The A₃ adenosine receptor agonist CF502 inhibits the PI3K, PKB/Akt and NF-κB signaling pathway in synoviocytes from rheumatoid arthritis patients and in adjuvant-induced arthritis rats

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ABSTRACT

The A₃ adenosine receptor (A₃AR) is over-expressed in inflammatory cells and was defined as a target to combat inflammation. Synthetic agonists to this receptor, such as IB-MECA and Cl-IB-MECA, exert an anti-inflammatory effect in experimental animal models of adjuvant- and collagen-induced arthritis.

In this study we present a novel A₃AR agonist, CF502, with high affinity and selectivity at the human A₃AR. CF502 induced a dose dependent inhibitory effect on the proliferation of fibroblast-like synoviocytes (FLS) via de-regulation of the nuclear factor-kappa B (NF-κB) signaling pathway. Furthermore, CF502 markedly suppressed the clinical and pathological manifestations of adjuvant-induced arthritis (AIA) in a rat experimental model when given orally at a low dose (100 μg/kg). As is typical of other G-protein coupled receptors, the A₃AR expression level was down-regulated shortly after treatment with agonist CF502 in paw and in peripheral blood mononuclear cells (PBMCs) derived from treated AIA animals. Subsequently, a decrease in the expression levels of protein kinase B/Akt (PKB/Akt), IκB kinase (IKK), I kappa B (IκB), NF-κB and tumor necrosis factor-alpha (TNF-α) took place. In addition, the expression levels of glycogen synthase kinase-3 beta (GSK-3β), β-catenin, and poly(ADP-ribose)polymerase (PARP), known to control the level and activity of NF-κB, were down-regulated upon treatment with CF502.

Taken together, CF502 inhibits FLS growth and the inflammatory manifestations of arthritis, supporting the development of A₃AR agonists for the treatment of rheumatoid arthritis.

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1. Introduction

The A$_3$ adenosine receptor (A$_3$AR) belongs to the family of the Gi-protein associated cell membrane receptors. Considerable evidence has been accumulated indicating that activation of the A$_3$AR results in an anti-inflammatory effect. The A$_3$AR was found to be highly expressed in inflammatory tissues, such as synovia and paw derived from an adjuvant-induced arthritis (AIA) model in rats, whereas low expression was found in relevant tissues from naïve animals. Interestingly, receptor up-regulation was also mirrored in the peripheral blood mono-nuclear cell (PBMCs) of the AIA rats reflecting receptor status in the inflamed tissue. Moreover, A$_3$AR up-regulation was found in PBMCs from rheumatoid arthritis (RA) patients compared to low levels in PBMCs from healthy subjects. Receptor over-expression was found to be a result of high-TNF-α levels in the cell microenvironment, known to induce up-regulation of the transcription factor NF-κB. DNA binding sites to NF-κB are present in the promoter of the A$_3$AR gene affecting its transcription. As is typical of other Gi-coupled protein receptors, A$_3$AR expression levels were found to be down-regulated upon activation with a specific agonist, such as CF101 (N^6-(3-iodobenzyl)adenosine-5′-N-methyluronamide, IB-MECA). This was noted both in the inflammatory tissues and in PBMCs, most likely due to receptor internalization and degradation [1–4].

Earlier studies showed that oral treatment of AIA rats with CF101 resulted in amelioration of the pathological and clinical disease manifestations, as well as prevention of bone destruction and cartilage damage. The mechanism of action involved with the anti-inflammatory effect of CF101 entails down-regulation of the PKB/Akt–NF-κB signaling pathway resulting in decreased levels of TNF-α, prevention of osteo-clasts formation and induction of inflammatory cell apoptosis [1–3]. CF101 was also found to be safe and well tolerated in Phase I/Phase IIa clinical studies and was able to improve indicators and symptoms of RA [5,6].

In addition to the anti-inflammatory effect, A$_3$AR agonists are known to be effective in protecting against lung and myocardial ischemia/reperfusion injury, colitis, sepsis-induced mortality, ischemic brain injury as well as protecting the myeloid system against chemotherapeutic damage. All the aforementioned studies were carried out utilizing either of the two prototypical A$_3$AR agonists, IB-MECA or its 2-chloro analogue Cl-IB-MECA. Both agonists possess high affinity and selectivity for the A$_3$AR and are known to have high-oral bioavailability [5,7–11].

Recently, a novel A$_3$AR agonist, CF502 (generically known as MRS3558) [(1′R, 2′R, 3′S, 4′R, 5′S)-4-{2-chloro-6-[(3-chloro-phenylmethyl)amino]purin-9-yl}-1-(methylaminocarbonyl)bi-cyclo [3.1.0]hexane-2,3-diol], was synthesized at the National Institutes of Health and displayed enhanced selectivity and specificity for the human A$_3$AR [12]. This agonist belongs to the class of (N)-methanocarba nucleosides, in which the ribose ring was substituted by an (N)-methanocarba ring system (Fig. 1), resulting in enhancement of the agonist affinity to the A$_3$AR. Full agonism of MRS3558 in activating the A$_3$AR was dependent on the presence of the 5′-N-methyluronamido moiety. Therefore, MRS3558 is at least 1000-fold selective for the human A$_3$AR compared to A$_1$, A$_{2A}$, and A$_{2B}$ AR subtypes [13,14].
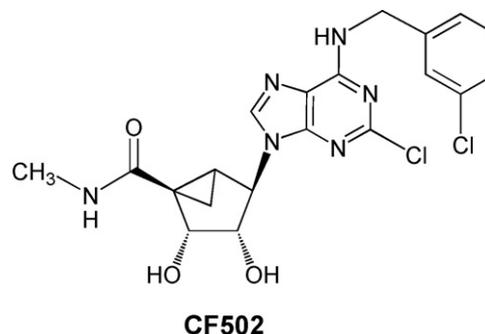

Fig. 1 – Chemical structure of the synthetic A$_3$AR-selective agonist, CF502, used in this study. The bicyclic ring system (fused cyclopentane and cyclopropane rings) in place of the ribose moiety maintains a nucleoside conformation that is preferred in the A$_3$AR binding site [12].

MRS3558 was tested earlier by Matot et al. for its *in vivo* activity. It was found that administration of the drug before reperfusion attenuated lung injury in cats. MRS3558 also protected the alveoli from apoptosis. Administration of an A$_3$AR antagonist (MRS1191) completely abolished the protection of the lungs induced by MRS3558, demonstrating the specific activation of the A$_3$AR by the agonist. The mechanism of action suggested by the authors includes up-regulation of the ERK pathway [14].

In this study we demonstrate the anti-inflammatory effect of CF502 on human FLS primary cultures as well as in an AIA model. Treatment with CF502 exerted a potent dose dependent anti-inflammatory effect both *in vivo* and *in vitro*, via a mechanism that includes down-regulation of the PKB/Akt–NF-κB signal transduction pathway.

2. Materials and Methods

2.1. Reagents

The A$_3$AR agonist, [(1′R, 2′R, 3′S, 4′R, 5′S)-4-{2-chloro-6-[(3 chlorophenylmethyl)amino]purin-9-yl}-1-(methylaminocar-bonyl)bicyclo[3.1.0]hexane-2,3-diol], designated CF502, was synthesized at the Molecular Recognition Section, Laboratory of Bioorganic Chemistry, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health (Bethesda, MD, USA). A stock solution of 10 mM was prepared in DMSO and further diluted in PBS or in culture medium.

Rabbit polyclonal antibodies against rat or human A$_3$AR and the signaling proteins PI3K (catalytic subunit), phoshory-lated (at serine 473) and non phosphrylated PKB/Akt, IKKα/β, p65 or p50 subunit of the NF-κB, TNF-α, phosphorylated and total GSK-3β, β-catenin and PARP were purchased from (Santa Cruz, CA, USA).

2.2. Binding assay

For human or rat A$_3$AR binding experiments, the procedures used were similar to those previously described [12]. Briefly, each tube contained 100 μl of membrane suspension, 50 μl of

[^{125}I]I-AB-MECA ([^{125}I]N^6-(4-amino-3-iodobenzyl)adenosine-5′-N-methyl-uronamide, final concentration 0.5 nM), and 50 μl of increasing concentrations of compounds in Tris·HCl buffer (50 mM, pH 7.4) containing 10 mM MgCl$_2$, 1 mM EDTA. Nonspecific binding was determined using 10 μM Cl-IB-MECA. The mixtures were incubated at 25 °C for 60 min. Binding reactions were terminated by filtration through Whatman GF/B filters under reduced pressure using a MT-24 cell harvester (Brandell, Gaithersburg, MD). Filters were washed three times with ice-cold buffer. Radioactivity was determined in a Beckman 5500B γ-counter. The binding of [^{3}H]CCPA ([^{3}H]2-chloro-N^6-cyclopentyladenosine, final concentration 0.5 nM) to recombinant human A$_1$ARs or native rat A$_1$ARs from rat cerebral cortex and the binding of [^{3}H]CGS21680 ([^{3}H]2-[p-(2-carboxyethyl)phenyl-ethylamino]-5′-N-ethylcarboxamidoadenosine, final concentration 5.0 nM) to recombinant human A$_{2A}$ARs or native rat A$_{2A}$ARs from rat striatum were performed as previously described [12]. Binding and functional parameters were calculated using Prism 4.0 software (GraphPAD, San Diego, CA, USA). IC$_{50}$ values obtained from competition curves were converted to K_i values using the Cheng–Prusoff equation [15]. Data were expressed as mean ± standard error.

2.3. Cyclic AMP accumulation assay

Chinese hamster ovary (CHO) cells expressing the recombinant human A$_3$AR or A$_{2B}$ receptors were cultured in Dulbecco-modified Eagle's medium (DMEM) and F12 (1:1) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin and 2 μmol/ml glutamine. Cells were plated in 24-well plates in 0.5 ml of medium. After 24 h, the medium was removed and cells were washed three times with 1 ml DMEM, containing 50 mM N-(2-hydroxyethyl)-piperazine-N′-2-ethanesulfonic acid (HEPES), pH 7.4. Cells were then treated with agonists in the presence of rolipram (10 μM) and adenosine deaminase (3 units/ml). After 45 min forskolin (10 μM) was added to the medium, and incubation was continued an additional 15 min. For the assay of the A$_{2B}$ receptor assay, agonists were incubated for 30 min under the same conditions. The reaction was terminated upon removal of the supernatant, and cells were lysed following the addition of 200 μl of 0.1 M ice-cold HCl. The cell lysate was resuspended and stored at −20 °C. For determination of cyclic AMP production, the method of Nordstedt and Fredholm was used [16]. Protein kinase A (PKA) was incubated with ^{3}[H]cyclic AMP (2 nM) in K$_2$HPO$_4$/EDTA buffer (K$_2$HPO$_4$, 150 mM; EDTA, 10 mM), 20 μl of the cell lysate, and 30 μl 0.1 M HCl or 50 μl of cyclic AMP solution (0–16 pmol/200 μl for standard curve). Bound radioactivity was separated by rapid filtration through Whatman GF/C filters and washed once with cold buffer. Bound radioactivity was measured by liquid scintillation spectrometry.

2.4. Human synovial cell cultures

Protocols for this study were approved by the hospitals' ethical committees. Synovial fluid samples were collected from RA patients after an informed consent was signed. The cells were enzymatically dissociated in a mixture of type I collagenase (4 mg/ml) and 0.25 (w/v) trypsin in DMEM for 4 h at 37 °C. After centrifugation for 10 min at 500 × g, the cells were resuspended and cultured in DMEM containing 15% FCS, 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin in a 37 °C, 5% CO$_2$ incubator. After overnight incubation the non-adherent cells were removed. The adherent cells (FLS) were sub-cultured at a 1:2 ratio, and the cells from passages 3 to 5 were used in the experiments. The cells were designated as RA FLS.

2.5. FLS proliferation assay

^{3}[H]Thymidine incorporation assay was used to evaluate the effect of CF502 on human RA FLS growth. The cells were serum starved overnight and then were incubated (2.5 × 10^4 cells/ml) with CF502 (0.1, 1, 10, 100 nM), in 96-well plates for 48 h. The selective A$_3$AR antagonist MRS 1191 (1,4-dihydro-2-methyl-6-phenyl-4-(phenylethynyl)-3,5-pyridinedicarboxylic acid, 3-ethyl-5-(phenylmethyl)ester) (Sigman RBI, Natick, MA, USA) was added at a concentration of 10 nM to the cell cultures 30 min prior to the introduction of CF502. TNF-α was added to the culture system at a dose of 0.5 ng/ml.

2.6. Experimental AIA model

Female Lewis rats, aged 8–12 weeks were obtained from Harlan Laboratories (Jerusalem, Israel). Rats were maintained on a standardized pelleted diet and supplied with tap water. Experiments were performed in accordance with the guidelines established by the Institutional Animal Care and Use Committee at Can-Fite BioPharma, Petach Tikva, Israel. The rats were injected subcutaneously (SC) at the tail base with 100 μl of a suspension composed of incomplete Freund's adjuvant (IFA) (Sigman RBI, Natick, MA, USA) with 10 mg/ml heat killed Mycobacterium tuberculosis (Mt) H37Ra (Difco, Detroit, USA). Each group contained 7–10 animals and the study was repeated four times.

CF502 treatment was initiated on day 14 after vaccination when the clinical arthritis became apparent. CF502 (at a dose of 1, 10 and 100 μg/kg) was orally administered by gavage. The animals were treated thrice daily in accordance with our former experience with another A$_3$AR agonist (IB-MECA designated as CF101) used in the same AIA model (un published data). The control group received vehicle only. The treatment lasted throughout the remaining days of the experiment.

The clinical disease activity score was assessed as follows: the animals were inspected every second day for symptoms of clinical arthritis. The scoring for each limb ranged from 0 to 4: 0: no arthritis; 1: redness or swelling of one toe/finger joint; 2: redness and swelling of more than one toe/finger joints; 3: involvement of the ankle and tarsal-metatarsal joints; 4: redness or swelling of the entire paw. The clinical score was calculated by adding the scores for four individual legs.

The histological score was assessed as follows: animals were sacrificed on day 28. The legs were removed up to the knee level, fixed in 10% formaldehyde, decalcified, dehydrated, paraffin-embedded, cut into 4 μm sections, and stained using Hematoxylin–Eosin.

The assessment of all pathological findings were performed using a semi-quantitative grading scale of 0–4 for the following parameters: the extent of infiltration of inflammatory cells to the joint tissues; synovial lining cell hyperplasia;

pannus formation; destruction of joint cartilage layers. Bone damage and erosion score was graded from 0 to 5: 0: normal; 1: minimal loss of cortical bone at a few sites; 2: mild loss of cortical trabecular bone; 3: moderate loss of bone at many sites; 4: marked loss of bone at many sites; 5: marked loss of bone at many sites with fragmenting and full thickness penetration of inflammatory process or pannus into the cortical bone. The mean of all the histological parameter scores were designated ''Histology Score''.

2.7. Immuno-histochemical staining of paraffin embedded slides of paws tissues derived from AIA rats

The paraffin of the slides was melted from the sections by immersing them in xylene, three times for 30 min each. The tissues were hydrated with serial dilutions of ethanol followed by retrieval of antigen upon heating with citrate buffer at 95 °C for 30 min. The slides were allowed to cool down and then washed three times in PBS. Endogenous peroxidase quenching was performed by washing the sections with fresh 20% hydrogen peroxide in methanol for 20 min. The sections were then blocked by incubating in 5% normal goat serum in PBS-BSA 0.1% for 2 h.

The primary antibody (Novus Biologicals, Inc., Littleton, CO) was diluted in 0.1% PBS-BSA and incubated overnight with the sections at room temperature. After three times washing in 1× PBS the slides were incubated in 0.5% biotinylated secondary antibody in PBS-BSA (0.1%) for 1 h at
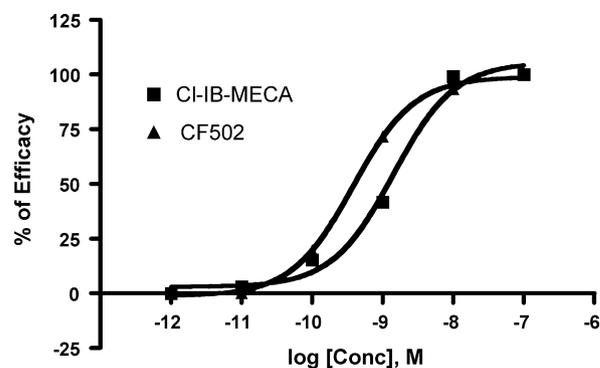
Fig. 2 – Inhibition of forskolin-stimulated adenylyl cyclase in human A_3ARs expressed in CHO cells. Curves are representative of three separate determinations.

room temperature and then subjected to a avidin–biotin complexation (ABC) After another wash the slides were incubated with DAB substrate, which was removed by washing in tap water and inactivated with bleach. A light Hematoxylin counter-staining was performed, the Hematoxylin was then removed by a rapid dip in acidic alcohol, and the slides were then exposed to Re-blue in ammonium hydroxide, dehydrated, and mounted with Permount. PBMCs from naïve, AIA and MRS3558-treated rats were fractionated from heparinized blood using a Ficoll-Hypaque gradient.
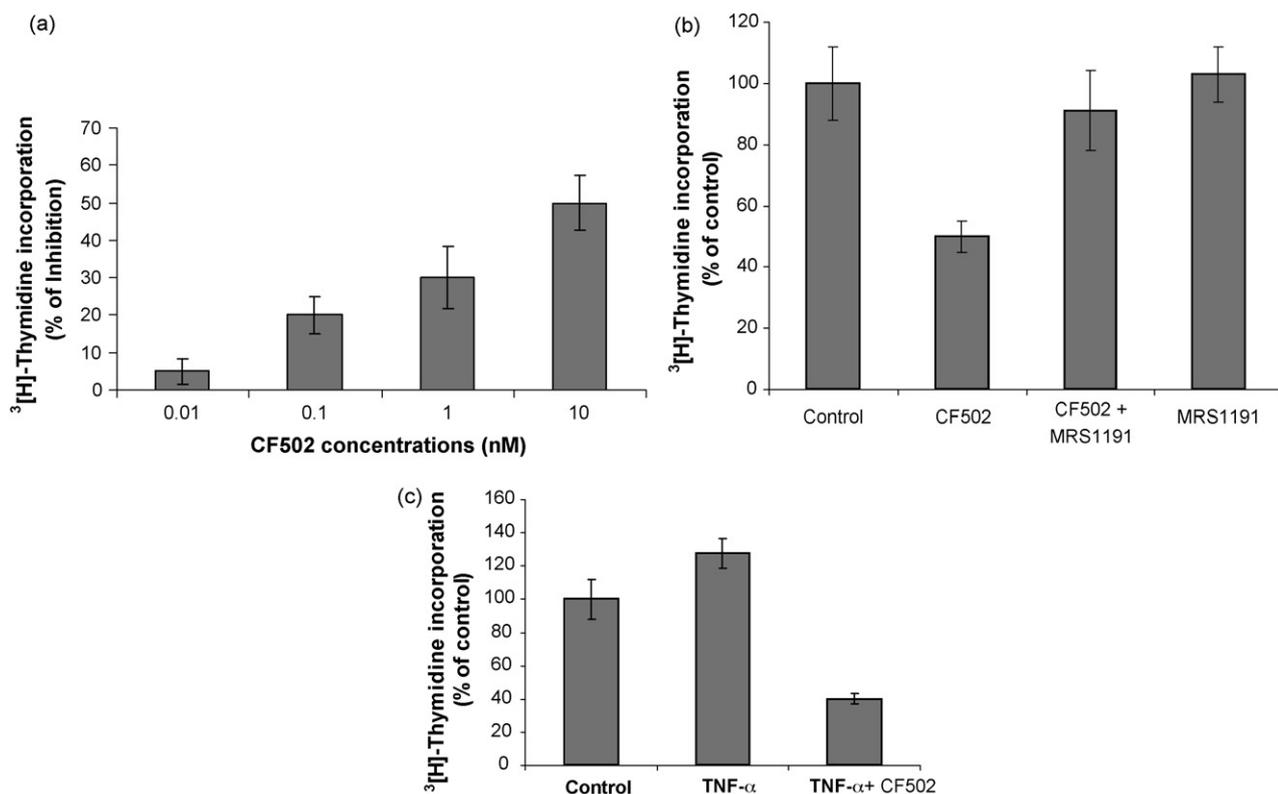
Fig. 3 – Effects of CF502 on the proliferation of FLS derived from human RA patients. The effect of CF502 on the proliferation of RA FLS was tested by ^{3}[H]thymidine incorporation assay. (a) CF502 inhibited RA FLS proliferation by a dose dependent manner. (b) CF502 inhibition effect was blocked by the administration of the A_3AR antagonist, MRS 1191 (10 nM). (c) TNF-α (0.5 ng/ml) induced RA FLS proliferation was decreased by the administration of CF502 (10 nM) to the culture system.

2.8. Protein extract preparation

The hind paws were dissected above the ankle joint. The bony tissue was broken into pieces, snap frozen in liquid nitrogen, and stored at −80 °C until use. The paw tissues were added to (4 ml/g tissue) RIPA extraction buffer containing 150 mM NaCl, 1% NP40, 0.5% deoxycholate, and 0.1% SDS, 50 mM Tris (pH 7.4). Tissues were homogenized on ice with a Polytron homogenizer and centrifuged, and the supernatants were subjected to Western blot (WB) analysis.

Blood samples were withdrawn from the heart of anesthetized rats and subjected to a Ficoll-Hypaque gradient. The PBMCs were then washed with PBS and stored at −80 °C until use.

FLS cells were removed from the flasks by trypsinization, washed with PBS and stored at −80 °C until use.

Samples were rinsed with ice-cold PBS and transferred to ice-cold RIPA buffer. Cell debris was removed by centrifugation for 10 min, at $7500 \times g$. Protein concentrations were determined using the Bio-Rad protein assay dye reagent.

2.9. Western blot analysis of A_3AR and additional signaling proteins

WB analysis was carried out according to the following protocol. Equal amounts of protein (50 μg) were separated by SDS-PAGE, using 10% polyacrylamide gels (Invitrogen). The resolved proteins were then electroblotted onto nitrocellulose membranes (Schleicher & Schuell, Keene, NH, USA). Membranes were blocked with 3% non-fat milk and incubated with the desired primary antibody (dilution 1:1000) for 24 h at 4 °C. Blots were then washed and incubated with a secondary
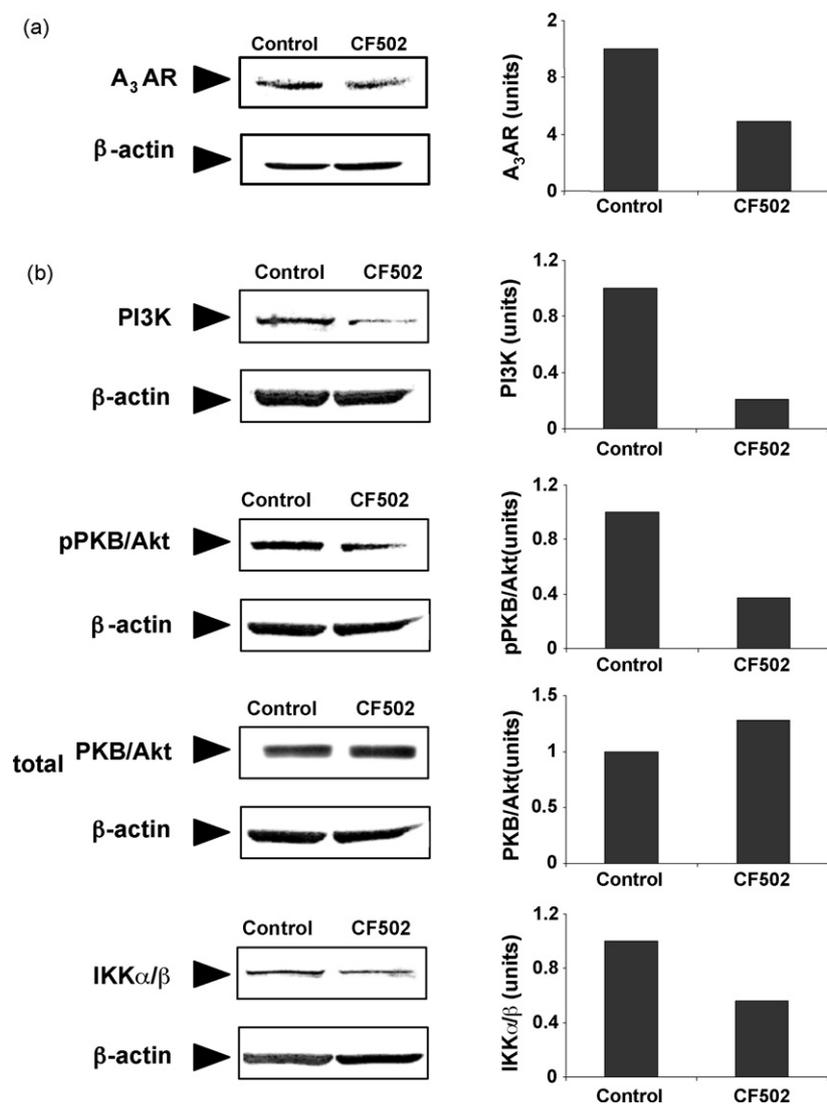

Fig. 4 – Effect of CF502 on the expression levels of the A_3AR and down-stream key signaling proteins in FLS cultured from RA patients. Synovial cells RA patients were cultured *in vitro* in the presence of CF502 and then subjected to WB analysis. (a) A_3AR expression level was down-regulated in the FLS upon treatment with 10 nM CF502. (b) The expression levels of PI3K, phosphorylated PKB/Akt, IKKα/β were decreased in the FLS upon treatment with 10 nM CF502. (c) Expression level and DNA binding activity of NF-κB were down-regulated upon CF502 treatment. (d) The expression level of phosphorylated GSK-3β was decreased while the expression level of the total GSK-3β was not affected by the CF502 treatment.
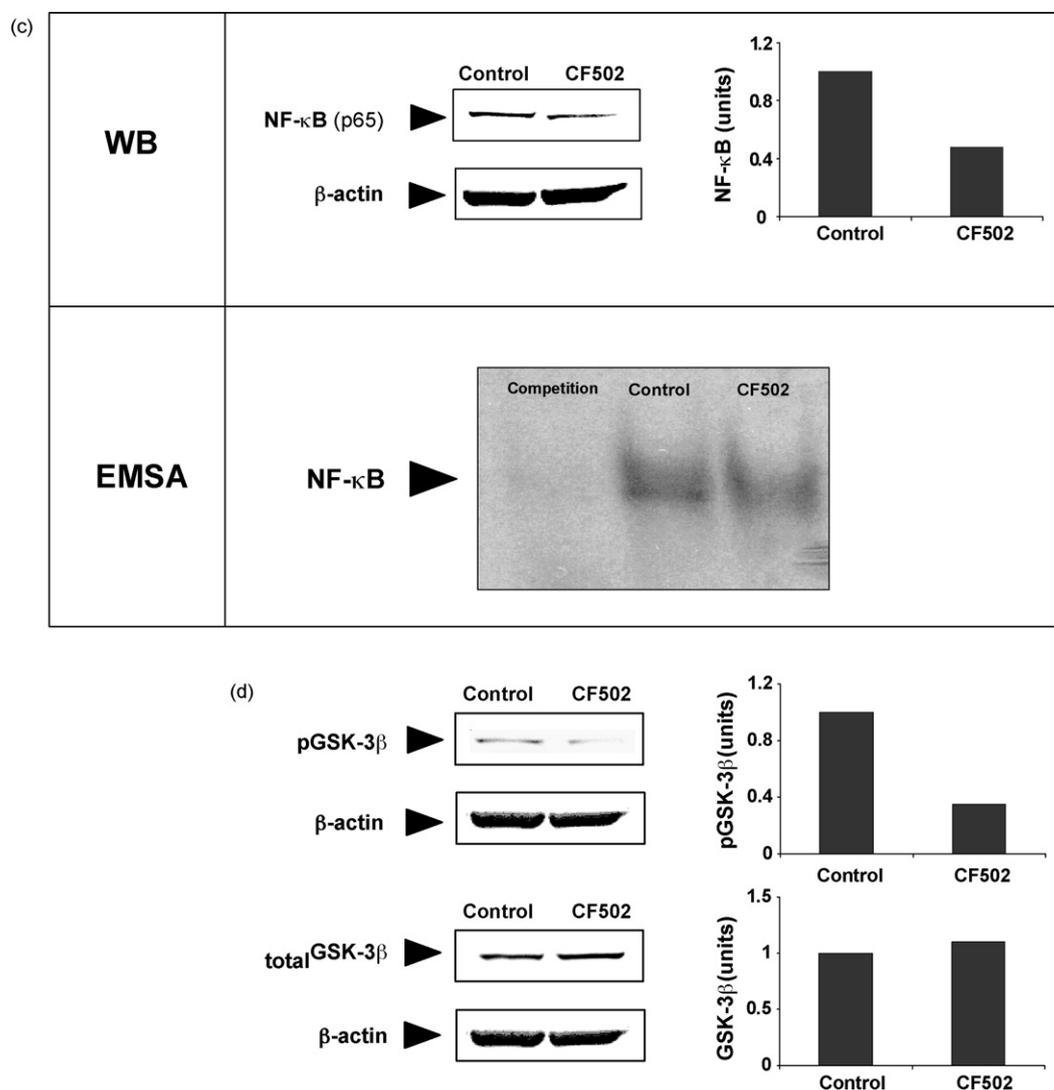
Fig. 4. (Continued).

antibody (0.1% non-fat milk) for 1 h at room temperature. Bands were recorded using BCIP/NBT color development kit (Promega, Madison, W1, USA). WBs were normalized against the housekeeping protein, β-actin. The optical density of the bands was quantified using an image analysis system and corrected by the optical density of the corresponding β-actin bands. The control values were designated as 1 unit. Data presented in the different figures are representative of at least four different experiments.

2.10. Preparation of nuclear extracts

Nuclear extract proteins from human FLS cultures treated and un-treated with CF502 were prepared by incubating the cells for 15 min on ice in a buffer containing 10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol (DTT), and 0.5 mM phenylmethylsulfonylfluoride (PMSF). Following incubation, Nonident P-40 (10%) was added, cells were vortexed for 10 s and centrifuged. The pellet was re-suspended in a buffer containing 20 mM HEPES (pH 7.9), 400 mM NaCl, 1 mM EDTA, 1 mM DTT and 1 mM PMSF, rocked on a shaker for 15 min at

41 °C and centrifuged. Protein was quantified utilizing Bio-Rad protein assay dye reagent.

2.11. Gel shift assay

Nuclear protein extracts from FLS treated and un-treated with CF502 were quantified and subjected to Gel shift assay. Double-stranded oligonucleotide for the consensus sequence for NF-κB were end-labeled with [γ-^{32}P]ATP (Amersham) using T_4 polynucleotide kinase (Promega). Protein extracts that were prepared from nuclear fraction (10 μg) and incubated for 30 min at room temperature with the end-labeled double-stranded oligonucleotides containing the NF-κB site (15,000 cpm each) in binding buffer containing 5 mM MgCl2, 250 mM NaCl, 2.5 mM DTT, 25 mM EDTA, 20% glycerol, 50 mM Tris–HCl pH 7.5 and 2 μg/sample of ploy(dI)*poly(dC) in a final volume of 25 μl. Competition with unlabeled oligonucleotide of NF-κB binding sequence at a 100-fold excess was used to analyze specific bands.

The sequence of the oligonucleotide was as follows: NF-κB, 5′-AGTTGAGGGGACTTTCCCAGGC-3′. It contained the con-

sensus NF-κB binding site in the IgL chain enhancer. The reaction product was analyzed by 6% non-denaturizing polyacrylamide gel electrophoresis. The specific bands were visualized by X-ray autoradiography.

2.12. Statistical analysis

The data of the in vitro proliferation assays represent a sum of three different assays in which each sample was repeated thrice. The western blots presented are derived from a representative study and the results shown are similar to results derived from other studies. The in vivo data present a sum of four different studies in which each group included between 7 and 10 animals.

The results were evaluated using the Student's t-test, with statistical significance set at $p < 0.05$. Comparison between the mean values of different experiments was carried out. All data are reported as mean ± S.D.

3. Results

3.1. Binding affinity and agonist efficacy of CF502

The multi-step synthesis of the (N)-methocarba nucleoside CF502 was carried out as reported, and the binding affinities at the ARs were evaluated as previously described [12]. We have expanded upon the previous pharmacological characterization of CF502. The K_i values at the human A_1, A_{2A}, A_{2B} and A_3ARs are $260 \pm 60(12)$, $2300 \pm 100(12)$, and 0.29 ± 0.04 nM, respectively. The EC_{50} value of CF502 to stimulate adenylyl cyclase in CHO cells expressing the human $A_{2B}AR$ was 11.4 μM. The binding affinity (K_i) at the rat A_1, A_{2A}, and A_3ARs are $105 \pm 20, 1080 \pm 180$, and $1.0 \pm 0.1(12)$ nM, respectively.

CF502 was further evaluated in a cyclic AMP functional assay in intact CHO cells stably expressing the human A_3AR. Fig. 2 shows that both CF502 and Cl-IB-MECA were fully efficacious, but CF502 was more potent as an A_3AR agonist. The EC_{50} values in the A_3AR-mediated inhibition of forskolin-stimulated adenylyl cyclase by CF502 and Cl-IB-MECA were 0.38 ± 0.12 and 1.4 ± 0.3 nM, respectively. The EC_{50} value of CF502 to stimulate adenylyl cyclase in CHO cells expressing the human $A_{2B}AR$ was 11.4 μM.

3.2. Effect of CF502 on human FLS

FLS were cultured from synovial fluids derived from RA patients. The cultures were incubated in the presence of various CF502 concentrations (0.1–100 nM). An ^{3}[H]thymidine incorporation assay was utilized to evaluate the effect of CF502 on FLS proliferation. CF502 exerted a concentra-
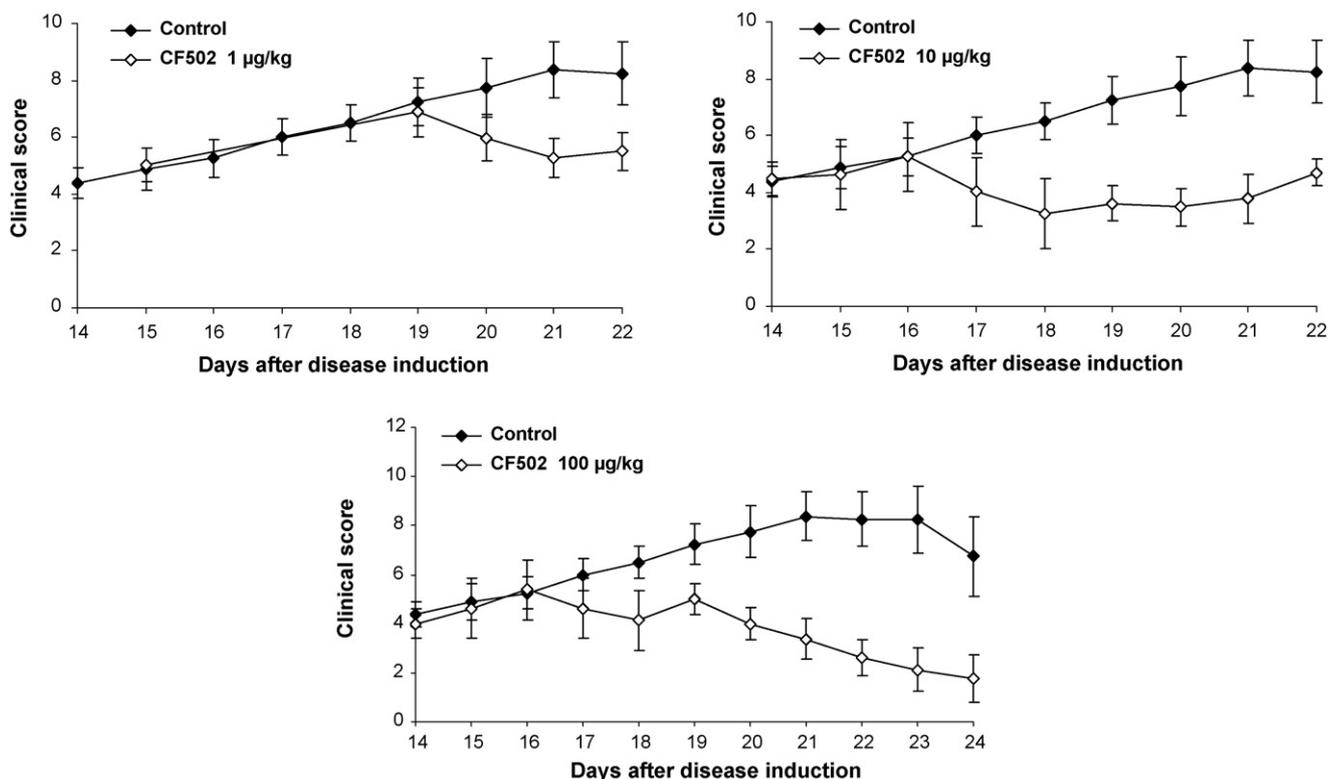

Fig. 5 – Effect of CF502 at various dosages on the clinical and pathological manifestations of AIA. Rats were immunized with a single injection of *Mycobacterium tuberculosis* in incomplete Freund's adjuvant. Treatment with CF502 was initiated upon the onset of arthritic disease. CF502 was administered orally, thrice daily. Clinical score of the arthritic disease during course of the studies was evaluated A significant dose-dependent decrease in the severity of the disease was noted with a maximal inhibition at the dose of 100 μg/kg of CF502 ($p < 0.05$).

tion-dependent inhibitory effect on the cell proliferation (Fig. 3a). Administration of MRS 1191 (10 nM), an A$_3$AR antagonist, counteracted the inhibitory effect of CF502, demonstrating the specificity of the response (Fig. 3b). Incubation of RA FLS in the presence of TNF-α (0.5 ng/ml), a pro-inflammatory cytokine, increased the cell proliferation rate by 28 ± 8.9%. Administration of CF502 (10 nM) to this culture resulted in a 60 ± 3% inhibition of cell proliferation (Fig. 3c).

Upon treatment of the FLS with 10 nM of CF502 A$_3$AR expression level was down-regulated (Fig. 4a) This was followed by decreased expression level of the catalytic subunit of PI3K and of the phosphorylated form of PKB/Akt, while the non phosphorylated PKB/Akt remained un-changed. There results demonstrate that not only the expression levels of these signal proteins were decreased, their activity was also down-regulated. The expression levels of the down-stream signal proteins IKKα/β and NF-κB (p65) also decreased in the FLS upon treatment with 10 nM CF502 (Fig. 4b and c). We further looked at the activity of NF-κB in CF502-treated FLS nuclei utilizing a band shift assay. Reduced DNA binding activity was noted (Fig. 4c). In addition the expression of phosphorylated GSK-3β known to control the activity of NF-κB was decreased upon treatment with CF502, while the level of the total GSK-3β remained unchanged (Fig. 4d).

3.3. CF502 ameliorated the clinical and pathological manifestations of AIA

Oral treatment with CF502 (1, 10, and 100 μg/kg, thrice daily) was initiated upon onset of disease and lasted till study termination. A statistically significant (which was noted starting days 18–20 after disease induction, $p < 0.05$) dose-dependent decrease in the severity of the disease, with the maximal anti-inflammatory effect at 100 μg/kg, was noted. Fig. 5 presents the clinical scores of the disease upon treatment with the various CF502 doses during course of the study.

In addition, pathological and histological evaluation, as well as analysis of key signaling proteins downstream to the A$_3$AR activation were conducted in paw and PBMCs from AIA
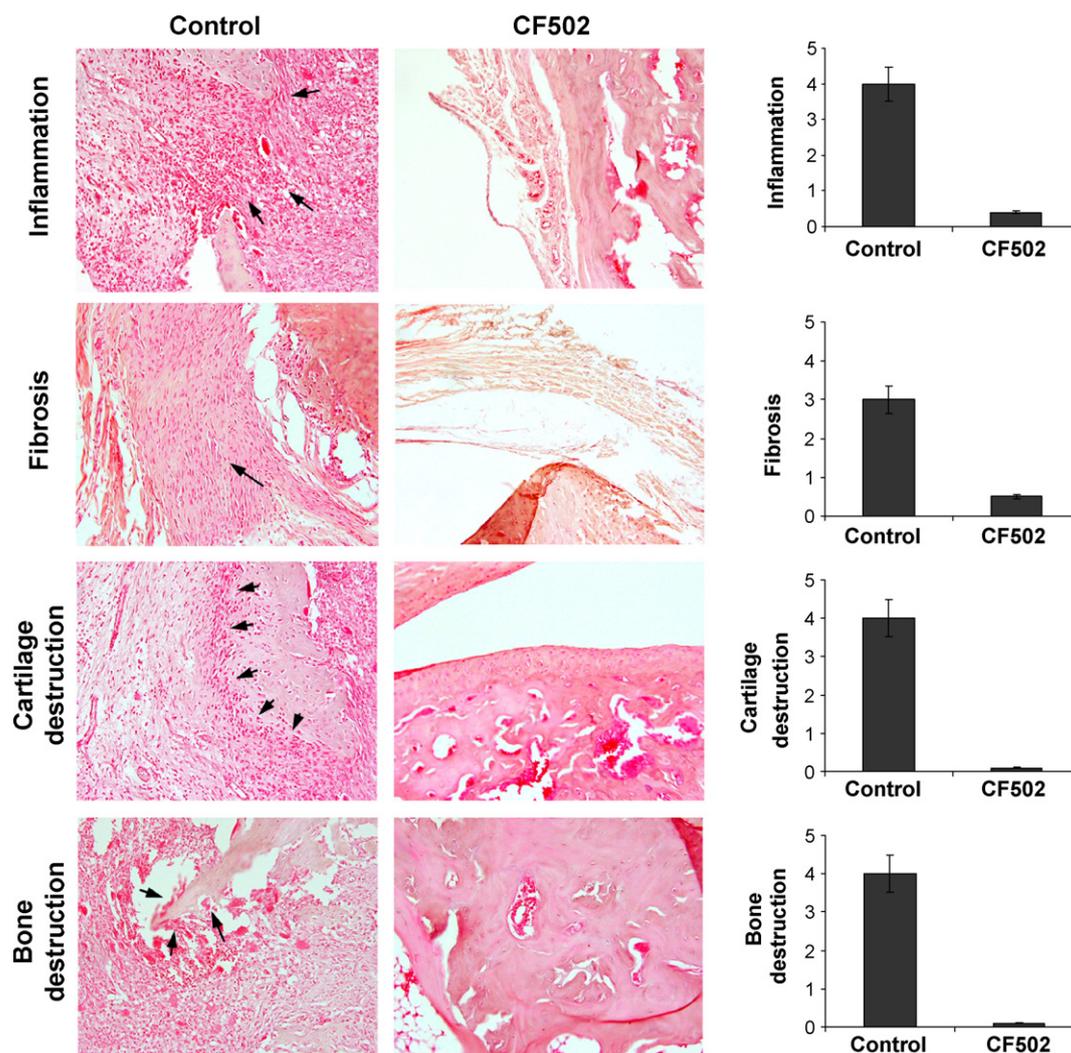

Fig. 6 – Histological evaluation of the effect of CF502 on the inflammation. Fibrosis and destruction of cartilage and bone was observed in Hematoxylin & Eosin-stained slides in arthritic joints from AIA rats ($p < 0.05$). Treatment with CF502 resulted in a significant suppression of these histopathological changes.

animals receiving a dose of 100 μg/kg CF502. Histological evaluation of Hematoxylin & Eosin stained slides from vehicle- and CF502-treated animals revealed signs of severe arthritis in the control animals. The arthritis was characterized by a robust inflammatory reaction that included response of lymphocytes and eosinophils, hyperplasia of synovial cells, moderate to severe fibrosis, pannus formation, and extensive cartilage destruction and bone lysis, which was accompanied by the presence of hyperplastic osteoclasts. Treatment with CF502 resulted in a significant suppression of these histo-pathological changes. Minimal inflammatory cells were found in CF502-treated animals, however granulation tissue remained in the synovia, suggesting that a transient inflammatory reaction was present in the past. The synovial membrane was not fibrotic and presented normal synoviocytes. The cartilage and bone were well preserved and very few osteoclasts were found (Fig. 6).

3.4. Analysis of A$_3$AR protein expression levels and down-stream signaling proteins involved with the anti-inflammatory activity of CF502

3.4.1. Analysis of paw extracts

A$_3$AR protein expression levels were down-regulated in paw extracts derived from the CF502-treated AIA rats in comparison to vehicle-treated animals (Fig. 7a). These data were supported by immuno-histochemical analysis of joint sections showing pronounced receptor exhibition in virtually all the inflammatory cells in the control group whereas A$_3$AR exhibition was found only in a very few inflammatory cells remaining in the CF502-treated group. In addition, hyperplastic synovial cells in the control group highly expressed A$_3$AR, compared with the CF502-treated group, in which only weak expression in a single layer of thin synoviocytes was found (Fig. 7b).
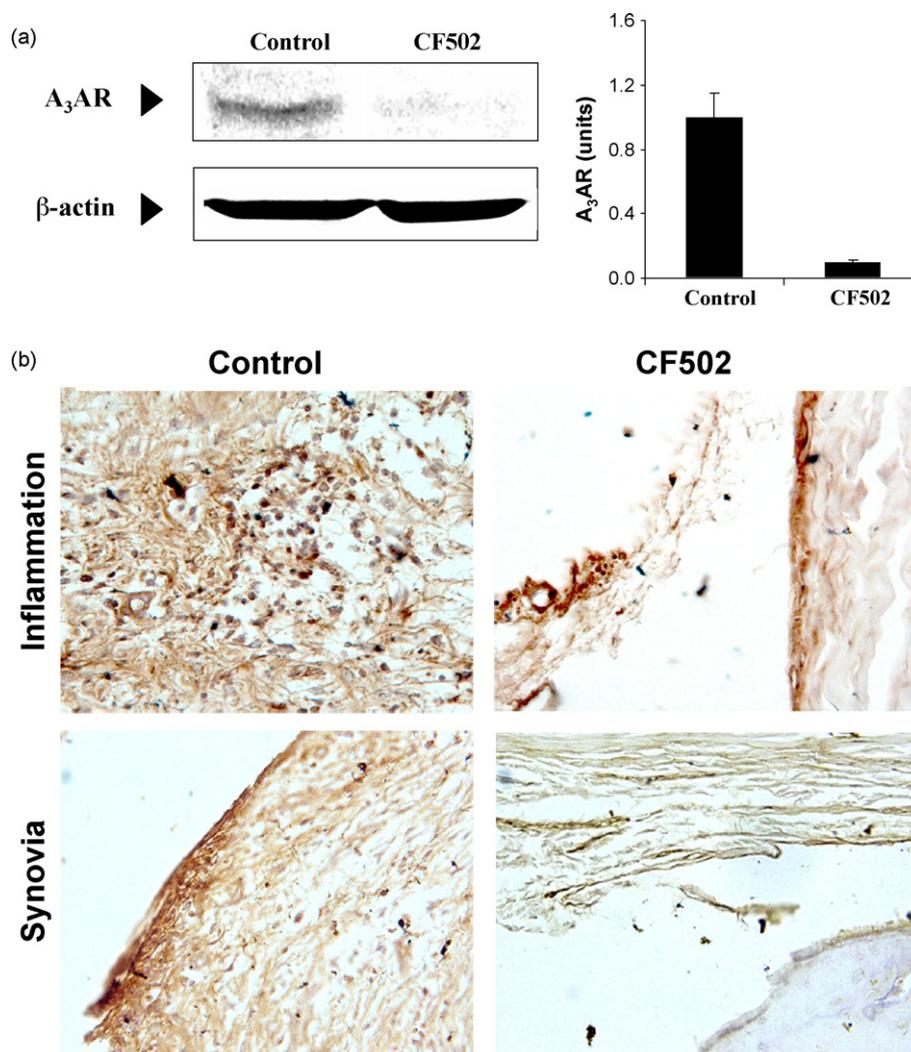

Fig. 7 – Analysis of A$_3$AR protein expression levels and exhibition upon treatment with CF502 in AIA rats. (a) A$_3$AR protein expression levels were down-regulated in paw extracts derived from CF502-treated AIA rats in comparison to vehicle-treated animals. (b) Immuno-histochemical analysis of joint sections exhibited pronounced receptor expression in virtually all the inflammatory cells in the control group whereas A$_3$AR exhibition was found only in very few inflammatory cells remaining in the CF502-treated group.
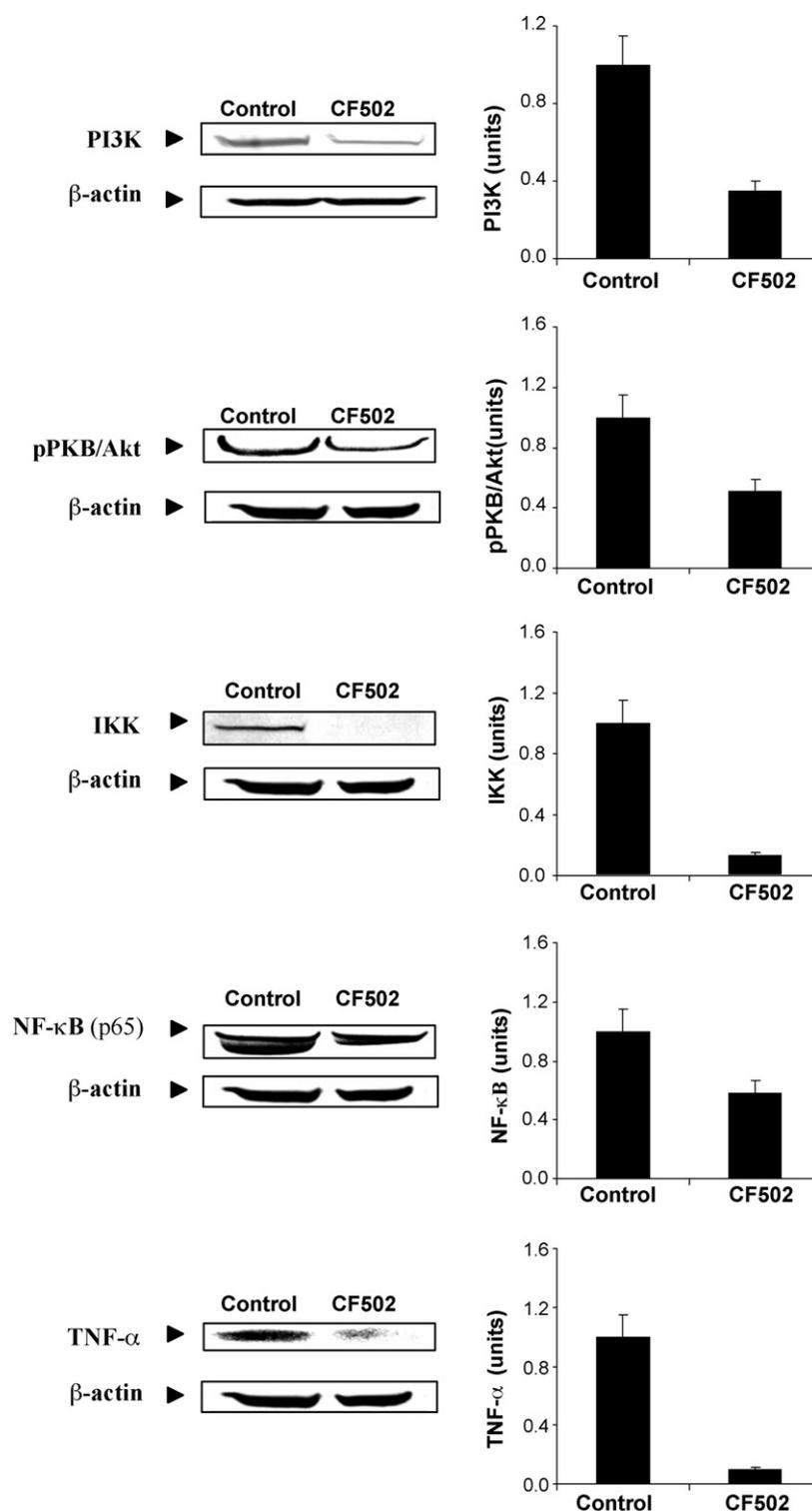
Fig. 8 – Analysis of signaling proteins expression levels involved with the anti-inflammatory activity of CF502. WB analysis of the paw extracts revealed a decrease in the protein expression levels of PI3K, PKB/Akt, IKK, NF-κB and TNF-α upon treatment with CF502.

WB analysis of the paw extracts revealed a decrease in the protein expression levels of PI3K, PKB/Akt, IKK, NF-κB (p65) and TNF-α (Fig. 8).

Further analysis showed that the levels of GSK-3β, β-catenin and PARP, known to control the expression and activity of NF-κB, were decreased upon treatment with CF502 (Fig. 9a–c).

3.4.2. Analysis of PBMCs

A marked reduction in the A$_3$AR expression levels was noted in PBMCs derived from the CF502-treated group in comparison to vehicle-treated animals (Fig. 10a). This was directly correlated to a reduction in the expression level of NF-κB (p50), known to act as a transcription factor of A$_3$AR (Fig. 10b).
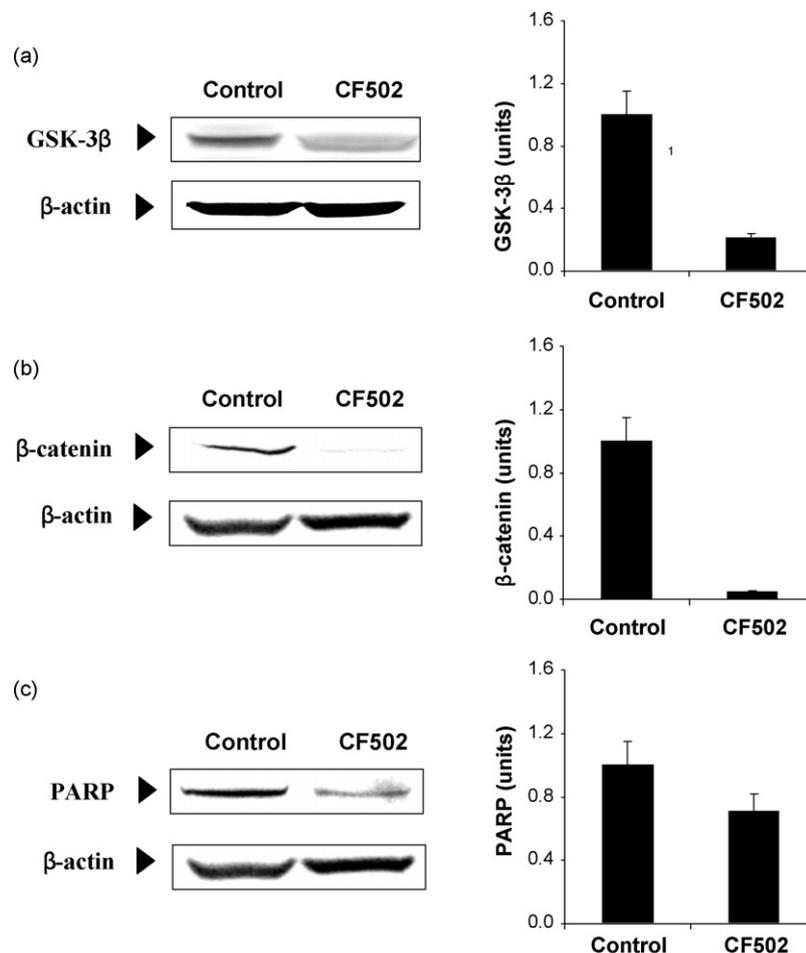
Fig. 9 – Effect of CF502 on the expression levels of signaling proteins controlling the expression and activity of NF-κB. Protein extracts were prepared from the hind paws of CF502-treated and untreated AIA rats and subjected to WB analysis. The expression levels of (a) GSK-3β, (b) β-catenin, and (c) PARP were down-regulated.

4. Discussion

The major finding of the present study is the dose-dependent anti-inflammatory effect of CF502 on human FLS derived from RA patients. In addition, CF502 treatment reduced the clinical and pathological manifestations of arthritis in an *in vivo* experimental model of AIA. Mechanistically, CF502 deregulated the NF-κB signaling pathway via the modulation of various intermediate signaling proteins.

Our previous studies as well as studies by others demonstrate that A_3AR activation plays an important role in attenuating anti-inflammatory responses [1–4,17–23]. The data produced in this study further support these findings utilizing a novel synthetic agonist with a profile different from those of previously used agonists.

CF502 possesses a K_i value of 0.3 nM at the human A_3AR (K_i of 0.2 nM), which is several-fold higher affinity than that reported earlier for CF101 [22]. At the same time, CF502 has a lower affinity than CF101 at the $A_{2A}AR$, granting the molecule higher selectivity toward the human A_3AR. The functionality of the agonist was evidenced *in vitro* by the adenylyl cyclase response, which resulted in a decrease of forskolin-stimulated cyclic AMP accumulation.

The functionality of CF502 was tested by its anti-inflammatory effects in an *in vitro* (utilizing human RA FLS) and *in vivo* (in a rat AIA) systems. CF502 inhibited in a concentration-dependent manner the proliferation of the human FLS in the presence and absence of TNF-α. This effect was counteracted by an A_3AR antagonist, confirming that the drug induced the effect via receptor activation. CF502 inhibitory effect on the FLS proliferation might be due to cell cycle arrest or apoptosis. A point to note is the linear concentration-dependent inhibitory effect that was exerted by CF502. Former studies with other A_3AR agonists yielded a bell-shaped response rather than a linear one [22]. This may be a result of the high affinity and selectivity of the CF502 to the human A_3AR.

In *in vivo* studies CF502 demonstrated a marked dose-dependent anti-inflammatory effect in the AIA rat model, manifested by marked improvement in the histology of the inflamed tissues. Interestingly, CF502 exerted a progressive dose-dependent anti-inflammatory response.

Shortly after treatment with CF502, A_3AR expression levels were down-regulated in the FLS as well as in paw and in PBMCs derived from AIA rats. This result was repeatedly shown in our previous studies with CF101. The down-regulation of the receptor represents a response of the cells
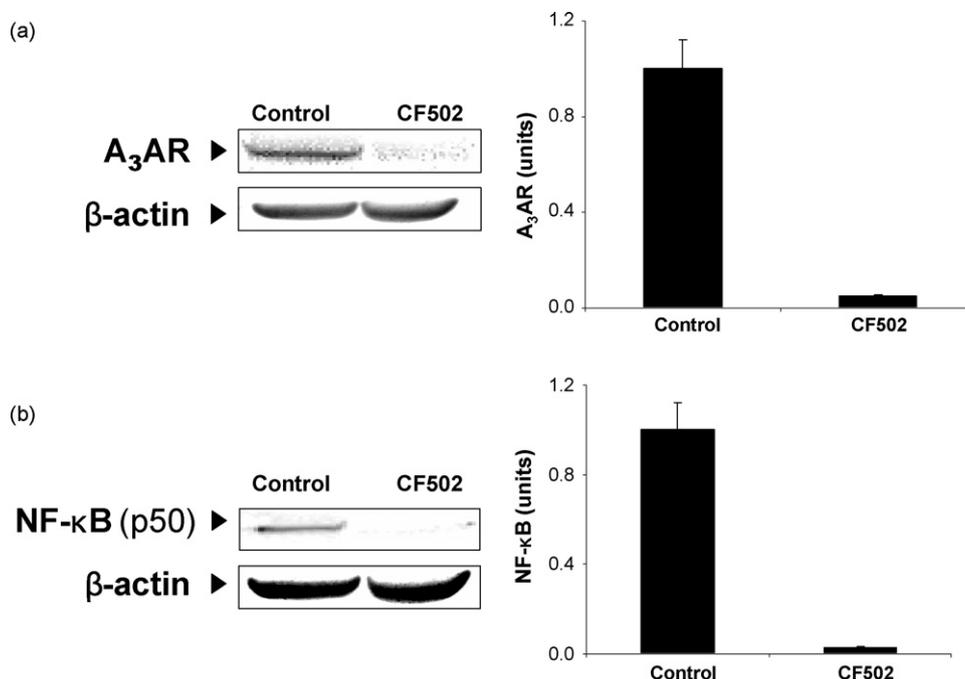
Fig. 10 – Analysis of the expression levels of the A$_3$AR and NF-κB in PBMCs derived from AIA rats. Protein extracts were prepared from the PBMCs of CF502-treated and untreated AIA rats and subjected to WB analysis. Marked reductions were noted in the expression level of: (a) the A$_3$AR, and (b) NF-κB, in PBMCs derived from the CF502-treated group in comparison to vehicle-treated animals.

to the drug, manifested by receptor internalization and degradation. In this study, we first demonstrate this phenomenon, not only at the level of expression of signaling proteins, but also by immunohistochemistry, supporting our former findings that, typical of G$_i$-protein coupled receptors in general, A$_3$AR is down-regulated upon agonist treatment [24,25]. Receptor down-regulation may also be a result of decreased NF-κB levels upon treatment with CF502. Bioinformatic studies revealed the presence of NF-κB in the A$_3$AR promoter supporting the role of this transcription factor in regulating A$_3$AR expression levels. Upon treatment with CF502, PI3K, PKB/Akt, IKK and IκB were down-regulated, leading to decreased NF-κB levels, which resulted in diminished A$_3$AR transcription and expression.

The de-regulation of the PKB/Akt–NF-κB pathway attributes as well to the anti-inflammatory effect of CF502. Earlier studies showed that CF101mediated the anti-inflammatory effect in the synovia, paw, lymph nodes and spleen via inhibition of the same pathway [1–3,23].

Similarly to IB-MECA, CF502 inhibited the protein expression level of PI3K, PKB/Akt and IKK in the paw extracts derived from the treated AIA rats. These data support the concept that A$_3$AR activation generates downstream signal transduction pathways leading to NF-κB inhibition.

NF-κB expression level and activity can also be mediated by additional signaling proteins. The first is GSK-3β, which is known to directly phosphorylate the p65 NF-κB subunit at Ser468 or Ser536. In addition, GSK-3β reduces the activation of NF-κB by preventing the association of the transcriptional co-activator CBP with p65. This in turn may result in a reduced formation of pro-inflammatory cytokines such as TNF-α and

IL-6 [26–29]. PARP-1 and β-catenin act as co-activators of NF-κB and may regulate inflammation via their capability to control its transcriptional activity [30–33].

Taken together, CF502 exerts an anti-inflammatory effect via modulation of signaling proteins that control the NF-κB pathway. It thus seems that NF-κB is involved with the regulation of A$_3$AR expression level and functionality. CF502, an orally bio-available small molecule that is highly selective and possesses high affinity to the A$_3$AR, appears to be a potential drug candidate to control inflammation.

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