Suppression of tumour necrosis factor production from mononuclear cells by a novel synthetic compound, CLX-090717

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Introduction

RA is a common autoimmune chronic inflammatory disease in humans. Progress has been made in understanding its pathogenesis and its treatment using molecular targeted therapy [1]. We found that TNF-α blockade has profound effects on all aspects of the disease, including dramatic protection of joints [2]. Currently, two anti-TNF-α monoclonal antibodies, infliximab (Remicade⁶) and adalimumab (Humira⁷) and a TNF receptor Fc fusion protein etanercept (Enbrel⁵) are routinely used as treatment modalities in RA [3]. Blocking antigen presentation with CTLA4-human Fc fusion protein abatacept (Orencia⁸) and targeted killing of B cells with anti-CD20 monoclonal antibody rituximab (Rituxan⁹, MabThera⁵) is also effective [4, 5], thus highlighting the contribution of both T- and B lymphocytes and antigen-presenting cells in the development and progression of RA [6].

Regrettably, all these effective therapeutics have drawbacks, as they are monoclonal antibodies or antibody receptor fusion proteins. They are injectables (either subcutaneously or intravenously), very costly, with consequent rationing and have a long half-life in circulation, so thus cannot be eliminated if there is toxicity. There is therefore a widespread search for possible other therapies, without these drawbacks; orally deliverable, easily cleared out and cheap.

Peroxisome proliferator-activated receptor gamma (PPAR-γ) agonists have been described to regulate macrophase and adipocyte function [7], and a novel agonist has been reported to reduce severity of CIA [8]. Calyx Therapeutics, Inc. (Hayward, CA, USA) had produced CLX-090717, a PPAR-γ agonist that had properties in a variety of assays, which suggested that it may be a potential therapeutic in chronic inflammatory autoimmune disorders.

The aim of the present study was to test the anti-inflammatory/immunomodulatory efficacy of CLX-090717 using several well-described human and mouse in vitro and in vivo model systems, as used previously for defining TNF-α as a therapeutic target [9, 10]. Clinical efficacy of the compound in vivo was tested therapeutically in an extensively studied animal model of RA, type II CIA in the DBA/1 mouse [11].

Our findings indicated that CLX-090717 significantly inhibited release of the pro-inflammatory cytokine TNF-α from both human and mouse mononuclear cells, and that this effect in human monocytes was in part modulated through nuclear factor-κB (NF-κB)-dependent pathway, as judged by sustained levels of IκBα in cytosolic extracts and a reduced level of LPS-induced NF-κB activity in nuclear extracts. CLX-090717 reduced clinical signs of arthritis and damage to joint architecture when administered therapeutically to arthritic mice. Mechanisms of action in CIA involved the reduction in proliferation of arthritic lymphocytes to antigen in vitro as well as reduced TNF-α release.

Conclusions. Our data suggest that the synthetic compound CLX-090717 has potential as a small molecular weight anti-inflammatory therapeutic for chronic inflammatory conditions.

Key words: Arthritis, Collagen, Cytokines, Monocytes, Synovium, Inflammation.

Objectives. To evaluate the clinical efficacy of a novel synthetic peroxisome proliferator-activated receptor gamma (PPAR-γ) agonist, CLX-090717, in several in vitro cell culture systems and murine CIA, an experimental model of RA.

Methods. Peripheral blood mononuclear cells purified by elutriation, and rheumatoid synovial cells isolated from clinical tissue were cultured with CLX-090717 and TNF-α release was measured. Molecular mechanism of action was analysed by western blotting and electrophoretic mobility shift assay. Thioglycollate-elicited murine peritoneal macrophages were cultured with CLX-090717 and lipopolysaccharide (LPS)-induced TNF-α release was assayed. Therapeutic studies were done in mice with established arthritis by evaluating clinical parameters and histology. In addition, type II collagen response of lymphocytes from mice with CIA was examined.

Results. CLX-090717 significantly inhibited spontaneous TNF-α release by RA synovial membrane cells, as well as LPS-induced TNF-α release from human and murine mononuclear cells. Inhibition of TNF-α in monocytes was mediated partially through a nuclear factor-κB (NF-κB)-dependent pathway, as judged by sustained levels of IκBα in cytosolic extracts and a reduced level of LPS-induced NF-κB activity in nuclear extracts. CLX-090717 reduced clinical signs of arthritis and damage to joint architecture when administered therapeutically to arthritic mice. Mechanisms of action in CIA involved the reduction in proliferation of arthritic lymphocytes to antigen in vitro as well as reduced TNF-α release.

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Materials and methods

Preparation of CLX-090717 solution for in vitro studies

The compound CLX-090717 (MW 517.57) from Calyx Therapeutics, Inc. was dissolved in DMSO (200 mM, Sigma Biosciences, Poole, UK). For use in in vitro culture systems (0.01–20 μM) serial dilutions were made in DMEM (PAA Laboratories, Paisley, UK). The lipopolysaccharide (LPS) preparation used in this study was Escherichia coli 026:B6 TCA extract (Sigma).

Culturing of human peripheral blood monocytes and rheumatoid tissue cells

Single donor platelethropheresis blood packs were obtained (North London Blood Transfusion service, Colindale, UK). Peripheral
blood mononuclear cells were separated from them on a density gradient material (Lymphoprep, specific density 1.077 g/ml, Nycomed Pharma A.S., Oslo, Norway). Monocytes were obtained by elutriation of the isolated buffy coats, as described [12]. Cells were plated (2 \times 10^5/100 \mu l/well), stimulated with LPS (10 ng/ml) in presence of CLX-090717 (0.01–10 \mu M) and 24 h culture supernatants harvested were assayed for TNF-\( \alpha \) release. Cells from surgical samples of synovium were obtained, isolated and cultured as per the manufacturer’s instructions (R&D Systems, Oxon, UK).

**Culturing of murine peritoneal macrophages**

Thioglycollate-elicited peritoneal macrophages were prepared and cultured (2 \times 10^7/100 \mu l/well) as described elsewhere [13]. Briefly, a 3% sodium thioglycollate solution was injected intraperitoneally into mice and on day 4 post-elicitations peritoneal exudate cells were harvested as a lavage in chilled medium. Cells were treated with CLX-090717 (0.01–20 \mu M), stimulated with LPS (10 ng/ml), 24-h culture supernatants harvested, and assayed for TNF-\( \alpha \). An ELISA kit was used for detection of TNF-\( \alpha \) as per the manufacturer’s instructions (R&D Systems, Oxon, UK).

**Western blotting and electrophoretic mobility shift assay**

Elutriated monocytes (5 \times 10^6/ml) were pre-incubated with CLX-090717 (10 \mu M) or medium at 37°C/30 min, then either stimulated with LPS (10 ng/ml) or left unstimulated as controls. Cytosolic and nuclear extracts were prepared; I\( \alpha \)Bo in cytosolic extracts was detected by western blotting, while 32P-NF-\( \kappa \)B oligonucleotide RNA probe (Promega, Southampton, UK) was added to nuclear extracts to detect NF-\( \kappa \)B using established protocols [16].

**Therapeutic treatment of CIA with CLX-090717 and culturing of lymph node cells for proliferation assays**

Arthritis induced in DBA/1 mice (Harlan Laboratories UK Limited, Bicester, Oxon, UK) was monitored daily by recording clinical scores and hind paw thickness according to our previously established protocol [17]. Treatments were given daily i.p., and commenced on the first day of detectable limb inflammation. CLX-090717 was administered at 10 mg/kg, while a vehicle mixture [DMSO: 40% polyethylene glycol (PEG-300) in PBS, pH 7.2, BDH Laboratory Supplies, Poole, UK, 1 : 1 v/v] was injected in a separate group. A group of arthritic mice were left as untreated controls. Local ethical committee approval was obtained for all treatment studies; these followed the Helsinki Declaration principles. Histology was done on hind feet specimens fixed, sectioned and stained with haematoxylin and eosin (H&E) dye. Images were captured by a camera loaded on Leitz Dialux 22 EB microscope using the Windows Spot Advanced software (version 4.5 Sterling Heights, USA.).

Pooled draining lymph node cultures from arthritic mice were set up on day 5, in the presence of CLX-090717 (0.02–20 \mu M),...
stimulated with antigen. Alternatively, lymph node cultures from vehicle or CLX-090717-treated mice were separately set up with antigen for 72 h. Tritiated thymidine (Amersham Biosciences, Buckinghamshire, UK) added and incorporated during turnover was measured as described earlier [18].

**Data analysis**

Data were analysed by GraphPad Prism Software (CA, USA). Clinical data were analysed by one-way analysis of variance with Tukey’s post test. Data on TNF-α levels were analysed by the Student’s t-test, unpaired, two-tailed test. P-values of ≤0.05 were considered statistically significant. Bars and symbols represent arithmetic mean values ± S.E.M.

**Results**

**Inhibition of TNF-α release from human monocytes and rheumatoid mononuclear cells**

Treatment of elutriated monocytes in vitro with CLX-090717 (0.01–10 μM) inhibited LPS-induced release of TNF-α in a concentration-dependent manner (Fig. 1A). At 1 μM a 66% and at 10 μM an 83% inhibition was seen (P < 0.001). Monocytes were viable at all concentrations of CLX-090717 assayed, by MTT assay (Fig. 1B). Efficacy of the compound in inhibiting release of spontaneous TNF-α from freshly isolated rheumatoid synovocytes was tested in this previously established in vitro cell culture system bearing the most direct clinical relevance. CLX-090717 down-modulated TNF-α release in a concentration (0.1–10 μM)-dependent manner (Fig. 1C). Spontaneous TNF-α release from different RA membrane cell cultures is highly variable. Addition of CLX-090717 to all five membrane cell culture preparations significantly diminished release of TNF-α in a concentration-dependent manner, by up to 76% in membrane 5 (Fig. 1C). Viability of cells isolated from all these membranes was maintained upon treatment with CLX-090717 (Fig. 1D).

**Analysis of mechanism of TNF-α inhibition**

Treatment of monocytes with CLX-090717 (10 μM) prior to LPS stimulation partially prevented IκBα degradation (Fig. 2A). Comparable IκBα band densities were recorded for 30–60 min treatment with CLX-090717 when compared with PSi, an NF-κB inhibitor that prevents proteosomal degradation of ubiquinated IκBα thereby blocking NF-κB release and its translocation from cytoplasm into the nucleus (Fig. 2A). Electrophoretic mobility shift assay (EMSA) on nuclear extracts from monocytes treated with CLX-090717 prior to LPS stimulation showed a reduction in translocation of NF-κB to the nucleus, with weaker NF-κB band intensities at both 30- and 60-min treatments compared with LPS controls (Fig. 2B). PSi showed comparable reduction in NF-κB band intensity to the CLX-090717-treated group when compared with cells treated with LPS alone for 60 min (Fig. 2B). The effect of CLX-090717 on preventing IκBα degradation and reduction of NF-κB in the nuclear compartment suggests that NF-κB inhibition contributes towards the reduction of TNF-α release from CLX-090717-treated monocytes.
Inhibition of TNF-α release from murine peritoneal macrophages

We investigated the effect of CLX-090717 on primary murine thioglycollate-elicited peritoneal macrophages, to verify that the anti-inflammatory effects of CLX-090717 were not species specific. A statistically significant dose-dependent inhibition of TNF-α release was observed. We observed a 50% inhibition at 2 µM (P = 0.0008), which increased to a 70% inhibition at 20 µM (P = 0.0002, Fig. 3A). At none of these concentrations was CLX-090717 toxic to cells, as assayed by Alamar Blue dye reduction test (Fig. 3B).

Treatment of established CIA with CLX-090717 arrests clinical progression of disease and protects joints

The anti-inflammatory/immunomodulatory efficacy of CLX-090717 was investigated in a well-established animal model of RA, the DBA/1 mouse CIA model [11]. Therapeutic treatment with CLX-090717 (10 mg/kg body weight, intraperitoneally) reduced the clinical severity of established disease, compared with vehicle-treated and untreated controls (Fig. 4A). On day 7 of established disease, the mean change in clinical scores ± S.E.M. from day 1 of arthritis in untreated, vehicle-treated and CLX-090717-treated mice were 2.317 ± 0.387, 2.005 ± 0.377 and 0.474 ± 0.102, respectively (Fig. 4A); a statistically significant reduction of 79.5% and 76.4% over the control groups (P < 0.001 and P < 0.05, respectively). Reduction of clinical scores closely mirrored the reduction achieved in hind paw swelling in CLX-090717-treated mice (Fig. 4B). On day 7 of disease, the mean paw swelling (Δ(mm)) ± S.E.M. for untreated, vehicle-treated and CLX-090717-treated mice were 0.350 ± 0.050, 0.318 ± 0.021 and 0.160 ± 0.022, respectively, a statistically significant reduction (54.3%, P < 0.001 and 49.7%, P < 0.05, respectively) (Fig. 4B). The vehicle used in our experiments had a partial effect on reduction of both clinical score and paw swelling, which was not statistically significant when compared with the untreated group of mice. There was also a good degree of joint protection of the hind feet. Proximal phalange and medial cuneiform joints are most representative for arthritic changes [17]. In untreated- and vehicle-treated mice extensive hyperplasia and infiltration of the synovium was seen, marked with an erosive pannus causing cartilage, subchondral and trabecular bone destruction; this was considerably reduced in CLX-090717-treated mice as seen by H&E-stained histology of hind feet sections (Fig. 5).
Hind feet were fixed in formalin, sectioned (6 treated controls (C) of 125 crystal-induced production of TNF-β-phorbol esters, but not LPS [22], negatively regulate macrophage production of monocyte inflammatory cytokines induced by ex vivo also prepared as control). Cell viability of cultures was unaffected in both groups, remained unaffected in both the groups (Fig. 6D).

Fig. 5. Histology of H&E-stained proximal phalange and medial cuneiform joint sections of mouse hind feet from untreated arthritic controls (A and B), vehicle-treated controls (C and D) and CLX-090717 (10 mg/kg)-treated mice (E and F). Hind feet were fixed in formalin, sectioned (6 μm thickness) and stained with H&E by Mayer’s protocol. Representative sections were shown at a magnification of 125×.

Suppression of antigen-specific murine lymph node T-cell responses

Addition of CLX-090717 (0.02–2 μM) to pooled lymph node cultures from arthritic mice suppressed the antigen-specific (bovine CII) response, as measured by incorporation of tritiated thymidine (Fig. 6A). An 86% inhibition of the bCII proliferation response was seen at 2 μM of CLX-090717 (P < 0.0001 vs vehicle control). Cell viability of cultures was unaffected in both groups, as judged by Alamar Blue dye (Fig. 6B). Lymph node cells were also prepared as ex vivo cultures from mice treated with CLX-090717 (10 mg/kg body weight, i.p., daily for 3 days), or vehicle post-onset of arthritis. Treatment with CLX-090717 suppressed proliferative response of lymph node cells to bCII by 71%, when compared with vehicle control cultures (Fig. 6C). Cell viability remained unaffected in both the groups (Fig. 6D).

Discussion

There is an unmet medical need for novel oral therapeutics with low toxicity and high disease-modulating efficacy for use in the management of chronic inflammatory conditions such as RA, psoriasis, inflammatory bowel disease and Crohn’s disease [19]. PPARs are a group of ligand-activated transcriptional factors belonging to the nuclear hormone receptor superfamily [20]. They are up-regulated in several inflammatory/immunomodulatory conditions [7, 21]. Here, we report the in vitro and in vivo anti-inflammatory/immunomodulatory properties of a novel synthetic compound, CLX-090717 (generated by Calyx Therapeutics Inc.).

CLX-090717 significantly suppressed LPS-mediated TNF-α release from human peripheral blood monocytes and was not toxic at concentrations tested. However, LPS-mediated IL-6 release from monocytes was unaffected (data not shown). PPAR-γ agonists like 15-deoxy-Δ12,14-prostaglandin J2 (15d-PGJ2), a prostaglandin D2 metabolite of the arachidonic acid pathway and troglitazone have been shown to inhibit production of monocyte inflammatory cytokines induced by phorbol esters, but not LPS [23], negatively regulate macrophage activation [23], and suppress monosodium urate monohydrate crystal-induced production of TNF-α and IL-1β from monocytes [24]. Differential effects of PPAR-γ ligands on pro-inflammatory cytokine release from monocytes have also been reported in gouty arthritis [25, 26], just as seen by us in our study. In addition, we did not observe any modulation of IL-10 levels from human peripheral blood monocyte cultures upon in vitro treatment with CLX-090717.

We explored the possible molecular mechanism of action of the compound CLX-090717. There was no effect on the activation of any MAPK (p38, JNK, ERK) family members. However, CLX-090717 inhibited the degradation of IκBα in the cytosol. This in turn was reflected by partial inhibition of nuclear NF-κB activity as measured by EMSA. PPAR-γ agonists, whose exact molecular mechanisms of inhibition of cytokine synthesis are as yet unknown, are believed to mediate their effects either transcriptionally or post-transcriptionally [20]. We have previously reported that inhibiting NF-κB by the use of adenoviruses overexpressing IκBα inhibits both TNF-α and IL-6 to the same degree [27]. The differential suppression of these two pro-inflammatory cytokines in our study thus indicates that there must be some other aspects of the mechanism of action apart from blockade of NF-κB, not known at present.

CLX-090717 significantly inhibited TNF-α release from primary murine peritoneal macrophages, while a modest inhibition was noted from RAW 264.7 cells (data not shown). We thus believe that the compound CLX-090717 has differential effects on suppression of pro-inflammatory cytokine production from different types of mononuclear cells, in both mouse and humans adding to the growing body of evidence that PPARs may be involved in regulating cytokine signals leading to monocyte/macrophage differentiation.

The significant down-modulation of LPS-driven TNF-α levels from monocytes and murine mononuclear cells prompted us to further explore a therapeutic role of CLX-090717 in two well-established systems reflecting benefit in RA, namely spontaneous release of TNF-α from cultured human RA synoviocytes [9], and the DBA/1 mouse CIA model [11].

We found that CLX-090717 significantly inhibits spontaneous TNF-α production (by up to 76%) from cultured synoviocytes obtained from five RA synovial membranes. At present, despite the lack of knowledge regarding specific cellular/molecular target for CLX-090717, this profound inhibition noted in the spontaneous TNF-α production in the synovium suggests therapeutic potential in rheumatoid patients.

Recently, a novel PPAR-γ agonist, THR0921, was reported to reduce clinical severity of CIA and protect joints [8]. We too found a similar amelioration of arthritis severity and joint protection with the use of CLX-090717. THR0921 acted in CIA through several mechanisms, such as reduced mRNA and protein levels of pro-inflammatory cytokines, proliferation of splenocytes and reduced circulating levels of antibodies to collagen [8]. We too in our CIA study have seen a significant reduction in anti-type II collagen immune responses. It is known that PPAR-γ ligands possess anti-inflammatory activity in adjuvant-induced arthritis (AIA) mediated through the inhibition of NF-κB pathway, reduced nitrotirosine formation in ankle joints and inhibition of protein expression of iNOS, COX-2 and ICAM-1 [28]. It has also been reported that PPAR-γ ligands troglitazone and 15d-PGJ2 induce RA synoviocyte apoptosis, and reduce severity of AIA in Lewis rats [29]. In our CIA model, the observed reduction in paw swelling in combination with histological benefit indicated effective control of joint inflammation. However, we have not monitored any long-term disease-modulating effects of treatment with CLX-090717 in our studies. We thus believe that CLX-090717 may be a useful therapeutic in RA.

Lymphocytes obtained from arthritic mice showed a significant reduction of their proliferative responses to the eliciting antigen (bovine type II collagen) in the presence of CLX-090717. In addition, proliferative responses of draining lymph node lymphocyte cultures to specific antigen (bovine type II collagen)
from arthritic mice treated with CLX-090717 were inhibited by 71%. PPAR-γ ligands have been shown to control chronic inflammation by down-modulating vascular cell adhesion molecule-1 (VCAM-1) expression on endothelial cells [30], thus suggesting that perhaps they could control lymphocyte trafficking to inflammatory sites.

We believe that the observed disease-ameliorating action of CLX-090717 in our CIA model could be mediated through a combination of its ability to suppress bovine type II collagen proliferative responses and partial inhibition of NF-kB-mediated inflammatory cytokine gene transcription from mononuclear cells.

Several results presented in this article suggest that CLX-090717 may be of benefit in human RA: it inhibited human peripheral blood monocyte and mouse macrophage TNF-α release as well as inhibiting spontaneous TNF-α production from human RA synovial membrane cell cultures and significantly ameliorated CIA with joint protection against arthritic changes when used after disease onset. However, clinical trials are needed to establish CLX-090717 as a useful therapeutic in chronic inflammatory disorders.

**Rheumatology key messages**

- The discovery of new therapeutics targeting inflammatory cytokine release is much needed.
- The PPAR-γ agonist CLX-090717 targets release of TNF-α from mononuclear cells in various inflammatory models.
- The compound CLX-090717 has potential as a small molecular weight therapeutic in RA.

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