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The anti-inflammatory effect of A_3 adenosine receptor agonists: a novel targeted therapy for rheumatoid arthritis

Sara Bar-Yehuda, Michael H Silverman, William D Kerns, Avivit Ochaion, Shira Cohen & Pnina Fishman†
†*Can-Fite BioPharma, 10 Bareket Street, PO Box 7537, Petach-Tikva 49170, Israel*

Targeting the A_3 adenosine receptor (A_3AR) to combat inflammation is a new concept based on two findings. First, A_3AR is highly expressed in inflammatory cells, whereas low expression is found in normal tissues. This receptor was also found to be overexpressed in peripheral blood mononuclear cells, reflecting receptor status in the remote inflammatory process. Second, A_3AR activation with a specific agonist induces de-regulation of the NF-κB signaling pathway in inflammatory cells, as well as initiation of immunomodulatory effects. The A_3AR agonist CF-101 (known generically as IB-MECA) induces anti-inflammatory effects in experimental animal models of collagen- and adjuvant-induced arthritis. Combined therapy with CF-101 and methotrexate in adjuvant-induced arthritis rats yielded an additive anti-inflammatory effect. Methotrexate induced upregulation of A_3AR, rendering the inflammatory cells more susceptible to CF-101. In Phase I and in Phase IIa human studies, CF-101 was safe, well tolerated and showed strong evidence of an anti-inflammatory effect in rheumatoid arthritis patients. In peripheral blood mononuclear cells withdrawn from the patients at base line, a statistically significant correlation between A_3AR expression level and response to the drug was noted. It is suggested that A_3AR may serve as a biologic marker to predict patient response to the drug. Taken together, this information suggests that A_3AR agonists may be a new family of orally bioavailable drugs to be developed as potent inhibitors of autoimmune–inflammatory diseases.

Keywords: A_3 adenosine receptor, biologic marker, CF-101, rheumatoid arthritis

Expert Opin. Investig. Drugs (2007) ***16****(10):1601-1613*

1. Introduction

Present treatments of rheumatoid arthritis (RA) and additional autoimmune–inflammatory diseases include NSAIDs used for the management of pain and inflammation; disease-modifying anti-rheumatic drugs (DMARDs) including methotrexate (MTX), hydroxychloroquine, sulfasalazine and leflunomide; and biologic-response modifiers, targeted agents that selectively inhibit specific molecules of the immune system and include infliximab, etanercept and adalimumab (all are inhibitors of TNF-α). This group of drugs also includes anakinra, a recombinant inhibitor of IL-1; abatacept, the first co-stimulation blocker; and rituximab, a chimeric anti-CD20 monoclonal antibody [1-4]. Although the biologic-response modifiers are the most efficient among the various drugs on the market today, their disadvantages include predisposition to adverse events such as tuberculosis, lymphoma and progressive multifocal leukoencephalopathy,

as well as their high cost. In addition, there is no way to select patients for successful treatment based on predictive tests prior to treatment initiation [5,6].

In this review, the authors present a new targeted therapy for autoimmune–inflammatory conditions aiming at the A_3 adenosine receptor (A_3AR), using synthetic agonists as drug candidates that bind with high affinity and selectivity to this receptor. The focus of this discussion will be RA, in which over-expression of the target was found in inflammatory cells as well as in peripheral blood mononuclear cells (PBMCs). Binding of the agonists to inflammatory cells induces down-regulation of the NF-κB signaling pathway, resulting in decreased TNF-α levels and inhibition of auto-reactive T-cell proliferation, leading to anti-inflammatory effects. Results from animal and human studies are discussed.

2. Adenosine mediates anti-inflammatory effects via its cell surface receptors

Adenosine (9-β-D-ribofuranosyladenine) is a purine nucleoside released into the extracellular environment under highly metabolic conditions, such as inflammation. It has been shown that adenosine binds to selective G-protein-associated membrane receptors, designated as A_1, A_{2A}, A_{2B} and A_3. The receptors consist of seven membrane-spanning proteins that couple to hetero-trimetric G proteins to access numerous intracellular signaling pathways. A_1AR and A_3AR are linked to inhibitory G proteins, whereas A_{2A}AR and A_{2B}AR are linked to stimulatory G proteins. Inflammatory cytokines can alter the expression of adenosine receptors in various cell types, which suggests that adenosine signaling parameters are dynamic in inflammatory environments and that attenuation of the inhibitory G-protein pathways by activation of the receptors could yield a net anti-inflammatory effect. Although activation of the A_{2A} and the A_{2B} receptors results in up-regulation of cAMP, activation of the A_1 and the A_3 receptors induce downregulation of cAMP [7-11]. The A_3AR was the last member of the adenosine receptor family to be cloned and recent studies show its involvement in mediating cardio-, neuro- and chemo-protection [12-16]. The A_3AR is commonly over-expressed in a number of malignancies [17,18]. In tumor cells, targeting the receptor with a specific highly selective agonist has been shown to generate downstream de-regulation of the Wnt and NF-κB signal transduction pathways. These chain of events result in tumor growth inhibition [19-27].

Adenosine induces anti-inflammatory effect via its capability to affect cytokine release, such as with TNF-α, IL-6 and MIP-1-α. However, due to its short half-life and its rapid metabolism into inosine, adenosine could not be considered as a drug candidate. Furthermore, inosine, which is known to act to some extent as an A_3AR agonist, can not be considered as a drug candidate due to problems with controlling its plasma concentrations. Previous studies have shown that the anti-inflammatory effect of adenosine is mediated via the A_1, A_{2A}, A_{2B} and the A_3 adenosine receptors [28-35]. Nevertheless, A_3AR was selected by the authors' group as a target to combat inflammation due to the following reasons: i) there is a debate in the literature regarding the cardiovascular effects mediated via A_{2A} receptor [36], whereas the A_3AR is definitely involved with cardio-protective effects; and ii) A_3AR was found to be overexpressed in inflammatory cells, whereas low expression is found in normal cells, suggesting it as a specific target [37-39].

The rationale of the authors was that targeting the receptor with a low dose of a highly selective and specific agonist will exclusively affect the A_3AR, which is highly expressed in the pathologic cells. The specificity of this target served as the rationale to assess the anti-inflammatory effects of synthetic A_3AR compounds in different experimental animal models.

3. CF-101: a highly selective A_3 adenosine receptor synthetic agonist

The drug that is most frequently used in the authors' animal studies, and that is presently in clinical development, is CF-101, generically known as IB-MECA. CF-101 is a metabolically stable adenosine receptor agonist, which binds to and activates A_3AR. It has a very high affinity to the human A_3AR, with inhibitor dissociation constant (K_i) values of 1.2 nM, as demonstrated by Fredholm *et al.* [40]. Nevertheless, data obtained by these authors via studies carried out by Cerep have shown that CF-101 has a K_i value of 0.47 nM at the A_3AR and a very high selectivity (of > 1000-fold) over A_1 and a K_i value of 560 nM at A_{2A}AR and 42,300 nM at A_{2B}AR (unpublished data). In addition, CF-101 is orally absorbed in animals and humans and was found to be stable with a half-life time of 9 h in humans [41].

CF-101 has a molecular weight of 510.29 Da and substitutions at the N6 and 5′ positions create metabolic stability and A_3AR selectivity. **Table 1** depicts the nomenclature and chemical formula of CF-101.

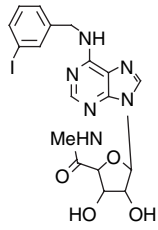
4. *In vivo* studies using highly selective A_3 adenosine receptor agonists

4.1 Therapeutic effect of CF-101 in experimental models of rheumatoid arthritis

CF-101 has been found to exert a marked anti-rheumatic effect in three different experimental animal models of polyarticular inflammatory arthritis. The anti-inflammatory effect of CF-101 was examined in rat adjuvant-induced arthritis (AIA), in mouse collagen-induced arthritis and in rat tropomyosin-induced arthritis. The animals were treated orally once daily with CF-101 and the effect on arthritis severity was assessed clinically and histologically in all models.

CF-101 10 or 100 μg/kg given once daily on day 7, markedly ameliorated the clinical and histologic features of arthritis in the various autoimmune models. CF-101 also inhibited

Table 1. Nomenclature and chemical formula of CF-101.

Chemical name	Methyl 1-[N^6-(3-iodobenzyl)-adenin-9-yl]-β-D-ribofuronamide
Chemical formula	

TNF-α expression levels in synovial tissue, lymph nodes and spleen derived from the arthritic animals. A point to note is that the 10-µg/kg dose was more effective than the 100-µg/kg dose of CF-101 [30]. It is difficult to interpret these data in light of the results discussed below, which show even more dramatic effects in animals treated three-times daily with the 100-µg/kg dose.

Additional experiments were conducted using the AIA rat model in which the animals were treated twice daily with CF-101 (10 µg/kg). The beneficial effect of CF-101 was inhibited by MRS-1220 (10 µg/kg), an A_3AR antagonist. MRS-1220 is actually a rat A_{2A}AR-selective antagonist, although it is selective for human A_3AR.The affinity of MRS-1220 for rat A_{2A}AR is 52 nM, whereas it does not have a significant effect on the rat A_3AR at concentrations up to 10,000 nM. In addition, the affinity of CF-101 for rat A_{2A}AR is ~ 50 nM [42,43]. However, in studies performed by the authors, MRS-1220 served as an A_3AR antagonist, which counteracted the anti-arthritic effect of CF-101, showing that the response was indeed mediated via CF-101 [28]. Interestingly, when AIA rats were treated three-times daily with CF-101, a dose-dependent inhibitory effect of the drug at a dose range of 0.1 – 100 µg/kg was observed (**Figure 1**).

In an additional set of experiments Szabo *et al.* showed that CF-101 (0.5 mg/kg/day) was effective in reducing the severity of joint inflammation in collagen-induced arthritis. The mechanism of action included inhibition of macrophage inflammatory protein 1-α, IL-12, nitrotyrosine and suppression of neutrophil infiltration in the paw [29].

4.2 Molecular mechanism: analysis of A_3 adenosine receptor protein expression levels and downstream signaling proteins involved with the anti-inflammatory activity of CF-101

In the rat AIA model, A_3AR is highly expressed in the synovia, paw and the draining lymph node tissues in comparison to that of naive animals. Shortly after CF-101 treatment, down-regulation of A_3AR expression level occurred [28,44]. This event is typical of G-protein-coupled receptors and is attributed to receptor internalization and degradation in response to agonist treatment [45]. Interestingly, the high receptor expression was reflected in the PBMCs of the AIA rats and was down-regulated on CF-101 treatment [28,37]. This observation shows that A_3AR analysis in PBMCs may reflect receptor status in the inflamed tissue. Thus, A_3AR analysis prior to CF-101 treatment may serve as a biologic marker to predict response to CF-101.

The expression level of key signaling proteins in the synovial tissue, paw and draining lymph node cells were examined downstream to receptor activation. The expression levels of phosphoinositide-3 kinase (PI3K), protein kinase B (PKB)/Akt, IKKα/β, NF-κB and TNF-α were down-regulated with CF-101 treatment. These data correlate to earlier studies showing that, in the synovial tissue of RA patients, activated PKB/Akt is highly expressed compared with its level in osteoarthritis patients. PKB/Akt controls apoptosis via the modulation of downstream key signaling proteins that include NF-κB and caspase 3. Indeed, CF-101 treatment diminished IKKα/β and NF-κB, whereas up-regulated protein expression levels of caspase 3 [28,30,44].

Inhibition of NF-κB in collagen-induced arthritis in rats enhanced apoptosis of synovial cells. Caspases, a family of cysteine proteases, are integral parts of the apoptosis procedure with central functions in inflammatory signaling pathways. In particular, caspase 3, when activated, has many cellular targets that produce the morphologic features of apoptosis [45-51].

RA synovial tissue contains macrophages, synovial fibroblasts and lymphocytes. Increased proliferation might contribute to increased numbers of synovial fibroblasts and chronic inflammatory cells in RA joints. However, no active locally proliferation of the macrophages (which are probably derived from peripheral blood monocytes) or lymphocytes (which undergo mitogen-induced maturation) is observed within the joints. Extended lifespan or inadequate apoptosis of the above inflammatory cells is one of the hallmarks of RA [52]. One of the molecular mechanisms that can contribute to the persistent inflammation in the joints is inhibition of apoptosis due to stimulation of the PI3K–PKB/Akt signaling pathway. PKB/Akt phosphorylates several proteins such as GSK-3β, FKHR and BAD, which then fail to induce apoptosis. It may also prevent the expression of caspase 9 and caspase 3, pivotal proteins in the apoptotic cascade. Over-expression and activation of PKB/Akt was defined as the main barrier of apoptosis in the inflamed RA tissues. Interestingly, down-regulation of phosphorylated PKB/Akt levels by wortmannin resulted in apoptosis of synoviocytes and macrophages in RA [53-57]. Thus, it seems that induction of apoptosis of macrophages, synovial fibroblasts or lymphocytes – either through suppression of signaling pathways or up-regulation in the expression of pro-apoptotic molecules – could be therapeutically beneficial in RA. Indeed, findings by the authors have demonstrated that PKB/Akt inhibition followed by an increase in caspase 3 levels in the CF-101-treated animals supports its role in ameliorating the inflammatory process (**Figure 2**).

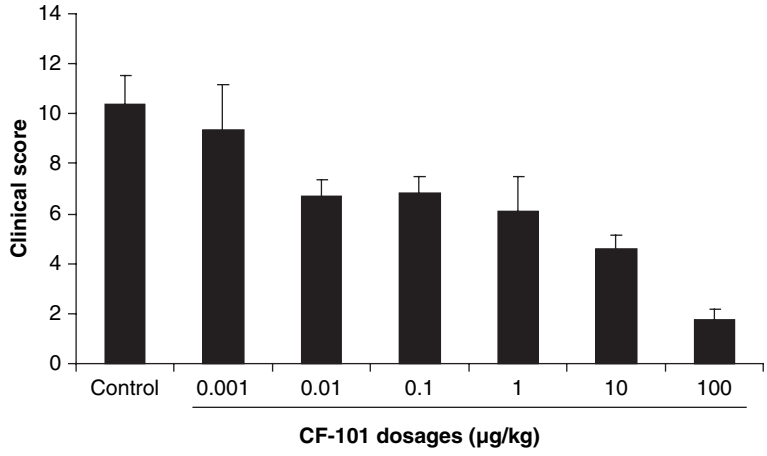


Figure 1. CF-101 induced a dose-dependent anti-arthritic effect in AIA. Rats were injected with emulsion composed of IFA and with heat-killed *Mycobacterium tuberculosis* 10 mg/ml. Treatment with CF-101 (0.001 – 100 µg/kg i.p. t.i.d.) was initiated on onset of disease. Clinical score after 14 days of treatment is presented.
AIA: Adjuvant-induced arthritis; IFA: Incomplete Freund's adjuvant.

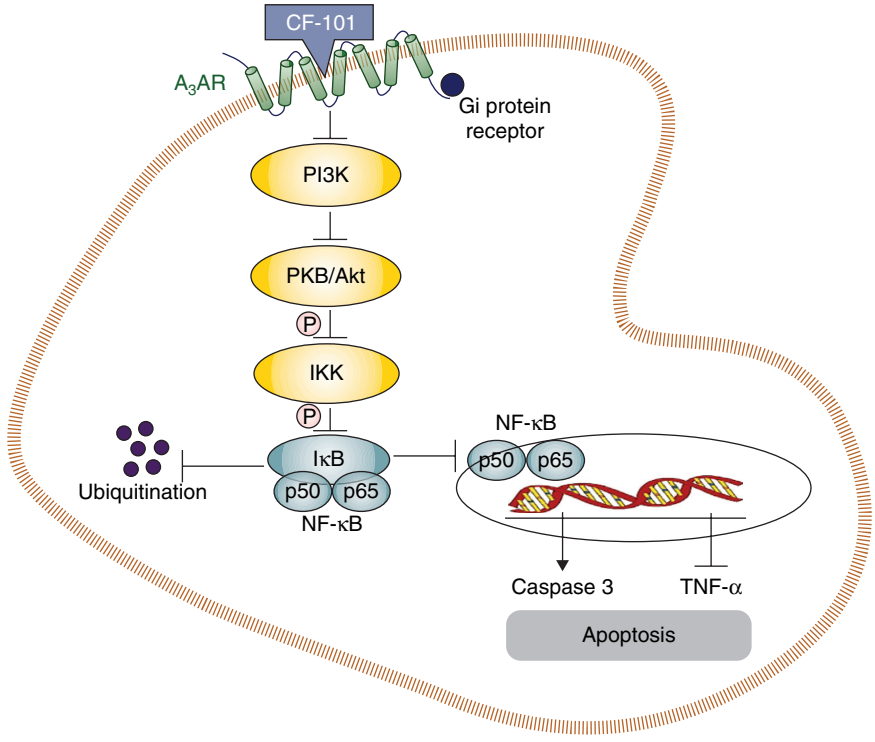


Figure 2. Effect of CF-101 on signaling proteins extracted from inflammatory tissues of AIA rats. CF-101 downregulates A_3AR expression level, generating downregulation of PI3K, PKB/Akt, IKK, NF-κB and TNF-α. Caspase 3 is upregulated, suggesting that cells are driven towards apoptosis.
A_3AR: A_3 adenosine receptor; AIA: Adjuvant-induced arthritis; PI3K: Phosphoinositide-3 kinase; PKB: Protein kinase B.

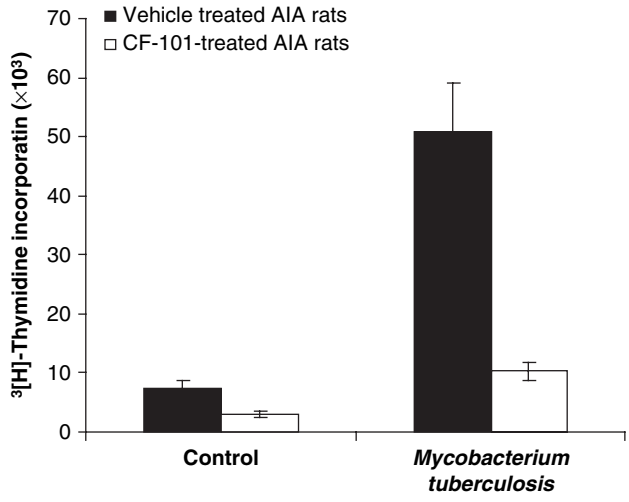


Figure 3. Effect of CF-101 treatment on the proliferation of splenocytes isolated from AIA rats. Splenocytes derived from vehicle- and CF-101-treated AIA rats were cultured in the presence or absence of *Mycobacterium tuberculosis*. The treatment with CF-101 markedly inhibited T-cell proliferation with or without *Mycobacterium tuberculosis* stimulation.
AIA: Adjuvant-induced arthritis.

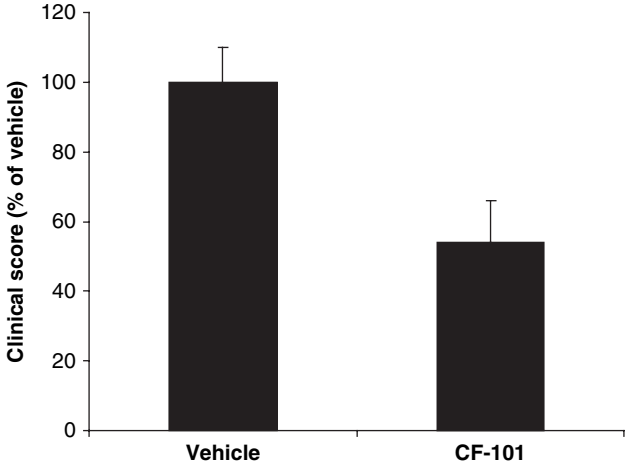


Figure 4. CF-101 inhibits adoptive transfer in AIA rats. Splenocytes derived from vehicle- and CF-101-treated AIA rats were stimulated *ex vivo* with concanavalin A and injected intravenously to naive rats. Severity of arthritis was evaluated by the clinical score. CF-101 treatment inhibited the development of arthritis in the engrafted rats.
AIA: Adjuvant-induced arthritis.

4.3 CF-101 exerts immunomodulatory effect on cellular immune response

RA is believed to be a T-cell activation-mediated disease. Moreover, in the AIA model, which is induced in Lewis rats by subcutaneous injection of a suspension of *Mycobacterium tuberculosis* in mineral oil, the reactivity of T cells to the mycobacterium antigens has been implicated in the pathogenesis of joint inflammation [58,59]. To assess cellular immunity effect of CF-101 in the AIA model, we looked at *ex vivo* T-cell activity. This was tested on *in vivo* treatment with CF-101 by measuring the proliferation of splenocytes derived from vehicle and CF-101-treated AIA rats. Splenocytes derived from CF-101-treated AIA rats showed a marked reduction in the proliferation rate in comparison to splenocytes derived from vehicle-treated AIA rats. This was also noted when *Mycobacterium tuberculosis* was added to the culture system (**Figure 3**).

Previous studies have shown that AIA can be transferred from one animal to the other on engrafting spleen or lymph node cells from the diseased animal to a naive one [60,61]. Interestingly, CF-101 treatment inhibited the transfer of arthritis (**Figure 4**), demonstrating that the drug induces an immunomodulatory effect beside its direct effect on the inflammatory cells.

4.4 CF-101 enhances the anti-inflammatory effect of methotrexate

MTX is the most commonly used DMARD to treat patients with RA and all the new therapies today are given as an add-on to MTX. MTX polyglutamates inhibit the enzyme amino imidazole carboxamido adenosine ribonucleotide (AICAR). The accumulation of AICAR has a direct inhibitory effect on adenosine deaminase and AMP deaminase, resulting in increased concentrations of adenosine. The latter was suggested to play a role in the anti-inflammatory effect of MTX, which is partially mediated via A_3AR [62-65]. Interestingly, in AIA rats, MTX treatment induced up-regulation of A_3AR expression level in the inflamed tissues, thereby preconditioning the cells to CF-101 treatment. This led to the hypothesis that a combined treatment of MTX and CF-101 may be beneficial. Indeed, an additive anti-inflammatory effect was observed in the combined treatment (**Figure 5**). In MTX-treated RA patients, A_3AR expression levels were found to be upregulated, supporting a combined treatment in this patient population [66]. A Phase IIb study to explore this effect is presently underway.

4.5 CF-101 prevents bone resorption in rats with adjuvant arthritis

RA is characterized by hyperplasia of stromal cells and a massive infiltration of hematopoietic cells into the joints, leading to chronic synovitis and destruction of cartilage, bone, tendons and ligaments. Patients with RA show a reduced bone volume and decreased bone turnover, which exacerbates the osteoporosis associated with the disease. This progressive joint damage results in functional decline and disability [67,68]. It is well documented that the bone destruction in RA is mediated by a variety of pro-inflammatory mediators, degradative enzymes and the activity of osteoclasts. A member of the TNF family, the receptor activator of NF-κB ligand (RANKL), is required

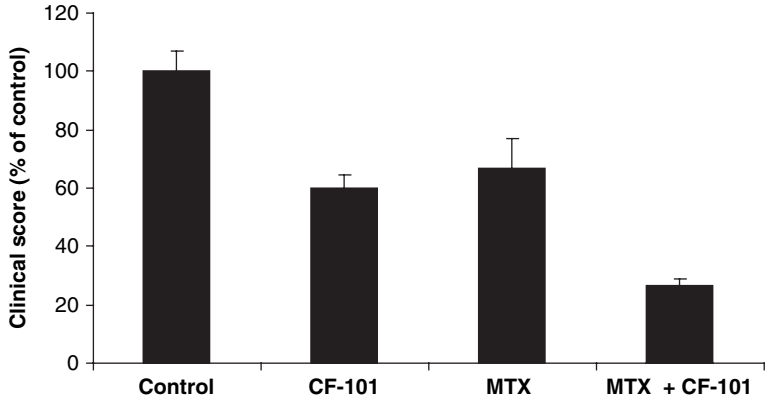


Figure 5. Effect of CF-101 and MTX treatment on the development of AIA. Rats were injected subcutaneously at the tail base with 100 µl of suspension composed of IFA with heat-killed *Mycobacterium tuberculosis* 10 mg/ml. The clinical score of combined treatment of MTX plus CF-101 exerts significantly lower valued then each of the treatments alone in comparison to score of the control group.
AIA: Adjuvant-induced arthritis; IFA: Incomplete Freund's adjuvant; MTX: Methotrexate.

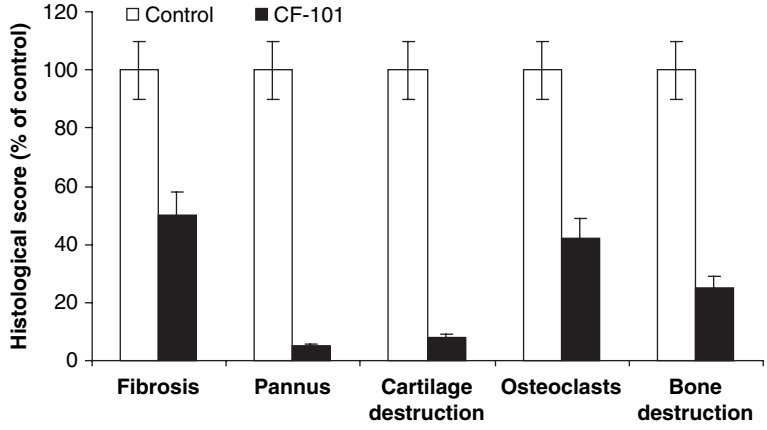


Figure 6. Effects of CF-101 on the histologic features of AIA. Histologic evaluation of joints derived from vehicle- and CF-101-treated AIA rats. In the control group, abundant fibroblasts, extensive pannus, severe cartilage damage, increased osteoclasts appearance and high incidence of bone destruction was seen while the morphohistologic status in the CF-101-treated animals seemed to be normal.
AIA: Adjuvant-induced arthritis.

for the differentiation of osteoclasts from their precursor cells, monocytes/macrophage, by activating osteoclastogenesis in inflammatory sites and promoting the osteoclast activity and survival. The expression level of RANKL is mediated via the pro-inflammatory cytokine TNF-α, which is also known to play an important role in the pathogenesis of RA [69-73].

In an AIA model, CF-101 ameliorated the clinical manifestations of arthritis, reduced pannus formation, attenuated cartilage and bone destruction and decreased the number of osteoclasts (**Figure 6**). The molecular mechanism involved down-regulation of the NF-κB signaling pathway, resulting in decreased RANKL expression level [44].

5. Role of A_3 adenosine receptor in mediating anti-inflammatory effects in additional experimental animal models

The anti-inflammatory effect induced by A_3AR agonists was also demonstrated in additional animal models of inflammatory diseases. In three animal models of colitis (rat chronic model of 2,4,6-trinitrobenzene sulfonic acid-induced colitis, dextran sodium sulphate-induced colitis and spontaneous colitis found in IL-10 gene-deficient mice), treatment with CF-101 (1.5 mg/kg b.i.d., 1 or 3 mg/kg/day accordingly) protected against colitis. Preliminary data from studies by the authors have shown that, in dextran sulfate sodium-induced colitis,

CF-101 treatment (0.01 mg/kg, b.i.d.) ameliorated the pathologic damage to the colon, prevented weight loss and prolonged survival. This was evident by the ability of CF-101 to ameliorate the increased levels of inflammatory cytokines and chemokines, as well as the infiltration of inflammatory cells, preventing the gut damage and weight loss [74,75]. Treatment with CF-101 in a bacterial lipopolysaccaride endotoxin-induced endotoxemia mice, experimental model enhanced the release of the anti-inflammatory cytokine IL-10. In addition, CF-101 inhibited the production of IFN-γ and nitric oxide product [76]. In a murine model of sepsis (achieved by cecal ligation and double puncture procedure) CF-101 treatment improved renal and hepatic as well as survival. Interestingly CF-101 was able to reduce mortality in A_1AR and A_{2A}AR-knockout mice, but not in A_3AR-knockout mice. Moreover, treatment with MRS-1191 (an A_3AR antagonist) worsened renal and hepatic function and increased mortality. The above data demonstrate the specificity of CF-101 in activating A_3AR to protect against sepsis [77].

In addition, three different inflammatory models (i.e., experimental autoimmune encephalomyelitis induced by MBP, concanavalin A-induced liver inflammation and mono-iodoacetate-induced osteoarthritis), CF-101 (0.01 or 0.1 mg/kg b.i.d.) given orally on onset of disease markedly improved the clinical and pathologic manifestations of these diseases (unpublished data).

6. A_3 adenosine receptor is over-expressed in inflammatory and peripheral blood mononuclear cells of rheumatoid arthritis patients

Upregulation of the A_3AR target has been shown in experimental animal models as the rationale to treat AIA rats with A_3AR agonists. Similar data were reported in RA patients showing A_3AR upregulation in the synovial tissue and in the PBMCs. To understand the molecular mechanism leading to A_3AR overexpression, an *in vitro* system of mitogen-activated PBMCs was used. Activation of PBMCs by PHA or lipopoysaccharide (LPS) resulted in upregulation of A3AR expression levels. NF-κB expression was also upregulated on cell activation. Antibodies against IL-2 abrogated the increase in A_3AR and NF-κB expression. In PBMCs activated with LPS, the levels of A_3AR and NF-κB were upregulated and antibodies against TNF-α induced downregulation of A_3AR and NF-κB. These data demonstrate that A_3AR overexpression is associated with an increase in the production of inflammatory cytokines (TNF-α and IL-2), which subsequently upregulate the expression level of NF-κB. Bioinformatics studies revealed the presence of NF-κB in the A_3AR promoter, demonstrating the role of this transcription factor in determining A_3AR expression level. Interestingly, a high NF-κB expression level was found in the PBMCs of RA patients, supporting its role in modulating A_3AR expression level [37,66].

As mentioned in Section 4.2, A_3AR expression levels in the PBMCs reflect the status of the receptor in the pathologic tissue. In addition, treatment with the A_3AR agonist, CF-101, resulted in downregulation of the receptor expression level, both in the inflammatory organ and in the PBMCs. This observation was followed by amelioration in the clinical and pathologic manifestations of the arthritic symptoms. Taken together, it is suggested that monitoring receptor expression level in the PBMCs of treated subjects could be useful biologic marker to follow up the response to the CF-101.

7. Preclinical studies

7.1 Bioavailability

CF-101 has relatively low clearance in preclinical species, suggesting that there is little first-pass effect and that absorption is the major limiting factor dictating bioavailability. The volume of distribution was variable in mice, dogs and monkeys, ranging from low in the monkey to high (2 – 3 × body water) in the mouse. The rate of oral absorption in the monkey was ~ 3-times slower than elimination and this most likely accounted for the dramatic difference in oral versus intravenous half-life in the monkey. Relatively low protein binding of ~ 90% in all species, including humans, suggests that, although bioavailability is low in animals, a significant amount of active non-protein bound drug is available to interact with the A_3AR.

7.2 Metabolism

In *in vitro* studies, CF-101 was slowly metabolized in mouse, rat, rabbit, monkey and human hepatocytes, with ~ 70 – 97% recovery after 4-h incubation. A total of nine metabolites was found. The major biotransformation pathways of CF-101 were *N*-deglycosylation, *N*-dealkylation (*N*-de-3-iodobenzylation) and mono-oxidation coupled with *N*-deglycosylation. Glucuronidation was a minor pathway except in rabbits. Ring oxidation (hydroxylation) seemed to be a minor pathway in all five species. In general, there were no major differences in the CF-101 metabolic profiles across species. All of the major and minor metabolites identified in the human hepatocyte system were also detected in the mouse, rat, rabbit and monkey systems. Studies with human hepatic microsomes indicate that drug interactions, related to inhibition of the CYP450 metabolism, are unlikely to occur [41].

7.3 Toxicity

The toxicity of CF-101 has been characterized following oral single-dose, 4-day, 28-day and 90-day repeated-dose toxicity studies in male and female mice, dogs (single dose only) and monkeys. Chronic toxicity studies in mice and monkeys are underway. Embryo–fetal development studies have been completed in mice and rabbits and chronic toxicity studies (90 days) have been completed in mice

and monkeys. Fertility studies have been completed in mice. Effects on cardiovascular parameters were evaluated in conscious instrumented monkeys and anesthetized dogs. Genotoxicity studies were conducted in *in vitro* bacterial and mammalian mutation assays and in an *in vivo* chromosomal aberration assay.

In mice, lethality was observed at doses ≥ 100 mg/kg administered as a Cremophor® RH40 (BASF, Germany) solution of CF-101 and no mortality was observed at 1000 mg/kg in a methylcellulose suspension administered orally. In monkeys, no effects were noted at the highest dose of 300 mg/kg, administered orally as a suspension.

In conscious cynomolgus monkeys using telemetry, CF-101 (10 and 100 mg/kg p.o.) had no effects on blood pressure or cardiac intervals (including QT), heart rate or body temperature and did not cause cardiac arrhythmias. In an intravenous study in anesthetized dogs, CF-101 did not cause any effects on cardiac intervals or heart rate and did not cause cardiac arrhythmias, at doses ≤ 10 mg/kg. In this study, CF-101 decreased mean arterial pressure transiently at 10 and 100 µg/kg and in a sustained manner (20 – 50%) at doses of 1000 and 10,000 µg/kg. There was no reflex tachycardia in this model.

In mice and monkeys, no toxicity was identified at 30 and 100 mg/kg/day for 28 or 90 days, respectively. In mice, intestinal erosion and inflammation were noted at 300 mg/kg. The therapeutic index in the rat adjuvant arthritis model is ~ 1000. AUC safety multiples (compared with human AUC values at 4 mg b.i.d.) ranged from × 6 in mice to × 31 in monkeys. Long-term toxicology studies are ongoing in the mouse and monkeys.

There was no evidence of genotoxicity in the Ames test, mouse lymphoma and mouse micronucleus assays. In embryo–fetal development studies in mice, cranio-facial and skeletal abnormalities were observed at 30 – 300 mg/kg. In a combined fertility and developmental toxicity study in mice, no effects on development were noted at 3 mg/kg. There were no effects on male or female fertility and no effects on embryo–fetal development in rabbits [41].

8. Clinical human studies

8.1 Phase I studies

8.1.1 Single-dose study

In a controlled, double-blind, single-ascending dose study designed to evaluate the safety, tolerability, pharmacokinetic behaviour and pharmacodynamic activity of CF-101 (oral solution and oral suspension) in man, normal volunteers received oral doses of placebo solution (4 subjects) or CF-101 1 mg in solution (4 subjects), 5 mg in solution (8 subjects), 5 mg in suspension (4 subjects) or 10 mg in solution (4 subjects). CF-101 was absorbed promptly with a T_{max} value of 1 – 2 h in all cases. Mean C_{max} and $AUC_{0\text{-}48}$ values were linearly related to the dose and the half-life of elimination ($t_{½}$) was ~ 9 h and was dose independent. The apparent plasma clearance was always 4 – 7 l/h and, again, dose independent. CF-101 is pharmacokinetically well behaved; there were linearly dose-related increases in C_{max} and AUC and elimination half-life of 8 h.

In the 5-mg group, 2 subjects experienced headache and 1 experienced lightheadedness. At the 10-mg dose, all 4 subjects experienced adverse events (3 reported headache, 2 reported nausea and 1 experienced vomiting and facial flushing). At this dose level, clinically notable increases in heart rate and systolic blood pressure were also observed in some subjects.

Single oral doses of CF-101 of 1 and 5 mg were generally safe and well tolerated. At the 10-mg dose, CF-101 was associated with adverse events in four out of four subjects, including asymptomatic sinus tachycardia and mild elevations of systolic blood pressure; these events are presumed to represent effects on cardiovascular adenosine receptors, which are most likely to be $A2_AAR$ at these high plasma concentrations [41].

8.1.2 Repeat dose study

In a subsequent trial of twice-daily repeat-dose testing of CF-101 4 mg every 12 h, the schedule was found to be well tolerated in male volunteers, with an adverse event profile comparable to placebo. A dose level of 5 mg every 12 h was associated with an increased frequency of generally mild and transitory adverse events and occurrences of mild, asymptomatic increases in heart rate. There was evidence of tolerance to the heart rate increase by day 7. Accordingly, a dose of 4 mg b.i.d. was judged to be the maximum tolerated dose for every 12 h in normal male volunteers. In this trial, it was demonstrated that CF-101 is well absorbed in man, with the T_{max} as 1 – 2 h; CF-101 pharmacokinetics were not changed after repeated 12-h dosing for 7 days; oral pharmacokinetics of CF-101 were dose proportional at steady-state; and the half-life of CF-101 was independent of the dose, ~ 9 – 10 h at steady-state [41].

8.1.3 Food-effect study

In a normal volunteer food-effect study, oral absorption of CF-101 was shown to be influenced by food intake. The mean $AUC_{0\text{-}t}$ and $AUC_{0\text{-}inf}$ for the fed treatment were 39 and 37% lower than the estimates for the fasted treatment, respectively (unpublished data).

8.1.4 Interaction of CF-101 and methotrexate

In patients with RA, there were no significant changes to plasma pharmacokinetics parameters of MTX or its metabolite 7-*OH* MTX after co-administration of CF-101 when compared with placebo. Similarly, no significant changes were found in the cumulated excretion of MTX or 7-*OH* MTX in urine (unpublished data).

8.2 Phase IIa study in rheumatoid arthritis patients

In a Phase IIa clinical study, the safety and efficacy of CF-101 in patients with active RA were examined. In addition, the correlation between A_3AR expression level

in PBMCs at baseline and response to the drug was examined.

The trial was a multicenter (10 sites), randomized, double-blind, parallel-group study and included 74 patients with active RA who failed between one and four DMARDs, excluding the biologic drugs. CF-101 was administered at doses of 0.1, 1.0 and 4.0 mg b.i.d. p.o., for 12 weeks. The primary efficacy end point was ACR 20 response (American College of Rheumatology outcome score for 20% improvement in a number of different measures) at week 12. Blood samples were withdrawn from 18 out of 74 patients at baseline. PBMCs were separated and western blot analysis of protein extracts was used to evaluate A_3AR protein expression level.

CF-101 reduced disease activity, showing maximal response at 1 mg, with a somewhat lower response at 0.1 and 4 mg. At week 12, there were 60, 36 and 12% of the patients receiving CF-101 1 mg who achieved ACR 20, 50 and 70 responses, respectively. The respective mean percentage reduction in the number of tender and swollen joints was ~ 80% in all dose groups. CF-101 was well tolerated with no dose-limiting toxic effects. A statistically significant direct correlation between A_3AR over-expression at base line and ACR 50 at week 12 was found.

It was concluded that CF-101 showed a clinical response in this Phase IIa study without dose-limiting side effects in patients with active RA. A_3AR levels may be a predictive surrogate marker of response to this therapy [78].

9. Expert opinion and conclusions

The field of adenosine receptors has grown exponentially during the last couple of years. Activation of A_{2A}AR with selective agonists have demonstrated their ability to inhibit the manifestations of inflammatory cell activity, including superoxide anion generation, cytokine production and adhesion molecule expression. However, tachycardia is observed due to the vasodilatation effect induced by those agonists [79,80].

The A_3AR was the last receptor to be cloned among the adenosine receptor family and the synthesis of agonists and antagonists to this receptor, a field pioneered by Jacobson *et al.* [7], led to the understanding of its role in different pathologies. The findings presented in this study enlighten the A_3AR as an attractive platform for drug development.

The A_3AR seems to be a highly specific target due to its differential expression in inflammatory (high expression) and normal cells (low expression). Moreover, the findings showing that A_3AR expression levels in PBMCs reflect receptor status in the remote inflammatory organ, suggest the use of the receptor as a biologic marker. This has enabled the development of tailor-made drugs, A_3AR agonists, with high selectivity and affinity to this specific target.

Additional findings demonstrating that there is a differential response of pathologic and normal cells to a given synthetic A_3AR agonist has encouraged the use of this target as a platform for the development of anti-inflammatory agents. In inflammatory cells, apoptosis occurred on treatment with CF-101, whereas normal cells were refractory to the effect of these drugs.

Moreover, the defined mechanism of action generated by CF-101 (i.e., de-regulation of the NF-κB signaling pathway) was already demonstrated for other effective anti-rheumatic drugs, supporting the use of A_3AR agonists as drug candidates. NF-κB is involved with the regulation of both receptor expression and functionality, being one of the transcription factors present in the promoter of the A_3AR gene.

It needs to be stressed that NF-κB is not the only one involved in mediation of A_3AR expression and functionality and studies are underway to look at the role of more transcription factors such as AP-1 and GATA that are present in the A_3AR promoter and that are known to control inflammation.

Furthermore, the mechanism of action through which CF-101 ameliorates the manifestations of arthritis is even broader and entails an immunomodulatory effect demonstrated by inhibition of T-cell proliferation playing a major role is the pathogenesis of inflammation.

The present therapy of RA includes treatment with DMARDs in early diagnosed disease aimed to prevent ultimate joint damage, such as sulfasalazine, hydroxychloroquine and MTX. However, side effects of these DMARDs have been reported, limiting its clinical use due to toxicity, rather than lack of efficacy. Side effects of MTX, for example, include nausea, changes in transaminases, stomatitis (which are often dose dependent) and others such as pneumonitis and hepatocellular changes. In ~ 30% of patients with RA, toxicity leads to discontinuation of MTX therapy within 1 year [81]. The newer agents used in treatment of RA, such as leflunomide and ciclosporin, induced side effects that include hypertension, gastrointestinal and dermatologic symptoms, severe weight loss, nausea, diarrhea for leflunomide and side effects for ciclosporin were reported to be increased serum, hypertension, infections and gingival hyperplasia were reported. The most concerned adverse effect of ciclosporin is the development of nephrotoxicity [82-84].

A recent advance in the management of RA is the use of biologic agents that block certain key molecules involved in the pathogenesis of the disease. Infliximab, etanercept and adalimumab are TNF-blocking agents. These biologic agents have been associated with a variety of serious and opportunistic infections including the development of active tuberculosis. In addition, reactivation of chronic hepatitis B, increased lymphoma risk, trends towards worse prognosis in heart failure patients, formation of auto antibodies and, in some rare cases of anplastic anemia, pancytopenia, vasculitis and demyelination have been described with the anti-TNF therapy [85,86]. Rituximab, which is an anti-CD20 agent, induced respiratory and urinary tract infections;

viral infections; mild-to-moderate headache, nausea and rigors; infusion reactions; hepatitis B reactivation; progressive multifocal leukoencephalopathy; and immune and pulmonary toxicity [87].

The biologic agents available so far are proteins and as such exert some difficulties associated with protein drugs, such as infusion administration (which needs hospitalization), high cost, anaphylactic shock and development of auto-antibodies. Exploring the molecular events contributing to the development of RA has led to a shift from conventional treatment with aggressive immune suppression to targeted biologic-based therapies that control the inflammatory pathways associated with the disease. Therefore, small-molecular orally active agents, which target specific pro-inflammatory pathways, could suggest an attractive alternative to biologic agents. A small-molecular agent inhibiting p38 MAPK has been characterized *in vitro* and so far several compounds have been advanced into clinical trials [88].

As described in this paper, CF-101 is a small orally bioavailable molecule that binds to a specific target: the A_3AR. Activation of this receptor induces inhibition in the NF-κB signaling pathway, resulting in various anti-inflammatory effects. At this stage, the data obtained from the Phase I and Phase II clinical studies support the general concept presented in this review. First, the high safety profile of CF-101 proves that the differential effect on normal and pathologic cells indeed takes place in human beings. The strong activity signal of CF-101 found in the RA Phase IIa clinical study suggests A_3AR as a possible target to be used in the management of RA and as a predictive biologic marker. This leads the way for the development of A_3AR agonists for additional autoimmune–inflammatory diseases.

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Affiliation

Sara Bar-Yehuda[1] PhD of Biology,
Head Research Laboratories,
Michael H Silverman[1] MD, Medical Director,
William D Kerns[1] MDV,
VP of Drug Development,
Avivit Ochaion[1,2], Head of Molecular Biology Laboratories,
Shira Cohen[1], Head of Animal Facilities &
Pnina Fishman[†1], Professor of Biology, CSO
[†]Author for correspondence
[1]Can-Fite BioPharma, 10 Bareket Street,
PO Box 7537, Petach-Tikva 49170, Israel
Tel: +972 3921 3501;
Fax: +972 3921 3567;
E-mail: pnina@canfite.co.il
[2]PhD Student, Bar Ilan University,
The Mina and Everard Goodman Faculty of Life Sciences, Ramat Gan, Israel