR. Results are expressed as fold induction compared with unstimulated cells (fold induction = 1). Bars show mean (n=3) and SEM. ($p < 0.05$, one way ANOVA with Dunn's multiple comparison test, tested against the stimulated control).

Life Technologies). For the measurement of mRNA for albumin, CRP, SAA, C3, fibrinogen- β , fibrinogen- γ , and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 1 μl of cDNA in duplicate was used for amplification by the Taqman real time PCR system (ABI Prism 7900HT Sequence Detection System, Applied Biosystems) with specific Taqman primers/probes. For SAA-1, suitable primers and probe were developed between the transit of exon 3 and 4 using the software program Primer Express 2.0 (Applied Biosystems) and the assay was ordered by Assay-by-Design.

Amplification was performed using standard conditions: denaturation at 95°C for 15 seconds, 40 cycles of amplification with annealing at 60°C for 1 minute, and extension at 50°C for 2 minutes. According to the comparative Ct (threshold cycle value) method described in the ABI manual, the resulting mRNA amount of the gene of interest was normalised to the housekeeping gene GAPDH, yielding the ΔCt value. The ΔCt value of unstimulated cells was subtracted

from the average ΔCt value of each sample, yielding the $\Delta\Delta\text{Ct}$. The amount of target, normalised to an endogenous reference (GAPDH) and relative to the control sample, is given by $2^{-\Delta\Delta\text{Ct}}$.

Statistics

One way analysis of variance (ANOVA) with Dunn's multiple comparison test was performed using GraphPad Prism version 3.00 for Windows, GraphPad Software (San Diego, CA, USA).

RESULTS

Activation of p38 MAPK and STAT-3

Phosphorylation of p38 MAPK and STAT-3 in the cell lines Hep3B and PLC/PRF/5 was analysed after stimulation with (combinations of) IL6, IL1 β , and TNF α for 30 minutes. Figure 1 shows that phosphorylation of p38 MAPK in

Table 1 Effect of RWJ 67657 pretreatment on fibrinogen production ($\mu\text{g/ml}$) in four hepatoma cell lines (mean of six experiments)

RWJ ($\mu\text{mol/l}$)	Unstimulated	IL1 β	IL6	IL1 β + IL6	TNF α	IL6 + TNF α
<i>PLC/PRF</i>						
0	2.77	2.13	10.33	5.92	2.35	8.77
0.01	2.47	1.77	10.42	5.57	2.05	8.00
0.1	2.68	1.65	8.67	5.12	1.87	7.20
1	2.35	1.45	8.23	4.57	1.48	5.58*
10	1.68	0.85	5.10*	2.48**	0.95	2.57***
<i>Hep3B</i>						
0	BD	BD	1.62	0.58	BD	0.35
0.01			1.25	0.62		0.30
0.1			0.80	0.43		0.20
1			0.6*	0.28		0.13
10			0.27***	0.10**		0.10
<i>HepG2</i>						
0	5.21	5.09	20.38	20.30	ND	ND
0.01	4.82	5.45	18.08	17.61		
0.1	4.30	5.40	17.97	16.58		
1	3.93	4.41	15.15	11.44*		
10	2.65	2.70	9.71**	5.73***		
<i>HuH7</i>						
0	2.88	2.90	10.93	5.77	ND	ND
0.01	2.23	2.53	9.82	5.56		
0.1	2.25	2.27	8.12	6.00		
1	2.08	1.81	7.70*	4.47*		
10	1.78	1.25	6.30***	2.56**		

Cells were unstimulated or stimulated with (combinations of) cytokines for 48 hours, with or without pretreatment with RWJ 67657 at 0.01, 0.1, 1, and 10 $\mu\text{mol/l}$. Fibrinogen levels were measured in cell supernatants by ELISA and expressed in $\mu\text{g/ml}$. The table shows the mean and SEM ($p < 0.05$, $p < 0.01$, $p < 0.001$, one way ANOVA with Dunn's multiple comparison test, tested against the stimulated control). BD, below detection limit; ND, not determined).

Hep3B occurred with all stimuli, including IL6, and inhibition of phosphorylation due to the p38 MAPK inhibitor was shown for MAPKAPK2, the downstream substrate of p38 MAPK. Strong activation of STAT-3 was demonstrated after IL6 stimulation, which was not affected by p38 MAPK inhibition.

Production of acute phase proteins by hepatoma cell lines

In the supernatants of the four different hepatoma cell lines the production of the acute phase proteins CRP, SAA, fibrinogen, and C3 was measured by ELISA. CRP (type-1 APP) production could not be detected in HepG2 and HuH7

Table 2 Effect of RWJ 67657 pretreatment on C3 production ($\mu\text{g/ml}$) in four hepatoma cell lines (mean of six experiments)

RWJ ($\mu\text{mol/l}$)	Unstimulated	IL1 β	IL6	IL1 β + IL6	TNF α	IL6 + TNF α
<i>PLC/PRF</i>						
0	0.72	1.57	2.03	1.62	1.22	1.47
0.01	0.73	1.28	1.37	1.37	1.25	1.52
0.1	0.65	1.20	1.65	1.33	1.12	1.45
1	0.60	1.07*	1.45	1.55	0.98	1.25
10	0.52	0.82***	1.55	1.12	0.72	0.88
<i>Hep3B</i>						
0	0.82	3.13	3.85	5.27	1.80	3.23
0.01	0.67	3.72	5.27	6.00	2.05	3.17
0.1	0.65	3.42	4.37	6.00	1.82	2.95
1	0.65	2.57	2.67	4.97	1.48	2.48
10	0.55	1.72**	2.45	2.28*	1.32	1.95
<i>HepG2</i>						
0	5.58	11.12	7.33	9.25	ND	ND
0.01	4.70	12.95	6.77	7.63		
0.1	5.22	11.53	8.30	7.45		
1	4.70	8.70	5.77*	6.88		
10	3.83	6.40*	4.97**	4.57*		
<i>HuH7</i>						
0	0.48	2.67	1.42	2.83	ND	ND
0.01	0.47	3.02	1.30	2.28		
0.1	0.58	2.87	1.42	2.53		
1	0.75	2.47	1.77	2.22		
10	1.23	1.52*	0.92	2.07		

Cells were unstimulated or stimulated with (combinations of) cytokines for 48 hours, with or without pretreatment with RWJ 67657 at 0.01, 0.1, 1, and 10 $\mu\text{mol/l}$. C3 levels were measured in cell supernatants by ELISA and expressed in $\mu\text{g/ml}$. The table shows the mean and SEM ($p < 0.05$, $p < 0.01$, one way ANOVA with Dunn's multiple comparison test, tested against the stimulated control). ND, not determined.

cell lines. In PLC/PRF/5, the highest CRP production was seen after combined IL1 β and IL6 stimulation, and to a lesser extent after TNF α stimulation alone or together with IL6. In Hep3B, CRP production was found exclusively after simultaneous stimulation with IL1 β and IL6 (fig 2A). In both cell lines, CRP production was significantly inhibited by RWJ 67657 both at 1 and 10 μ mol/l.

High production of SAA was detected in all hepatoma cell lines after combined stimulation with IL1 β and IL6 (fig 2B). Inhibition due to p38 MAPK inhibition was seen only at 10 μ mol/l in Hep3B and HuH7. No significant inhibition was seen in PLC/PRF/5 and HepG2.

Table 1 shows the results of fibrinogen production. Fibrinogen is constitutively produced in the liver and in cultured hepatoma cell lines as well. Fibrinogen is a type 2 APP, and after stimulation with IL6 a maximal fourfold increase of production is seen. In Hep3B, fibrinogen production was not constitutive and could only be detected after IL6 or combined stimulation. Significant inhibitory effects of p38 MAPK at 10 μ mol/l were seen in all four cell lines after stimulation with IL6 alone or in combination with IL1 β . In Hep3B, HepG2, and HuH7 inhibition was seen at 1 μ mol/l, but not in PLC/PRF/5.

In table 2, we show the results of C3 production in the hepatoma cell lines. Like fibrinogen, C3 is also constitutively produced, and production is increased after stimulation with all cytokines and combinations. Significant inhibition with RWJ 67657 at 10 μ mol/l was seen in all four cell lines after IL1 β stimulation and in Hep3B and HepG2 after IL1 β + IL6 stimulation. In HepG2, there was also significant inhibition of IL6 induced C3 production at 1 μ mol/l. Pretreatment of hepatoma cells with 0.1% dimethylsulphoxide had no significant effect on any acute phase protein production (data not shown).

mRNA analysis of acute phase proteins

With quantitative RT-PCR, mRNA expression of CRP, SAA-1, fibrinogen- β , fibrinogen- γ , complement C3, and albumin was measured in Hep3B and PLC/PRF/5. In HepG2 and HuH7, no CRP mRNA could be detected, as was seen for CRP protein production. Figure 3 shows the results of mRNA expression of three different experiments in Hep3B cells after 24 hours' stimulation. Results of mRNA analysis in PLC/PRF/5 were comparable. CRP and SAA-1 mRNA expressions were abundantly induced, especially after combined stimulation with IL6 and IL1 β . Pretreatment with RWJ 67657 at 0.1, 1 or 10 μ mol/l reduced CRP mRNA expression in a dose dependent way but not SAA-1 mRNA expression. Reduction of CRP mRNA at 10 μ mol/l was significant both after IL6 and combined IL6/IL1 β stimulation. Fibrinogen and C3 mRNA are constitutively expressed, but were increased by cytokine stimulation. Fold inductions after IL6, combined IL6/IL1 β , and combined IL6/TNF α stimulation for fibrinogen- β and fibrinogen- γ were 45.0, 21.0, 7.7 and 13.8, 5.3, 2.5, respectively. mRNA induction for both fibrinogen chains was significantly reduced by treatment with RWJ 67657 at 10 μ mol/l after IL6 stimulation. C3 mRNA expression was induced after all stimulations, but there was no inhibitory effect detectable due to pretreatment with RWJ 67657. Albumin mRNA expression was reduced in all stimulated cells, and was further reduced by p38 MAPK inhibitor at 10 μ mol/l in IL6 stimulated cells (data not shown).

DISCUSSION

In this study, the effect of p38 MAPK inhibition on the acute phase response was investigated in hepatoma cell lines. We found significant inhibition of CRP and fibrinogen production with RWJ 67657 at 1 μ mol/l, which is considered a pharmacologically relevant concentration.

Knowledge of the central role of IL1 and TNF α in inflammation has led to the successful treatment by cytokine blocking agents in RA. Also the use of anti-IL6 antibody intended to block the JAK/STAT pathway seems promising.¹⁷ The interest in the regulation of cytokine production by signal transduction pathways and transcription factors has led to new therapeutic targets.¹⁸ For RA several p38 MAPK inhibitors have been designed, which have been tested preclinically and in clinical studies.¹¹⁻¹⁴ In clinical trials, responses are evaluated using the American College of Rheumatology response criteria and several laboratory markers, including acute phase proteins. Recent studies investigating prognostic factors in RA,¹⁹ but also work from our group,⁴ have demonstrated that increased levels of CRP, especially, are predictive for continuing joint damage. It is important to know whether the levels of acute phase proteins reflect disease activity during treatment, or whether the production itself is influenced by treatment.

By western blotting it was demonstrated that p38 MAPK could be activated after IL6 stimulation and that MAPKAPK2 phosphorylation effectively was blocked by p38 MAPK inhibition. A study by Zauberman *et al* demonstrated that the p38 MAPK inhibitor SB 203580 reduced IL6-induced MAPKAPK2 activation in HepG2 cells and that haptoglobin and α_1 acid glycoprotein levels were also reduced by p38 MAPK inhibition.¹⁰

In contrast with others,²⁰⁻²¹ it was not possible for us to detect CRP production or mRNA expression by HepG2 and HuH7 cells, making these cell lines less valuable as models to study acute phase response reactions. Induction of CRP production and mRNA expression, however, was found in Hep3B and PLC/PRF/5 cells, which could be reduced significantly by pretreatment with the p38 MAPK inhibitor at 1 μ mol/l. The study by Parasrampur *et al* demonstrated that after a single oral dose of RWJ 67657, ranging from 0.25 to 30 mg/kg, a plasma concentration of 0.01 to 6 μ mol/l of the p38 MAPK inhibitor could be reached in humans,²² so 1 μ mol/l is a pharmacologically relevant concentration. All four cell lines showed high production of SAA, and inhibition by RWJ 67657 was seen only at 10 μ mol/l. Strong induction of SAA-1 mRNA was demonstrated in PLC/PRF/5 and Hep3B after combined IL6 and IL1 β stimulation. Our results confirmed the data reported by Hagihara *et al*,²³ concerning the critical role of IL6 in the synergistic induction of SAA gene expression in hepatoma lines. Inhibition by RWJ 67657 pretreatment could not be detected.

Fibrinogen synthesis is mainly mediated by IL6,³ as confirmed in our studies in the four hepatoma cell lines. Combined stimulation induces lower fibrinogen production, owing to cross talk between IL6 and IL1 β signalling pathways, leading to IL1 β dependent down regulation of STAT-1 phosphorylation.²⁴ p38 MAPK treatment additionally reduces fibrinogen production whichever stimulus is used, apparently by another mechanism. Fibrinogen has an important role in the coagulation cascade, but is also an important acute phase protein, and the often used erythrocyte sedimentation rate largely depends on the fibrinogen concentration. The fact that fibrinogen production is indeed reduced by p38 MAPK treatment could have important implications for the use of erythrocyte sedimentation rate as marker of disease activity.

CRP and SAA are the major acute phase reactants in RA, but they have a different function and are differentially regulated,³ although both CRP and SAA are potentially induced by combined IL6 and IL1 β stimulation. The inhibition of CRP production by RWJ 67657 at the pharmacologically relevant concentration of 1 μ mol/l, whereas SAA production is not inhibited, might be explained by differences in the acute phase responsive elements in the promoter regions of the two

APPS.²⁵ Cunnane *et al* demonstrated that SAA is the best marker for the assessment of inflammatory joint disease,²⁶ while Rau *et al* demonstrated that in acute pancreatitis SAA had a wider dynamic range, but measurement of CRP provided an earlier differentiation between patients.²⁷ The results presented in this study suggest that SAA measurement should be included in clinical trials when p38 MAPK inhibitors are used.

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