Antiinflammatory Effect of A3 Adenosine Receptor Agonists in Murine Autoimmune Arthritis Models

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ABSTRACT. Objective. CF101, an A3 adenosine receptor (A3AR) agonist, is a small orally bioavailable molecule known to suppress in vitro the production of tumor necrosis factor-α (TNF-α). We evaluated its therapeutic potential and antiinflammatory effects in 3 murine models of adjuvant induced arthritis (AIA).

Methods. The antiinflammatory effect of CF101 was examined in rat AIA, in mouse collagen induced arthritis, and in tropomyosin induced arthritis. The clinical effect of another A3AR agonist, CI-IB-MECA, was examined in rat AIA. The effect of low dose (10 or 100 mg/kg/day) A3AR agonists administered orally once daily on arthritis severity was assessed clinically and histologically. The effect of CF101 on the protein expression level of TNF-α in the synovial tissue, draining lymph nodes, and spleen cells was determined by Western blot.

Results. CF101 and CI-IB-MECA markedly ameliorated the clinical and histological features of arthritis in the 3 models when administered orally at a low dose of 10 mg/kg body weight in the 3 autoimmune arthritis models. The lower dose of 10 mg/kg of either CF101 or CI-IB-MECA had better antinflammatory effect than the higher 100 mg/kg dose. Decreased expression of TNF-α was noted in protein extracts of synovia, draining lymph nodes, and spleen tissues.

Conclusion. The results provide evidence that A3AR agonists exert significant antirheumatic effects in different autoimmune arthritis models by suppression of TNF-α production. The beneficial activity of the drugs at the low dose demonstrates that the effect is A3AR mediated. (J Rheumatol 2005;32:469–76)

Key Indexing Terms:
A3 ADENOSINE RECEPTOR AGONIST
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Rheumatoid arthritis (RA) is a chronic autoimmune disorder with predominant involvement of the joints. The etiology of RA is unknown but it is evident that tumor necrosis factor-α (TNF-α) plays a key role in the development and persistence of the disease. Transgenic mice hyperexpressing the human TNF-α gene develop spontaneous arthritis resembling RA. Recently, anti-TNF-α treatments have been found to be highly effective. These drugs modify the disease course and in the majority of patients prevent periarticular bone damage. TNF-α production is regulated mainly by nuclear factor-κB (NF-κB), a cytoplasmic protein, which upon translocation to the nucleus induces transcription of TNF-α gene.

Adenosine (9-β-d-ribofuranosyladenine), the purine nucleoside, mediates various physiological cellular activities, such as cell growth, differentiation, and cell death. Adenosine is released into the extracellular environment from activated or metabolically stimulated cells and binds to selective G-protein-associated membrane receptors, designated A1, A2A, A2B, and A3. The cumulative effects of activated adenosine A2 and A3 receptors (A2AR, A3AR) on mononuclear cells of the immune system are considered antiinflammatory. Modification of monocyte/macrophage function by adenosine is expressed by decreased production of proinflammatory cytokines, such as TNF-α, interleukin 6 (IL-6), and IL-8, and the increase of the antiinflammatory cytokine IL-10. Moreover, the cytokine milieu alters the expression and function of adenosine membrane receptors on inflammatory cells. In addition, adenosine was found to act as an antirheumatic agent in autoimmune arthritis mod-
Intraperitoneal injections of pertussis toxin 200 ng/100 µl PBS were admin-istered to the animals to induce arthritis. Adjuvant induced arthritis (AIA) in rats and collagen induced arthritis (CIA) in mice are considered classic murine models for RA. The third experimental model is an autoimmune condition resembling Behçet’s disease and is characterized by T cell dependent joint inflammation. Indeed, the data show that the 2 agonists markedly inhibit the manifestations of autoimmune arthritis, suggesting that A3AR may serve as a target to downregulate the inflammatory process.

**MATERIALS AND METHODS**

**Drugs.** The A3AR agonist CF101, a GMP grade of the compound known generically as 1-deoxy-1-β-[3-(iodobenzyl)adenosine-9-H]-N-methyl-d-ribofuranuronamid (IB-MECA), was synthesized for Can-Fite BioPharma by Albany Molecular Research Inc. (Albany, NY, USA). The additional A3AR agonist, 2-chloro-N6-(3-iodobenzyl)-adenosine-5’- N-methyl-uronomide (CHB-MECA) and MRS 1220, a highly selective A3AR antagonist, were purchased from RBI/Sigma (Natick, MA, USA). For both agonists and antagonists a stock solution of 10 mM was prepared in DMSO and further dilutions in culture medium or phosphate buffered saline (PBS) were performed to reach the desired concentration. Methotrexate (MTX) was purchased from Abic (Petch-Tikva, Israel).

**Animal models.** AIA. Female Lewis rats aged 8–12 weeks obtained from Harlan Laboratories (Jerusalem, Israel) were injected subcutaneously (SC) at the tail base with 100 µl of suspension composed of incomplete Freund’s adjuvant (IFA) with 10 mg/ml heat killed Mycobacterium tuberculosis H37Ra (Difco, Detroit, MI, USA).

CIA. Male DBA mice aged 10 weeks were injected SC with 100 µl of Type II collagen 200 µg in complete Freund’s adjuvant (CFA). On Day 21 a booster injection of the same emulsion was administered. Mice were inspected daily for symptoms of clinical arthritis.

**Tropomyosin induced arthritis (TIA).** TIA was induced by vaccinating the female Lewis rats at tail base SC with 100 µl emulsion composed of 100 µg bovine tropomyosin dissolved in PBS, emulsified in an equal volume of IFA containing 4 mg/ml heat killed M. tuberculosis H37Ra. Intraperitoneal injections of pertussis toxin 200 ng/100 µl PBS were administered on Day 0 (induction) and on Day 21. Animals received standardized pelleted diet and tap water ad libitum.

Experiments were performed in accordance with the guidelines established by the Institutional Animal Care and Use Committee at Can-Fite BioPharma.

Each group contained 10 animals and each experiment was conducted at least 3 times.

**Treatment protocols.** Drugs were orally administered by gavage, once daily. The positive control received vehicle only (DMSO in a dilution corresponding to that of the drug), while the treatment groups received 10 µg/kg or 100 µg/kg of CF101 or CHB-MECA. In the AIA and TIA models treatment was initiated on Day 7 after vaccination, while in the CIA model, mice were treated at onset of clinical arthritis. To compare the effect of CF101 to the standard therapy, we treated an additional group of animals with MTX. The drug (3 mg/kg) was given intraperitoneally twice weekly, starting on Day 3 after immunization.

Clinical disease activity score. In the AIA and TIA models, the animals were inspected every second day for clinical arthritis. The scoring system ranged from 0 to 4 for each limb as follows: 0: no arthritis; 1: redness or swelling of one toe/finger joint; 2: redness and swelling of more than one toe/finger joint; 3: ankle and tarsal-metatarsal joint involvement; and 4: entire paw redness or swelling. The arthritis score was calculated by adding the 4 individual leg scores to a maximum of 16.17,18

Inflammatory intensity in the CIA model was determined in accord with the increase in hindpaw diameter, measured by calipers. The mean score in each experimental group was designated as the clinical score.

**Histology score.** Animals were sacrificed and the legs were removed up to the knee level, fixed in 10% formaldehyde, dehydrated, embedded in paraffin, cut into 4 µm sections, and stained by hematoxylin-eosin.

Assessment of all pathologic findings was performed blind (by LRW and MH) using semiquantitative grading scales of 0 to 4 for the following measures: (1) extent of inflammatory cell infiltration to the joint tissues; (2) synovial lining cell hyperplasia; (3) pannus formation; (4) joint cartilage layer destruction; and (5) bone damage and erosion score, graded 0 to 5 as follows: 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.16,19 The mean of all the histological measure scores was designated the histology score.

**Measurement of TNF-α expression.** TNF-α was measured in synovia, draining lymph nodes, and spleen tissues derived from control and CF101 treated rats with AIA. This experiment was repeated 3 times, and in each experiment protein extracts were prepared by pooling the relative organ sample from 5 different animals.

To detect the level of expression of TNF-α, Western blot analysis was performed. Spleen and draining lymph node mononuclear cells and synovial tissue were rinsed with ice-cold PBS and transferred to ice-cold lysis buffer (TNN buffer, 50 mM Tris buffer, pH 7.5, 150 mM NaCl, NP 40 0.5%) for 20 min. Cell debris was removed by centrifugation for 10 min at 7500 g. The supernatant was utilized for Western blot analysis. Protein concentrations were determined using the Bio-Rad protein assay dye reagent. Equal amounts of the sample (50 µg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis using 12% polyacrylamide gels. Resolved proteins were then electroblotted onto nitrocellulose membranes (Schleicher & Schuell, Keene, NH, USA). Membranes were blocked with 1% bovine serum albumin and incubated with polyclonal goat anti-rat TNF-α antibodies (dilution 1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 24 h at 4°C. Blots were then washed and incubated with rabbit anti-goat polyclonal antibodies for 1 h at room temperature. Bands were recorded using BCIP/NBT color development kit (Promega, Madison, WI, USA). Densitometry of protein expression was normalized against β-actin and expressed as percentage of control.

Statistical analysis. Results were evaluated using the Student t test. Comparison between the mean values of different experiments was carried out. The criterion for statistical significance was p < 0.05.

**RESULTS**

**Effects of A3AR agonists on development of arthritis.** Adjuvant induced arthritis. In the control group, arthritis appeared on Day 15, disease incidence was 70%, and the mean of the maximal clinical arthritis score (6.3 ± 1.56) was observed on Day 21. In the 10 µg/kg and 100 µg/kg CF101 treated groups, the incidence of disease was only 20%, and the maximal clinical arthritis score was delayed to Day 26 and had lower values of 1.0 ± 0.7 and 2.7 ± 0.9, respectively (Figure 1A). The effect of CF101 (10 µg/kg) was similar
Figure 1. Effect of CF101 on development of adjuvant arthritis. Rats were injected with CFA and treatment with CF101 (10 or 100 µg/kg) was initiated 7 days later; an additional group was treated with MTX (3 mg/kg) twice daily starting on Day 3 after immunization. A. Effect of CF101 on disease clinical score. B. Hind paws of representative rat from group treated with 10 µg/kg CF101 and control, on Day 26. C. Effect of CF101 on histology features of joint destruction: representative section was obtained after rats were sacrificed on Day 26 (left panel 40x magnification, right 20x). D. Mean histology score.
to that of MTX. Interestingly, when treatment with CF101 (100 µg/kg) started on Day 14, the effect was even better than in the group in which treatment was initiated on Day 7 (maximal clinical score 0.9 ± 0.12).

Figure 1B depicts the paw of the vehicle treated rat (left panel) that is markedly swollen and red, while the leg of CF101 treated rat (10 µg/kg) appears completely normal (right panel).

Figure 1C shows representative hind foot joint histology. In the control, prominent infiltration of predominant mononuclear inflammatory cells is visible. The joint cleft was obliterated by these cells as well as cell debris. In addition, there is destruction of the cartilage and bone degradation. In contrast, the treatment with CF101 preserved the normal histology of rat hind limb joints. No damage to cartilage and bone was detected in the treated groups. Figure 1D shows the mean histology score, which is significantly reduced in the 2 CF101 treated groups.

In another set of experiments, the effect of an additional A3AR agonist, Cl-IB-MECA (10 and 100 µg/kg/day), on the development of AIA was examined. In the control group the incidence of disease was 80%, the arthritis started on Day 14, and the maximal clinical arthritis score (6.3 ± 1.56) was observed on Day 22. In both CI-IB-MECA treated groups the incidence of disease was 60%, and the maximal arthritis score was delayed to Day 26 and had a statistically significant lower value [1.4 ± 0.66 (p < 0.01) and 3.4 ± 1.23 (p < 0.05), respectively; Figure 2].

Since similar or even better results were obtained with the lower dose (10 µg/kg/day) than with the higher dose (100 µg/kg/day), the subsequent experiments were conducted utilizing 10 µg/kg/day CF 101 only.

Collagen induced arthritis. In the following experiments, the effect of CF101 (10 µg/kg/day) on the development of CIA was evaluated. DBA mice developed the disease several days after the second immunization with Type II collagen/CFA emulsion. In each individual mouse, CF101 was administered at the onset of disease. In the control group maximal hindpaw swelling was observed 6 days after disease onset, whereas in the CF101 treated group it was noted on Day 14 (Figure 3A). The intensity of the arthritis was reduced throughout the experiment period, reaching a maximum on Day 21 (p < 0.02). In the CF101 treated group histology sections showed minimal inflammatory changes compared to the extensive changes in the control group (Figure 3B). In addition, the histology score was markedly lower in the CF101 treated group compared to the control group (3.87 ± 1.2 vs. 1.0 ± 0.6; p < 0.01; Figure 3C). These clinical measurements combined with the histology evidence reinforce the previous conclusion that the activation of A3AR has an antirheumatic effect, demonstrated in different experimental models of RA.

Tropomyosin induced arthritis. CF101 tested in TIA induced a remarkable antiinflammatory effect, expressed by the reduced maximal clinical arthritis score (9.3 ± 1.62 vs 4.1 ± 0.72; p < 0.001; Figure 4A). In pathological sections of controls the inflammation and the joint structure destruction were prominent, whereas in CF101 treated mice the joint structure and synovial tissue were preserved (Figure 4B). The histology score supports the clinical observation that mild inflammation of joints was visualized in the treated rats (Figure 4C). Overall, these results demonstrate that the antiinflammatory effect of CF101 is not limited to a specific murine model of arthritis.

Measurement of TNF-α. We measured TNF-α expression by Western blot analysis of protein extracts from draining lymph nodes, splenocytes, and synovial tissue. Figure 5 shows representative data of one experiment (this experi-
ment was repeated 3 times). In each experiment protein extracts were prepared by pooling the relative organ samples from 5 different animals. A decreased level of the cytokine was found in the different tissues.

**DISCUSSION**

Our series of experiments revealed a remarkable antiinflammatory effect mediated by administering A3AR agonists to different mouse/rat experimental models of inflammatory arthritis. The incidence of animals affected with arthritis was reduced significantly and the maximal and mean joint inflammation intensity was decreased.

CF101 and Cl-IB-MECA reduced the inflammatory cell infiltration and synovial involvement in the inflammatory process. Moreover, minimal damage of the joint, cartilage, and bone was detected in the majority of the treated animals with AIA. These protective effects are typical of disease modifying antirheumatic drugs, which in addition to their ability to negate the inflammatory manifestations of arthritis can reduce the accumulative structural damage to the joints’ hard tissues.

These findings indicate that CF101 exhibits a general antiinflammatory effect in autoimmune models, and the effect was observed in rat as well as in mouse models.

Interestingly, CF101 and Cl-IB-MECA had significant antiinflammatory effects at the low doses of 10 µg/kg and 100 µg/kg, respectively, in the AIA model, suggesting that the antiinflammatory effect of CF101 is A3AR mediated. We have reported similar data in various murine tumor models in which the animals were treated with IB-MECA or Cl-IB-MECA at the same doses, resulting in tumor suppression²⁰-²². It was hypothesized that efficacy at the low dose was attributed to the high selectivity of CF101 and Cl-IB-MECA to A3AR (Ki 1.1 nM and 0.33 nM, respectively). Since the concentration of the agonists was very low (10 µg/kg) in the present study, it could target the A3AR exclusively. It is reasonable that at the low agonist concentration A3AR is predominantly activated, whereas at higher doses other adenosine receptors may be involved, resulting in a lower antiinflammatory response.

Supporting the role of A3AR in mediating antiinflammatory effects are the studies showing that in A3AR knockout...
mice, selective agonists to the receptor did not inhibit lipopolysaccharide induced TNF-α production. Moreover, Montesinos, et al showed that MTX treatment in A3AR knockout mice did not prevent the accumulation of inflammatory cells in an air-pouch model in comparison to its effect in wild-type mice.

Szabo, et al showed the efficacy of IB-MECA in ameliorating the manifestations of CIA in mice, utilizing high dose agonist (500 µg/kg). They concluded that the anti-inflammatory effect was attributed to activation of both A2AR and A3AR. In our study the effect is probably A3AR mediated due to the low dose of agonist.

Macrophage and T cell derived cytokines such as TNF-α appear to play a critical role in the induction and perpetuation of chronic inflammatory processes in rheumatoid joints as well as in the systemic manifestations of this disease. TNF-α is overproduced in joints of RA patients, and is known to trigger synovioyte proliferation and a cascade of secondary mediators involved in the recruitment of inflammatory cells and activation of osteoclast that mediates joint destruction. Accumulated data indicate that in RA patients as well as in animal models, there is increased activity of NF-κB, the transcription factor of TNF-α, and additional proinflammatory mediators. Moreover, in mouse strains that are genetically prone to develop spontaneous autoimmune diseases, higher levels of nuclear active NF-κB have been detected, leading to greater expression of the proinflammatory cytokine IL-12.

We found diminished TNF-α in the synovial tissue, splenocytes, and draining lymph node cells derived from CF101 treated rats. TNF-α inhibition upon A3AR activation has previously been observed in monocytes and macrophages. In these studies, both TNF-α mRNA and protein expression levels were downregulated, supporting our data and the suggested mechanism. The anti-inflammatory effect of CF101 is probably related to its ability to regulate TNF-α levels.

MTX, the widely used RA disease modifying agent,

![Graph](image-url)

**Figure 4.** Effect of CF101 on development of tropomyosin induced arthritis. Rats were immunized with bovine α-tropomyosin and treatment with CF101 (10 µg/kg) was started 7 days later. A. Effect of CF101 on disease clinical score. B. Effect of CF101 on histology features: representative sections were obtained after rats were sacrificed on Day 28 (both 40× magnification). C. Mean histology score.
acts by increasing the extracellular adenosine concentration\textsuperscript{35}. In this pathway MTX inhibits the enzyme 5-aminomimidazole-4, carboxamide ribonuclease (AICAR) transformylase, and as a consequence intracellular AICAR accumulates, resulting in increased adenosine concentration\textsuperscript{36}. This means that at least part of the mechanism of MTX is mediated by the metabolite adenosine. Furthermore, Montesinos, \textit{et al}\textsuperscript{23} showed that in A3AR knockout mice, MTX lost its antiinflammatory effect, indicating that A3AR activation is an essential step in mediating the antirheumatic properties of MTX. Chronic treatment with MTX has toxic effects in the liver, lungs, hematopoietic system, skin, and mucosal membranes, and its use is forbidden during pregnancy and lactation\textsuperscript{37,38}. Thus, utilizing CF101, an oral nontoxic drug, may be advantageous.

Lately, a new class of antirheumatic biologic drugs, the monoclonal antibodies against the cytokines TNF-\textgreek{a} and IL-1, has been developed. These drugs have shown beneficial effects in about 80\% of patients treated for severe disease or multiple antirheumatic drug failure\textsuperscript{39-41}. The anti-TNF-\textgreek{a} antibodies are very costly, require parenteral administration, and have been associated with both acute adverse reactions and longer-term sequelae\textsuperscript{42}, including serious infectious complications such as tuberculosis, histoplasmosis, increased incidence of squamous cell carcinoma of the skin, and hematological neoplasias\textsuperscript{43-45}. Thus, despite recent advances in the treatment of RA, there is still a need for convenient, safe, and cost-effective medications.

The beneficial clinical and histological effects of CF101 in experimental autoimmune arthritis models, its suppressive effect on TNF-\textgreek{a} production, and its high oral bioavailability suggest CF101 as an attractive adjuvant therapy for autoimmune arthritis.

REFERENCES


