Inhibition of Inflammatory Cytokine Production from Rheumatoid Synovial Fibroblasts
by a Novel IκB Kinase Inhibitor

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List of non-standard abbreviations: ACHP, 2-amino-6-[2-(cyclopropylmethoxy)-6-hydroxyphenyl]-4-piperidin-4-yl nicotinonitrile; ATL, adult T-cell leukemia; ChIP, chromatin immunoprecipitation; CHPD, (7-[2-(cyclopropylmethoxy)-6-hydroxyphenyl]-5-[(3S)-3-piperidinyl]-1,4-dihydro-2H-pyrido[2,3-d][1,3]oxazin-2-one hydrochloride); ELISA, enzyme-linked immunosorbent assay; IKK, IkB kinase; IL, interleukin; MKK, mitogen-activated protein kinase kinase; MMP, matrix metalloproteinase; NF-κB, nuclear factor kappa B; PBS, phosphate-buffered saline; PI3K, phosphatidylinositol 3-kinase; RA, rheumatoid arthritis; RSF, rheumatoid synovial fibroblasts; RT, reverse transcription; TBS-T, Tris-buffered saline with Tween 20; TNFα, tumor necrosis factor α; VCAM, vascular cell adhesion molecule.

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Abstract

Nuclear factor kappa B (NF-κB) is involved in pathophysiology of Rheumatoid arthritis (RA) and is considered to be a feasible molecular target in treating patients. In the RA joint tissues activation of NF-κB is often observed together with high amounts of proinflammatory cytokines, TNFα and IL-1β. TNFα and IL-1β are known to stimulate NF-κB signalling and produced as the effect of NF-κB signalling, thus forming a vicious cycle leading to a self-perpetuating nature of rheumatoid inflammation and expansion of such inflammatory response to other joints. Since a kinase called IκB kinase (IKK) is involved in the NF-κB activation cascade, we examined the effect of a novel IKK inhibitor, (7-[2-(cyclopropylmethoxy)-6-hydroxyphenyl]-5-[(3S)-3-piperidinyl]-1,4-dihydro-2H-pyrido[2,3-d][1,3]oxazin-2-one hydrochloride) (CHPD), on the production of inflammatory cytokines from rheumatoid synovial fibroblasts (RSF). TNFα stimulation induced production of inflammatory cytokines such as IL-6 and IL-8 in RSF and the extents of IL-6 and IL-8 induction were dramatically reduced by CHPD under non-cytotoxic concentrations. Similarly, expression of il-6 and il-8 genes was significantly reduced by CHPD. In addition, ChIP assays revealed that the DNA-binding of NF-κB (p65) to il-8 promoter in RSF was induced after TNFα stimulation, and upon CHPD treatment to RSF for 1 h the NF-κB binding to il-8 promoter was significantly decreased. In this paper, we have demonstrated that an IKKβ inhibitor, CHPD, acts as an effective inhibitor for the production of inflammatory cytokines in response to proinflammatory cytokines. These findings indicate that such IKKβ inhibitor could be a feasible candidate of anti-rheumatic drug.
Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disease that affects systemic synovial joints (Firestein, 2003). In RA, proliferation of synovial cells and infiltration of activated immuno-inflammatory cells including T cells, macrophages and plasma cells (Firestein, 2003) leads to progressive destruction of cartilage and bone. Various cytokines, including interleukin-1β (IL-1β), tumor necrosis factor α (TNFα), IL-6, IL-8, IL-17 and macrophage colony stimulating factor, are present in the synovial fluid and tissue of RA patients (Okamoto, 2006).

Synovial hyperplasia in RA is considered to be due to the impairment of apoptosis (Huber et al., 2006). Most of the above mentioned pathophysiological features of RA can be explained by activation of a transcription factor nuclear factor kappa B (NF-κB) (Feldmann, 2001; Okamoto, 2005; Okamoto, 2006), which is highly activated in the synovial lining cells of RA joint tissue (Sakurada et al., 1996; Huber et al., 2006). NF-κB induces both TNFα and IL-1β gene expression while TNFα and IL-1β stimulate NF-κB signalling, forming a vicious cycle that can perpetuate and expand the inflammatory responses. Thus, blocking this cascade by inhibiting NF-κB signalling is considered feasible for the treatment of RA.

Interestingly, some of the drugs for RA were shown to block either the NF-κB-activation cascade or its action (Yang et al., 1995; Yamamoto and Gaynor, 2001; Okamoto, 2006). For example, monovalent gold compounds, often used for RA treatment, could inhibit the DNA-binding activity of NF-κB through oxidation of the cysteins associated with zinc (Yang et al., 1995; Yoshida et al., 1999b). Similarly, methotrexate is known to suppress NF-κB activation (Majumdar and Aggarwal, 2001). In addition, intervention therapies using anti-TNFα antibody and soluble TNFα receptor exhibited dramatic therapeutic efficacies by blocking the vicious cycle of NF-κB activation cascade mentioned above (Elliott et al., 1993;
Moreland et al., 1997). However, these drugs are expensive and have to be administered by injection and various adverse effects were reported, thus warranting necessity of effective small molecular compounds.

NF-κB is a hetero- or homodimer consisting of Rel family proteins, p65 (RelA), RelB, c-Rel, p50/p105 and p52/p100, and normally present in the cytoplasm in association with its inhibitor, IκB (Zabel and Baeuerle, 1990). Stimulation by the proinflammatory cytokines such as TNFα and IL-1β results in the activation of IκB kinase (IKK) complex (Nakano et al., 1998), which consists of three subunits, IKKα, IKKβ and IKKγ/NEMO. Activated IKK complex, mainly through the IKKβ activity (Mercurio et al., 1997; Zandi et al., 1997; Li et al., 1999b; Karin and Delhase, 2000), phosphorylates IκBα leading to ubiquitination and degradation of IκB. NF-κB, then, translocates to nucleus and binds to the κB site of target genes. In this regard, IKKβ is a reasonable molecular target for blocking NF-κB signalling upon inflammatory stimuli.

NF-κB is highly activated not only in the synovial tissue of patients with RA, but also in some types of neoplasm such as multiple myeloma and adult T-cell leukaemia (ATL) (Mori et al., 1999; Sanda et al., 2005; Sanda et al., 2006). In these cells, NF-κB is constitutively activated as evidenced by (1) the continuous phosphorylation of IκBα and p65 subunit of NF-κB, (2) activation of NF-κB DNA binding and (3) upregulation of various target genes that are responsible for inhibition of apoptosis. Moreover, a specific IKK inhibitor, ACHP (2-amino-6-[2-(cyclopropylmethoxy)-6-hydroxyphenyl]-4-piperidin-4-yl nicotinonitrile) (Murata et al., 2004), could inhibit cell growth and induce apoptosis of multiple myeloma and ATL cells (Sanda et al., 2005; Sanda et al., 2006) by inhibiting the phosphorylation of IκBα and p65.

In this study, we examined the effects of a novel IKK inhibitor, CHPD (7-[2-(cyclopropylmethoxy)-6-hydroxyphenyl]-5-[(3S)-3-piperidinyl]-1,4-dihydro-2H-pyrido[2,3-
d][1,3]oxazin-2-one hydrochloride) (Ziegelbauer et al., 2005), a chemical derivative of ACHP, on the cytokine production of rheumatoid synovial cell. ACHP was initially synthesized by Murata et al. (Murata et al., 2004) based on a massive screening while CHPD was identified among the synthesized derivatives of ACHP with highest selectivity for IKKβ and IKKα (IC₅₀ values for IKKβ and IKKα are 2 and 135 nM, respectively, by in vitro kinase assays) over other kinases (Ziegelbauer et al., 2005). In addition, CHPD showed good aqueous solubility and cell-permeability, thus demonstrating a very high oral bioavailability in mice and rats (Ziegelbauer et al., 2005). However, effects of CHPD on the production of inflammatory cytokines have never been examined. Here we show that CHPD could effectively block NF-κB pathway in rheumatoid synovial fibroblasts (RSF) and inhibit the production of IL-6 and IL-8 from these cells upon induction of NF-κB by TNFα. Future perspective of this compound in the treatment of RA is discussed.
Methods

Reagents:

A relatively selective IKKβ inhibitor, CHPD (7-[2-(cyclopropylmethoxy)-6-hydroxyphenyl]-5-[(3S)-3-piperidinyl]-1,4-dihydro-2H-pyrido[2,3-d][1,3]oxazin-2-one hydrochloride) (Ziegelbauer et al., 2005) was a kind gift from Dr. T. Murata of Bayer Yakuhin Inc. (Kyoto, Japan). The chemical structure of CHPD is shown in Fig. 1a. Human recombinant TNFα was purchased from Roche (Mannheim, Germany) and used at 1.0 ng/ml for NF-κB stimulation. Antibodies for IκBα (sc-371), p65 (sc-372) and α-tubulin (sc-8035) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA), while the antibody for phospho-IκBα (Ser32) (#9241L), JNK (#9252), phospho-JNK (Thr183/Tyr185) (#9251), ERK1/2 (#9102) and phosphor-ERK1/2 (Thr202/Tyr204) (#9191) was purchased from Cell Signaling Technology (Beverly, MA). Murine monoclonal TNFα antibody (MAB610) used in the neutralization assay (Fig. 1b) was commercially obtained (R&D Systems, Minneapolis, MN) and used at 10 μg/ml. Horseradish peroxidase-conjugated secondary antibodies were obtained from Amersham Biosciences (Little Chalfont, United Kingdom).

Patients:

RSF cultures were isolated from fresh synovial tissue biopsy samples from six RA patients at total knee arthroplasty or arthroscopic synovectomy as previously reported (Sakurada et al., 1996; Yoshida et al., 1999a; Yoshida et al., 1999b). Diagnosis of RA was made according to the clinical criteria of the American College of Rheumatology (Arnett et al., 1988). These RA patients included five females and one male, from 37 to 63 years old, and all patients had active RA at various clinical stages and classes. The mean disease
duration was 7.7 ± 6.7 year with a range of 1.4-18.0 years. Clinical characteristics of each
donor are shown in Table1. None of the patients had been treated with any biologic agents
such as Infliximab and Etanercept. Informed consents were obtained from each patient in
conformity with the requirements of the ethics committee of the Nagoya City University
Graduate School of Medical Sciences.

**Cell culture:**

RSF cultures were performed as previously reported (Sakurada et al., 1996; Yoshida et
al., 1999a; Yoshida et al., 1999b). Briefly, fresh synovial tissue biopsy samples were minced
into small pieces and treated with 1 mg/ml collagenase/dispase (Roche Diagnostics GmbH,
Mannheim, Germany) for 10-20 min at 37 °C. The cells were cultured in F-12 (HAM)
(Invitrogen Co.) supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml of
penicillin, 100 μg/ml of streptomycin and 0.5 mM mercaptoethanol. The culture medium was
changed every 3-5 days and nonadherent lymphoid cells were removed during the initial
passages. All the experiments were conducted using synoviocyte cultures during the third to
eighth passages. To characterize the cytological phenotype, the cells were stained with mouse
monoclonal antibodies against human HLA-DR, von Willebrand factor, desmin, smooth
muscle actin, CD1a, CD68 and 5B5 (DAKO, Glostrup, Denmark). Only 5B5 was positive for
RSF, indicating their fibroblast-like phenotype, consistent with our previous findings
(Sakurada et al., 1996; Yoshida et al., 1999a; Yoshida et al., 1999b). There was some
heterogeneity in cell growth property of each RSF preparation, however, there was no
quantitative difference that warrant aggressive nature of synovial cells. Human embryonic
kidney 293 cells were grown at 37 °C in Dulbecco’s modified Eagle’s medium (Sigma) with
10% heat-inactivated fetal bovine serum, 100 U/ml of penicillin and 100 μg/ml of
streptomycin.
Cytokine assays:

The cytokine concentrations were determined using cytokine-specific ELISA kits for IL-6 and IL-8 (Quantikine ELISA kits; R&D Systems) in RSF and 293 cells culture supernatant with experimental procedures recommended by the manufacturer. Triplicates were used for each test condition in the three independent cultures.

Cell proliferation assay:

In order to examine the cytotoxicity of CHPD, the cell proliferation of RSF upon treatment with CHPD at various concentrations was determined using WST-1 (Roche Diagnostics) according to the manufacturer's protocol. In brief, RSF cultures were incubated with CHPD in a 96 well plate for 24 h, incubated further for 4 h in the presence of WST-1, and the dissolved formazan was measured at 450 nm by spectrophotometry.

RT-PCR:

To measure mRNA expression of various genes, 2.0 x 10^5 RSF cells were cultured at 37 °C in CO₂ incubator, washed once with PBS, homogenized with QIAshredder (Qiagen, Alameda, CA), and total RNA was purified using RNeasy (Qiagen) according to manufacturer’s protocol. After incubation with DNase I (Invitrogen), 1.0 μg of total RNA was reverse transcribed using SuperScript First-Strand synthesis System (Invitrogen). The cDNA was then amplified from each RNA sample with HotStarTaq Master Mix Kit and gene-specific primers. The number of cycles was selected to allow linear amplification of the cDNA under study. The PCR products were analyzed by agarose gel electrophoresis. The oligonucleotide primers were as follows: *il-6*, sense 5’-TCT CAG CCC TGA GAA AGA AGA C-3’ and antisense 5’-GAA GAG CCC TCA GGC TGG ACT G-3’; *il-8*, sense 5’-GCA GCT CTG TGT GAA GGT GC-3’ and antisense 5’-TCC TTG GGG TCC AGA CAG AG-3’; *β-actin*, sense 5’-CCA GGC ACC AGG GCG TGA TG-3’ and antisense 5’-CGG CCA GCC
AGG TCC AGA CG-3’; matrix metalloproteinase (mmp)-3, sense 5’-GGA GGA AAA CCC ACC TTA CAT AC-3’ and antisense 5’-AGT GTT GGC TGA GTG AAA GAG AC-3’; vascular cell adhesion molecule (vcam)-1, sense 5’-GTC TGC ATC CTC CAG AAA TTA C-3’ and antisense 5’-TAA AAT CGA GAC CAC CCC-3’. The relative amount of each PCR product was quantified by densitometric scanner using Image J software. (http://rsbweb.nih.gov/ij/download.html).

Immunoblot analyses:

For detection and analyses of various proteins, RSF cells were maintained with or without CHPD at 37 °C. These cells were washed once with cold phosphate-buffered saline (PBS) and resuspended in 50 μl of lysis buffer containing 20 mM HEPES-NaOH (pH7.9), 150 mM NaCl, 1 mM EDTA, 1 mM Na3VO4, 5 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 0.2% Triton X-100 and protease inhibitors cocktail (Roche Diagnostics). After 15 min of incubation on ice, the samples were centrifuged at 15,000 rpm for 10 min and the supernatant was collected as “whole cell extract”. Protein concentration was measured using detergent-compatible protein assay (Bio-Rad, Hercules, CA). Equal amounts of the proteins were electrophoresed on 10% SDS-polyacrylamide gel and transferred onto a polyvinylidene fluoride membrane (Millipore Corporation, MA). The membranes were blocked with Tris-buffered saline with Tween 20 (TBS-T) (10 mM Tris-HCl [pH 8.0], 150 mM NaCl, 0.1% Tween 20) containing 5% nonfat milk for 1 h at room temperature, and incubated with TBS-T containing 5% nonfat milk and 1:1,000 diluted specific antibodies overnight at 4 °C. After incubation, the membranes were rinsed three times with TBS-T and further incubated with horseradish peroxidase-conjugated secondary antibodies in TBS-T with 5% nonfat milk at room temperature for 1 h. Each protein was detected by chemi-luminescence using SuperSignal (Pierce, Rockford, IL).
Transfection and luciferase assay:

The 293 cells (1.0 x 10^5/well) were transfected with reporter plasmids using FUGENE 6 transfection reagent (Roche Applied Science) according to the manufacturer’s instructions. For each transfection, 0.03 μg of 4kB-luc, where luciferase gene expression is under the control of NF-κB, and 0.01 μg of the internal control plasmid, pRL-TK, expressing Renilla luciferase under the control of TK promoter, were used (Yang et al., 1999). Twenty-four hours after transfection, the cells were treated with CHPD for 30 min and stimulated with TNFα (1.0 ng/ml) for 24 h. The transfected cells were then harvested, and the extracts were subjected to luciferase assay using the Luciferase Assay System (Promega). The luciferase activity was normalized with Renilla luciferase activity as an internal control to assess the transfection efficiency.

ChIP assays:

Chromatin immunoprecipitation (ChIP) assays were performed using ChIP assay kits (ChIP-IT™ Express; Active Motif, Carlsbad CA) according to the protocol as previously reported (Imai and Okamoto, 2006) with minor modifications. In brief, 1.0 x 10^6 RSF cells either with or without CHPD treatment or TNFα stimulation were cross-linked by adding formaldehyde to the medium (1% final concentration) and incubated at room temperature for 10 min. The cells were then washed with cold PBS containing protease inhibitors and the fixation reaction was stopped by adding 10 ml “glycine stop-fix” solution. Samples were lysed for 30 min in lysis buffer on ice and the chromatin was sheared by sonication 40 times for 30 s each time at the maximum power with 30 s of cooling on ice between each pulse with a sonicator (Bioruptor; COSMO Bio, Tokyo, Japan). Cross-linked and released chromatin fractions were immunoprecipitated with magnetic beads and specific antibodies on a rolling shaker overnight at 4 °C. Cross-linking of the immunoprecipitates containing fragmented DNA was chemically reversed. Then, PCR was performed with a HotStarTaq Master Mix kit.
(QIAGEN). The PCR primers used for amplifying promoters containing the NF-κB binding sites included human *il-8* promoter (-121 to +61), 5’-GGG CCA TCA GTT GCA AAT C-3’ and 5’-TTC CTT CCG GTG GTT TCT TC-3’; and human *β-actin* promoter (-980 to -915), 5’-TGC ACT GTG CGG CGA AGC-3’ and 5’-TCG AGC CAT AAA AGG CAA-3’. The relative amount of *il-8* promoter DNA bound to p65 was quantified by densitometric scanner using Image J software that was downloadable from (http://rsbweb.nih.gov/ij/download.html).

**Statistical analysis:**

The statistical significances of difference were evaluated by the Steel-Dwass’s test with p<0.05 considered statistically significant.
Results

CHPD inhibited the spontaneous IL-6 production from RSF -

To investigate the inhibitory effect of CHPD on the production of IL-6 upon stimulation of RSF with TNFα (1.0 ng/ml), involving NF-κB activation, cytokine concentration was measured in the cell culture supernatant 24 h after stimulation. As shown in Fig. 1b, the extent of spontaneous production of IL-6 from RSF was much higher than that from 293 cells (data not shown), a human epithelial cell line and downregulated by CHPD but not by neutralizing antibody to TNFα. The extent of inhibition of the spontaneous production of IL-6 by 1.0 μM CHPD was 41%. In addition, a neutralizing antibody against IL-1β did not change the levels of IL-6 spontaneously produced from RSF (data not shown). The representative data with RSF1 are shown in Fig. 1b and similar results were observed with other RSF cultures (data not shown). These data suggest that this spontaneous production of cytokines might be due to other factors involving IKKβ. In addition, we did not detect detectable levels of TNFα or IL-1β production in this study (data not shown) which we have reported previously (Sakurada et al., 1996).

CHPD inhibited the IL-6 and IL-8 production induced by TNFα from RSF -

In Table 2, we have measured the levels of IL-6 and IL-8 production in the culture supernatant of RSF cultures obtained from 6 individual patients with RA. As shown, the extent of augmentation of production of inflammatory cytokines after 24 h TNFα stimulation were 1.8-7.1 fold and 19.6-35.7 fold for IL-6 and IL-8, respectively. The concentrations of spontaneous production of IL-6 without TNFα stimulation in
the absence and the presence of 1 μM CHPD were 2.3 ± 0.9 and 1.8 ± 0.6 ng/ml, respectively. Similarly, we measured the extents of inhibition of production of these cytokines upon TNFα stimulation by CHPD (as indicated by IC50 values). We noted a slight heterogeneity in the responsiveness to the TNFα treatment and CHPD in these synovial cell cultures. Interestingly, when RSF cultures from 6 RA patients were pretreated with CHPD for 1 h before TNFα treatment, the extents of IL-6 and IL-8 induction were significantly reduced by CHPD in a concentration-dependent manner (Fig.2a, b). Although there was heterogeneity in the responsiveness to CHPD, a concentration-dependent suppression of cytokine production by CHPD was noted. IL-8 appeared to be preferentially inhibited by CHPD. The extents of inhibition by 1.0 μM CHPD of the TNFα-induced production of IL-6 or IL-8 were 55% (Fig.2a) and 90% (Fig.2b) in average for IL-6 and IL-8, respectively. CHPD at these concentrations did not show significant cytotoxicity (Fig. 2c). These results indicate that CHPD suppressed the IL-6 and IL-8 cytokine levels that were induced by TNFα without significant inhibition of cell proliferation or cytotoxicity.

**CHPD inhibited TNFα-induced il-6 and il-8 mRNA expression -**

To further examine whether CHPD suppresses the TNFα-induced gene expression of *il-6* and *il-8*, we semi-quantitatively detected the mRNA levels of *il-6* and *il-8* using RT-PCR. As demonstrated in Fig. 3a, the *il-6* and *il-8* mRNA expression levels in RSF were increased as early as 1 h until 16 to 24 h after TNFα stimulation (at 1.0 ng/ml) and then gradually decreased. When RSF was pretreated with 1.0 μM CHPD for 1 h before the treatment with TNFα, the extent of induction of *il-6* and *il-8* mRNA were significantly reduced by CHPD (Fig. 3b). These results indicate that CHPD could inhibit *il-6* and *il-8* mRNA synthesis that were induced by
TNFα. In addition to il-6 and il-8, gene expressions of vcam-1 (Iademarco et al., 1992) and mmp-3 (Borghaei et al., 2004), also under the control of NF-κB, were induced by TNFα and effectively inhibited by CHPD.

**CHPD inhibited TNFα-induced IκBα phosphorylation and degradation**

TNFα stimulation activates IKK complex and the activated IKKs induce IκBα phosphorylation, leading to ubiquitination and subsequent degradation of IκBα by the 26S proteasome (Okamoto, 2006). We then examined the inhibitory effect of CHPD on TNFα-induced IκBα phosphorylation and degradation by immunoblot analysis. As shown in Fig. 4a, TNFα stimulation caused IκBα phosphorylation ("P-IκBα"), which was observed at 5 min after stimulation of RSF. However, treatment with CHPD reduced IκBα phosphorylation in a concentration-dependent manner (Fig. 4b). CHPD at 1.0 μM appeared to prevent the phosphorylation of IκBα in all the RSF cultures tested (data not shown). We then proceeded to examine whether CHPD could block IκBα degradation as well. As also shown in Fig. 4, the IκBα protein committed nearly complete degradation in RSFs 15 min after TNFα stimulation and blocked by 1.0 μM CHPD. In contrast, α-tubulin levels were unchanged, indicating equal loading of proteins in the gel. Furthermore, although MAPK family members including JNK and ERK were reported to be activated by TNFα stimulation (Firestein, 2003), CHPD failed to inhibit these kinases (Fig. 4c, d). In addition, although there are two JNK molecular species, p54 and p46, we could detect only p46 protein. The representative results with RSF3 are shown here and similar results were observed with other RSF cultures (data not shown). These results suggest that CHPD exhibited abrogation in the TNFα-induced NF-κB activation through inhibition of IKKβ without inhibiting MAPK activities.
CHPD inhibited gene expression driven by TNFα and analysis with chromatin immunoprecipitation (ChIP) assay.

We then examined the effect of CHPD on NF-κB dependent gene expression using luciferase assay with the 4xκB-luc reporter plasmid, where luc gene expression depends on NF-κB (Yang et al., 1999). Since RSF cultures were hardly susceptible to gene transfection even using various modifications of lipofection, a human cell line derived from embryonic kidney, 293 cells, that is more susceptible for gene transfection, were utilized instead. As shown in Fig. 5a, TNFα stimulated expression of NF-κB dependent gene such as 4xκB-luc by 17.0 ± 3.8 fold, which was similarly observed as in the case of TNFα-induced IL6/IL-8 production (Fig. 2, Table 2). When cells were pretreated with CHPD, however, the gene expression was inhibited in a concentration-dependent manner (Fig. 5a). Approximately 80% of this activity was inhibited at 2.0 μM of CHPD.

To further confirm the nuclear translocation and promoter binding of NF-κB, we have adopted ChIP assay and examined the inhibitory effect of CHPD on the TNFα-induced activation of NF-κB-DNA binding. As shown in Fig. 5b, ChIP assays revealed that the DNA-binding of the p65 subunit of NF-κB in il-8 promoter was induced after 60 min of TNFα stimulation. When RSF was pretreated with CHPD for 1 h, the recruitment of NF-κB to the il-8 promoter was significantly inhibited. No amplification in the absence of p65 antibody nor NF-κB-binding to β-actin promoter (internal control) was observed, confirming the specificity of the DNA immunoprecipitation and ChIP assays.
Discussion

The intervention therapy using blocker of TNFα, IL-1β and IL-6 has been developed and demonstrated remarkable therapeutic efficacies (Elliott et al., 1993; Moreland et al., 1997; Bresnihan et al., 1998; Nishimoto et al., 2004). These findings indicate that these proinflammatory cytokines, which eventually lead to activation of NF-κB, play crucial roles in the pathogenesis of RA among other signalling cascades (Firestein, 2003; Okamoto, 2006). However, these cytokine blockers currently used are considered “biologic agents” requiring intradermal or intravenous injections, inducing allergic reactions as well as adverse effects and consuming substantial medical resources. Thus, development of small molecular weight chemical compounds which share a similar molecular target is desperately needed.

The biological cascade involving NF-κB forms a positive feedback loop, or “vicious cycle”, that can perpetuate by itself and expand the inflammatory responses to other joints and tissues (Okamoto, 2006). Thus, inhibiting NF-κB signalling by blocking this cycle is considered to be a feasible treatment strategy of RA (Feldmann, 2001; Okamoto, 2006). There are multiple steps by which NF-κB is activated by extracellular signals: (1) binding of proinflammatory cytokines to their receptors; (2) signal transduction near the cytoplasmic membrane through signal transducers such as TRADD, TRAF2 and RIP; (3) upstream kinases including Phosphatidylinositol 3-kinase (PI3K), Akt and p38 mitogen-activated protein kinase; (4) the enzymatic activation of IKK complex; (5) proteasome mediated IκB degradation; (6) nuclear transport; and (7) the DNA-binding of the liberated NF-κB (Okamoto, 2006). On the other hand, a number of specific NF-κB inhibitors have been developed with these
signalling steps as targets. For example, dehydroxymethylepoxyquinomicin (DHMEQ) appears to inhibit the nuclear translocation of NF-κB (Wakamatsu et al., 2005) and PS341 (Bortezomib) was identified to inhibit proteasome (Adams et al., 1999). Among these target molecules, however, IKK complex appears to be specific for NF-κB activation and is the converging molecule of a number of distinct NF-κB-activating agents such as TNFα, IL-1β, B-cell activating factor, lymphotoxin β, CD40 and Toll-like receptor signalling (Okamoto, 2006). In addition, there are a number of compounds exhibiting the IKK inhibition activity. As previously reported, ACHP, CHPD and IMD-0560 inhibited IKKβ (Murata et al., 2004; Okazaki et al., 2005; Ziegelbauer et al., 2005) and the NF-κB essential modulator-binding peptide could dissociate IKKγ from the IKKα-IKKβ complex, thus inhibiting IKK activity (Jimi et al., 2004). Among these IKK inhibitors, CHPD appears to have higher specificity to IKKβ (in vitro IC₅₀ values for IKKβ and IKKα were 2 nM and 135 nM, respectively, whereas other kinases including IKKγ, MKK4, MKK7, ERK-1, Syk, Lck, Fyn, PI3Kγ, PKA and PKC were not inhibited at over 10 μM) (Ziegelbauer et al., 2005). Furthermore, CHPD inhibited the TNFα-induced NF-κB activation in 293 cell cultures without significant inhibition of cell proliferation.

In cell culture experiments, IC₅₀ and CC₅₀ values were 0.27-0.51 μM and 47.5 μM, respectively, with a therapeutic window of 93-176. However, considering the significant difference from the IC₅₀ value of IKKβ inhibition in vitro and in vivo (Ziegelbauer et al., 2005), we suggest that this compound needs further modification for efficient incorporation into human cells. It was also shown that CHPD could inhibit the lipopolysaccharide-induced production of TNFα in mice when administered orally without any systemic side effects such as weight loss and lethargy.
(Ziegelbauer et al., 2005). In addition, CHPD did not exert any inhibitory effect on JNK and ERK in RSF (Fig. 4d) and AP-1 in normal human lung fibroblast MRC5 (Ziegelbauer et al., 2005). These characteristics indicate that CHPD is one of the feasible candidates for therapeutic IKKβ inhibitor, although further improvements are needed as therapeutic compounds. Since the inhibitory effects of CHPD were observed with synovial cell cultures that showed relatively rapid cell growth ex vivo, further studies in this area are warranted.

As previously discussed, IKKβ represents the major effector kinase in the canonical pathway of NF-κB activation, IKKα on the other hand is primarily involved in the non-canonical pathway (Okamoto, 2006). Although IKKβ knockout mice exhibited early embryonic death because of the massive apoptosis in liver (Li et al., 1999a), gene knockout of IKKα showed only impairment in skin and digit abnormality such as syndactyly (Hu et al., 1999). Moreover, recent experimental evidences (Enzler et al., 2006; Okamoto, 2006) have indicated the crucial importance of IKKα in autoimmunity. However, considering the crosstalk between the canonical and non-canonical pathways, such as phosphorylation of p65 at Ser536 and subsequent induction of the transcriptional activity of NF-κB (Jiang et al., 2003a; Jiang et al., 2003b), application of IKKβ inhibitor for the treatment of rheumatic diseases should be considered.

We noticed the production of inflammatory cytokines such as IL-6 from rheumatoid synovial cell cultures without stimulation with TNFα (Fig. 1b). Since neutralizing antibodies to TNFα and IL-1β did not but CHPD did block the production of these cytokines, other factors that can stimulate the NF-κB cascade involving IKKβ, such as oxidative or environment stress and irradiation, are considered to be involved in such background activity. In addition, we could not find
any correlation between the clinical characteristics and the extent of TNFα-mediated cytokine production or the responsiveness to CHPD. Similar findings were reported by Miyazawa et al. (Miyazawa et al., 1998) where constitutive IL-6 production was observed without any stimulation in rheumatoid synoviocytes from 11 RA patients whereas no such background production of inflammatory cytokines was detected in dermal fibroblasts and synoviocytes from osteoarthritis.

In conclusion, NF-κB activation in synovial cells and production of inflammatory cytokines by proinflammatory cytokines such as TNFα and IL-1β plays a crucial role in rheumatoid arthritis. Our observations clearly indicate that the IKK inhibitors such as CHPD have therapeutic efficacy in the inflammatory processes associated with rheumatoid arthritis. Further development of IKK inhibitors are needed for the development of feasible and affordable drug therapy against RA.
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References


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Legends for Figures

Figure 1. Inhibition of the spontaneous IL-6 production by CHPD in rheumatoid synovial fibroblasts.

(a) Structure of CHPD. IC50 values for various kinases are described. “Other kinases” include IKKγ, MKK4, MKK7, ERK1, Syk, Lck, Fyn, PI3Kγ, PKA and PKC (28). Mw, molecular weight (Daltons). (b) Amounts of IL-6 produced in the absence of TNFα. RSF cells were cultured with or without 1.0 μM CHPD and 10 μg/ml TNFα antibody, and the concentrations of IL-6 in the culture supernatant were measured using ELISA. The symbol “-” indicates that only DMSO was added in all the experiments of Figs. 1, 2, 3, 4 and 5. Anti-TNFα, anti-TNFα antibody.

Figure 2. Inhibition of the TNFα induced IL-8 and IL-6 production by CHPD in rheumatoid synovial fibroblasts.

(a) Summary of IL-6 production by TNFα and its inhibition by CHPD. Amounts of IL-6 produced in the presence of TNFα as in Fig. 1b. RSF cell cultures from six patients with RA were individually stimulated with 1.0 ng/ml TNFα in the presence or absence of CHPD and the concentrations of IL-6 in the culture supernatant were measured using ELISA. (b) Summary of IL-8 production by TNFα and its inhibition by CHPD. Amounts of IL-8 produced in the presence of TNFα. RSF cell cultures were stimulated with TNFα in the presence or absence of CHPD. The concentrations of IL-8 in the culture supernatant were measured. (c) Cell proliferation of CHPD on RSF cultures. Cell proliferation of three RSF cultures in the presence of various concentrations of CHPD were determined by the WST-1 method. The mean 50% cytotoxic concentration (CC50 value) of 47.5 μM was extrapolated from this measurement.
Figure 3. Inhibitory effects of CHPD on NF-κB-driven gene expression.

(a) Time course of il-6 and il-8 mRNA expression induced by 1.0 ng/ml TNFα stimulation. The total RNA was prepared from RSF cultures and examined for expression of the NF-κB-dependent genes such as il-6 and il-8. The relative amounts of PCR products were quantified by densitometric scanning using Image J software. (b) Effects of CHPD on the expression of il-6, il-8, mmp-3, vcam-1 genes induced by 1.0 ng/ml TNFα. RSF cultures were stimulated by TNFα for 16 h with or without pre-treatment of 1.0 μM CHPD that was added 1 h before. The representative data with two independent RSF cultures are shown.

Figure 4. Inhibition of TNFα induced IκBα phosphorylation and degradation by CHPD.

(a) Time course of IκBα phosphorylation and its degradation induced by TNFα stimulation. RSF cultures were stimulated by 1.0 ng/ml TNFα for indicated time. Whole cell extracts were prepared and subjected to immunoblotting with the indicated antibodies. (b) Inhibition of IκBα phosphorylation and its degradation by CHPD. RSF were stimulated by TNFα for indicated time with or without pre-treatment of various concentrations of CHPD. Whole cell extracts were prepared and subjected to immunoblotting. The IκBα proteins were visualized by immunoblotting with anti-IκBα and anti-phospho-IκBα (Ser32) antibodies. P-IκBα, IκBα proteins phosphorylated at Ser32. (c) Time course of JNK and ERK phosphorylation upon stimulation with TNFα. RSF cultures were stimulated by 1.0 ng/ml TNFα for indicated time. Whole cell extracts were prepared and subjected to immunoblotting with the indicated antibodies. (d) Inhibition of JNK and ERK phosphorylation by CHPD. RSF were stimulated by TNFα for 15 minutes with or without pre-treatment of various concentrations of CHPD. Whole cell extracts were prepared and subjected to immunoblotting. The JNK and ERK
proteins were visualized by immunoblotting with specific antibodies. P-JNK, JNK proteins phosphorylated at Thr183/Tyr185; P-ERK, ERK proteins phosphorylated at Thr202/Tyr204. The representative data with RSF3 are shown in Fig. 4c and d and similar results were observed with other RSF cultures (data not shown).

Figure 5. Luciferase and chromatin immunoprecipitation (ChIP) assays.

(a) Effects of CHPD on the NF-κB dependent gene expression. 293 cells were transfected with the 4κB-luc reporter plasmid together with the internal control plasmid, pRL-TK, expressing *Renilla* luciferase. Stimulation with 1.0 ng/ml TNFα was carried out 24 h after the transfection. CHPD was added 1 h before the treatment with TNFα. (b) RSF-1 culture was stimulated with 1.0 ng/ml TNFα and the p65 bound to the κB site within the *il-8* promoter was detected by ChIP assay. It is noted that preincubation of cells with 1.0 μM CHPD 1 h before the TNFα stimulation suppressed the p65 (NF-κB)-DNA binding. The experimental details are described in Materials and Methods. Similar observations were obtained reproducible at least for three times. Representative results are shown. The relative amount of *il-8* promoter DNA bound to p65 was quantified by densitometric scanner using Image J software. n.d., not detectable.
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RSF, rheumatoid synovial fibroblasts; PSL, prednisolone; SASP, salazosulfapyridine; DS, diclofenac sodium; MTX, methotrexate; Bu, bucillamine; Dex, dexamethasone; Ind, indomethacin.
Table 2 - Effects of CHPD on production of inflammatory cytokines.

RSF cell cultures from six patients with RA were individually stimulated with 1.0 ng/ml TNFα in the presence or absence of CHPD and the concentrations of IL-6 and IL-8 in the culture supernatant were measured using ELISA (n=6).

<table>
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<tr>
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<th>IL-6</th>
<th>IL-8</th>
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<tr>
<td>Basal level (ng/ml)</td>
<td>2.3 ± 0.9</td>
<td>0.58 ± 0.25</td>
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<tr>
<td>Induced level (ng/ml)</td>
<td>8.8 ± 3.2</td>
<td>16 ± 6.1</td>
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<td>Fold induction</td>
<td>4.2 ± 1.7</td>
<td>28 ± 5.3</td>
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<td>CHPD IC50 (μM)</td>
<td>0.51 ± 0.20</td>
<td>0.27 ± 0.16</td>
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7-[2-(cyclopropylmethoxy)-6-hydroxyphenyl]-5-[(3S)-3-piperidinyl]-1,4-dihydro-2H-pyrido[2,3-d][1,3]oxazin-2-one hydrochloride (CHPD) (Mw.=431.92)

IC$_{50}$ = 2 nM (IKK$\beta$)
IC$_{50}$ = 135 nM (IKK$\alpha$)
IC$_{50}$ > 10 $\mu$M (other kinases)

**Fig. 1a, b**
Fig. 2a, b, c

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Fig. 3a, b
Fig. 4a, b, c, d
Fig. 5a, b

a

![Graph showing relative luc activity (fold) versus CHPD (μM) and TNFα.](image)

- CHPD (μM): -0.5, 1, 2, 0.5, 1, 2
- TNFα: -0, +, +, +

Relative luc activity (fold):
- 0
- 5
- 10
- 15
- 20
- 25

b

RSF1

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