Anti-Inflammatory Effects of ABT-702, a Novel Non-Nucleoside Adenosine Kinase Inhibitor, in Rat Adjuvant Arthritis

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ABSTRACT

Adenosine (ADO) is a homeostatic inhibitory autacoid that is released at sites of inflammation and tissue injury, and exerts anti-inflammatory effects via multiple interactions at ADO receptor subtypes. Inhibition of ADO kinase (AK) increases extracellular ADO concentrations and AK inhibitors have demonstrated ADO-mediated anti-inflammatory effects in acute models of inflammation. To evaluate the potential utility of this approach in chronic inflammation, a novel, potent, and selective non-nucleoside AK inhibitor, ABT-702, was tested in the rat adjuvant arthritis model. Animals were immunized with complete Freund’s adjuvant on day 0 and were treated with vehicle or ABT-702 (20 mg/kg/b.i.d. p.o.) beginning on day 8. ABT-702 significantly inhibited arthritis as determined by paw volume. In addition, histologic and radiographic evidence of bone and cartilage destruction was significantly decreased in the treated group. Coadministration of the ADO receptor antagonist theophylline attenuated the anti-inflammatory effects of ABT-702, suggesting that this action was mediated through endogenous ADO release. To evaluate the mechanism of chondroprotection, Northern blot and electrophoretic mobility shift assays were performed on joints samples. These studies demonstrated that ABT-702 suppressed collagenase and stromelysin gene expression in treated animals. In addition, the activator protein-1 and nuclear factor-κB binding activity was also decreased. Therefore, ABT-702 inhibited clinical, radiographic, and histologic evidence of chronic inflammatory arthritis. The mechanism of joint protection is likely related to suppressed transcription factor activation and matrix metallocproteinase gene expression.

The anti-inflammatory effects of adenosine (ADO) have been documented in a variety of settings. For instance, ADO suppresses proinflammatory cytokine production by macrophages and decreases neutrophil superoxide release, degranulation, and adhesion to endothelial cell (Cronstein et al., 1983, 1986, 1992; Roberts et al., 1985; Schrier and Imre, 1986). ADO receptor agonists have also demonstrated efficacy in animal models of neutrophil adhesion and tissue infiltration (Grisham et al., 1989; Schrier et al., 1990; Nolte et al., 1991; Asako et al., 1993). However, the use of ADO receptor agonists is often limited by cardiovascular side effects, and this problem has hindered the clinical development of ADO receptor agonists (Belardinelli et al., 1989).

Since the actions of endogenous ADO are highly localized to tissues and cellular sites where it is released (Moser et al., 1989), inhibition of the primary metabolic enzyme for ADO, adenosine kinase (AK; ATP:adenosine 5'-phosphotransferase, EC 2.7.1.20), represents an alternative strategy to taking advantage of the beneficial actions of ADO by selectively increasing local concentrations of endogenous ADO in a site- and event-specific manner. AK inhibitors increase extracellular ADO concentrations in vitro (Golembiowska et al., 1996) and selectively increase ADO concentration in vivo in traumatized neural tissue (Britton et al., 1999). AK inhibitors also elevate extracellular ADO concentrations more effectively than adenosine deaminase inhibitors (Golembiowska et al., 1996). GP515, a nucleoside-like AK inhibitor, blocks neutrophil adhesion to endothelial monolayers in vitro via an adenosine-mediated mechanism. GP515 also inhibits inflammatory responses in several acute animal models, including the mouse air-pouch model, murine endotoxic shock, rat carrageenan paw edema, and the rat skin lesion model (Firestein et al., 1994a; Cronstein et al., 1995; Rosengren et al., 1995).

ABT-702, a novel and selective non-nucleoside AK inhibitor (Fig. 1), is an orally active agent that reduces both hyperalgesia and acute inflammation following intraplantar carrageenan administration into the rat hind paw (Kowaluk et al., 2000). To further characterize the efficacy of ABT-702...
in chronic inflammation, the novel AK inhibitor was evaluated in rat adjuvant arthritis. In this model of chronic inflammation, animals develop a destructive polyarthritis that mimics rheumatoid arthritis. Our studies demonstrated that oral administration of ABT-702 significantly suppressed chronic arthritis. The mechanism was related to adenosine receptor stimulation and was associated with improvement in bone destruction as well as decreased expression of matrix metalloproteinase (MMP) genes in the joint.

**Materials and Methods**

**Animals.** Male Lewis rats weighing 150 to 200 g were purchased from Charles River (Boston, MA). These were group housed (four per cage) in American Association for the Accreditation of Laboratory Animal Care-approved facilities at University of California, San Diego, in a temperature-regulated environment with lights on between 7:00 AM and 8:00 PM. Food and water were available ad libitum during testing. All animal handling and experimental protocols were approved by the University of California, San Diego, Animal Subjects Committee (Institutional Animal Care and Use Committee).

**Test Compounds and Reagents.** All chemicals were obtained from Sigma Chemical Co. (St. Louis, MO) unless otherwise stated. ABT-702 (Fig. 1) is a novel, potent (IC₅₀ 1.7 nM), and selective AK inhibitor (Jarvis et al., 2000). ABT-702 was synthesized as described (C.-H. Lee, M. Jiang, S. S. Bhagwat, M. Cowart, G. Gfesser, R. Perner, K. H. Ki, Y. G. Gu, A. O. Stewart, M. Williams, M. F. Jarvis, and E. A. Kowaluk, submitted).

**Adjuvant Arthritis Model.** Rats were immunized with complete Freund’s adjuvant on day 0. Arthritis typically began on day 10 and was maximum on day 16 to 20. Paw swelling was determined by water displacement plethysmometry. A synovial histology score was determined on H&E-stained sections using a semiquantitative scale that measures synovial inflammation (0–2+), cartilage integrity (0–2+), bone erosions (0–2+), marrow infiltration (0–2+), and extra-articular inflammation (0–2+) (maximum score = 10). Roentgenograms were obtained of the hind paws to assess bone changes using a semiquantitative scoring system [demineralization (0–2+), ankle and mid-foot erosions (0–2+), calcaneal erosion (0–1+), heterotopic bone formation (0–1+) (maximum possible score = 6)].

**Northern Blot Analysis.** Adjuvant arthritis rats were sacrificed on day 20 after immunization. The skin was removed from excised ankles and the joint samples were snap frozen and pulverized. Total RNA was obtained from paws with RNA STAT-60 (Tel Test, Friendswood, TX), according to the manufacturer’s instructions. RNA was quantified spectrophotometrically and the concentrations were normalized. RNA loading buffer (Bio-Rad, Hercules, CA) containing ethidium bromide was added and the pooled RNA was fractionated on a 1.2% formaldehyde agarose gel. The RNA was transferred to 0.45-μm nylon filter membrane. The blot was prehybridized in 50% formamide, 5× saline phosphate-EDTA (SSPE), 5× Denhardt’s solution, 1% sodium SDS, 200 μg/ml single-stranded DNA, and 50 μg/ml tRNA. Mouse collagenase-3 (MMP13) or ratstromelysin cDNA was denatured and labeled by incorporation (Random Primed Labeling kit; Boehringer-Mannheim, Indianapolis, IN) of [α-32P]dATP (New England Nuclear, Providence, RI). The probe was denatured at 100°C and the blot hybridized overnight at 42°C. The membrane was washed in 2× SSPE and 0.1% SDS at 37°C and exposed to Kodak X-Omat AR film (Rochester, NY) with an intensifying screen for 1 day at -80°C. Membranes were then stripped by washing in 50% formamide, 1% SDS, and 2× SSPE for 30 to 60 min at 65°C and reprobed as needed.

**Preparation of Nuclear Extracts.** Nuclear extracts were prepared as previously described (Han et al., 1998). Limbs were cut just above and below the ankle and the skin was removed. The joints were then snap-frozen in liquid nitrogen and pulverized. The samples were homogenized with 1 ml of buffer A (10 mM HEPES pH 7.4, 1.5 mM MgCl₂, 10 mM KCl, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 0.1% Nonidet P-40), incubated on ice for 15 min, and centrifuged at 850g at 4°C. The supernatants were discarded and the pellets resuspended 4 ml of buffer A without Nonidet P-40. The samples were centrifuged again and the supernatant was discarded. One hundred microliters of buffer C [25% (v/v) glycerol, 20 mM HEPES pH 7.9, 0.42 M NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA pH 8.0, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride] was added to the pellets and the samples were rocked at 4°C for 30 min. Samples were centrifuged in a microfuge at 4°C for 30 min and the supernatants were aliquoted and stored at -80°C.

**Electrophoretic Mobility Shift Assay.** The Bandsift kit (Promega, Madison, WI) was used according to the manufacturer’s instructions. Consensus and control oligonucleotides (Santa Cruz Biotechnology, Santa Cruz, CA) were labeled by polynucleotide kinase incorporation of [γ-32P]ATP (New England Nuclear, Boston, MA). Oligonucleotides sequences included the AP-1 consensus or mutant binding sequence and the NF-κB consensus or mutant sequence as previously described (Han et al., 1998). After the oligonucleotide was radiolabeled, the pooled nuclear extracts were mixed with 20 pmol of the appropriate 32P-labeled consensus or mutant oligonucleotide for 30 min at room temperature. Joint nuclear extracts were pooled for each time point and 4 μg of protein was incubated with the oligonucleotide. Samples were then resolved on a 4% polyacrylamide gel.

**Statistics.** Statistical analysis was performed using the Student’s t test or ANOVA.

**Results**

**ABT-702 Inhibits Paw Swelling in Adjuvant Arthritis.** ABT-702 was evaluated in adjuvant arthritis, a chronic model of inflammatory joint disease. Animals were immunized on day 0 with complete Freund’s adjuvant and treatment was begun on day 8. A representative experiment is shown in Fig. 2 (n = 3 separate experiments), demonstrating that ABT-702 significantly decreased paw swelling (n = 8 animals/group; p < 0.01). Histologic evaluation of the ankles was performed on paraffin-embedded sections. ABT-702 significantly decreased histologic damage to the joints (Fig. 3A) as assessed using a semiquantitative scoring system that...
includes synovial inflammation, extra-articular inflammation, bone marrow infiltration, and cartilage damage ($p < 0.05$; $n = 8$ animals/group). Finally, radiographic analysis of the hind paws demonstrated that ABT-702 significantly decreased bone destruction in adjuvant arthritis (Fig. 3B; $n = 8$ animals/group; $p < 0.05$). These data demonstrate that chronic administration of ABT-702 in adjuvant arthritis suppresses clinical, histologic, and radiographic signs of inflammatory arthritis.

**ABT-702 in Established Adjuvant Arthritis.** Having demonstrated the beneficial effect of ABT-702 in early adjuvant arthritis, an additional experiment was performed to evaluate its utility in established disease. Animals were immunized on day 0, and treatment with ABT-702 (20 mg/kg b.i.d.) was begun on day 20. As shown in Fig. 4, an anti-inflammatory effect was observed within a few days and persisted until the end of the experiment ($p < 0.01$).

**ABT-702 Acts through an Adenosinergic Mechanism.** To determine whether the effect of ABT-702 was mediated through adenosine receptor stimulation, rats were immunized with complete Freund’s adjuvant on day 0 and treated with either ABT-702 (20 mg/kg b.i.d.) or vehicle beginning on day 8. Simultaneously, each treatment group was divided into groups treated with either 20 mg/kg i.p. daily theophylline or a similar volume of vehicle. As shown in Fig. 5, ABT-702 significantly decreased paw swelling at all time points ($p < 0.01$ by ANOVA). However, the compound had only a marginal effect on paw swelling in animals treated with theophylline ($p > 0.10$ by ANOVA). These data suggest that ABT-702 acts through an adenosinergic mechanism.

**ABT-702 Decreases Synovial Transcription Factor Activation and MMP Gene Expression.** To determine the mechanism of the anti-inflammatory and chondroprotective effects of ABT-702, synovium was harvested from vehicle and ABT-702-treated rats with adjuvant arthritis on day 20 ($n = 8$ animals/group). Northern blot analysis on pooled synovial RNA demonstrated that ABT-702 decreased expression of MMP3 (stromelysin) and MMP13 (collagenase-3) (Fig. 6). Electrophoretic mobility shift assay studies on pooled nuclear extracts were then performed to determine the effect of ABT-702 on the transcription factor AP-1, which is a key regulator of MMP expression. As shown in Fig. 7, ABT-702 decreased AP-1 binding in nuclear extracts of arthritic joints. In addition, NF-$\kappa$B activation, which regulates an array of proinflammatory cytokines, was modestly decreased by ABT-702. Therefore, ABT-702 suppressed the machinery responsible for both joint destruction and inflammation in adjuvant arthritis.
ADO inhibits a variety of inflammatory processes that are relevant to arthritis. Neutrophil superoxide production (Cronstein et al., 1983, 1985; Roberts et al., 1985), degranulation (Schrier and Imre, 1986), and adhesion are inhibited. The effect of increased local ADO on T cells is complex, but generally inhibitory of activation (Apasov et al., 2000). ADO receptor agonists suppress T-cell and macrophage production of proinflammatory cytokines, including tumor necrosis factor-\(\alpha\) (Sajjadi et al., 1996), interferon-\(\gamma\), and interleukin-12 (Hasko et al., 1998). Local administration of ADO to the microvasculature inhibits leukocyte rolling and adhesion induced by platelet-activating factor (Asako et al., 1993) or by reperfusion injury (Grisham et al., 1989; Nolte et al., 1991). ADO receptor agonists have also demonstrated efficacy in animal models of acute inflammation (Schrier et al., 1990), although this approach has been hampered by cardiovascular side effects such as hypotension and bradycardia (Belardinelli et al., 1989).

The use of AK inhibitors represents one possible way to enhance tissue levels of adenosine to achieve a therapeutic effect while minimizing hemodynamic toxicity. One such agent, GP515, has been investigated in tissue culture adhesion assays and several animal models of acute inflammation (Firestein et al., 1994; Cronstein et al., 1995; Rosengren et al., 1995). In these experiments, the therapeutic efficacy was associated with increased ADO release and was blocked by ADO receptor antagonists. Other studies with ABT-702 in acute models of hyperalgesia and inflammation using receptor-selective antagonists indicate that both \(A_2\) and \(A_{2A}\) receptor activation contributes to the effects of ABT-702 in carrageenan-induced paw edema (Kowaluk et al., 2000).

Several antirheumatic drugs currently used in rheumatoid arthritis also have apparent ADO-regulating capacity that might contribute to their anti-inflammatory actions. For instance, methotrexate increases ADO release by endothelial cells and suppresses inflammation in the mouse air-pouch model through an ADO mechanism (Cronstein et al., 1991). More recently, Cronstein and colleagues demonstrated that the therapeutic effect of methotrexate in adjuvant arthritis was blocked by coadministration of the adenosine receptor antagonist theophylline (Montesinos et al., 2000). Other anti-inflammatory drugs, including sulfasalazine and aspirin, might also increase endogenous ADO release (Cronstein et al., 1999).

In the present report, we describe a novel non-nucleoside AK inhibitor that suppresses chronic inflammatory arthritis. Not only did ABT-702 inhibit joint swelling in this model, but histologic evidence of synovial inflammation and cartilage destruction was also significantly decreased. ABT-702 also suppressed joint destruction as determined by radiographic...
criteria. The mechanism of the anti-inflammatory effect is likely due to increased release of endogenous ADO. Because it has a very short half-life, measurements of ADO in tissues or blood are technically difficult and are frequently unreliable. Therefore, the ability of ADO receptor blockade to interfere with the therapeutic effect is often used to infer this mechanism of action. Because of the chronic nature of adjuvant arthritis and the short half-life of selective inhibitors, theophylline was used to block ADO receptors in this model. Coadministration of ABT-702 and theophylline abrogated the therapeutic benefit of the AK inhibitor, suggesting an ADO receptor-mediated mechanism. Although this does not prove that ADO receptor stimulation is involved in the mechanism of action, it is certainly consistent with this hypothesis and is similar to the effects observed with other ADO-mediated therapeutic agents. The specific ADO receptor subtypes involved have not been determined in this model. A3 adenosine receptor agonists have demonstrated anti-inflammatory effects in collagen-induced arthritis in mice, but since theophylline does not inhibit rat A3 receptors it is more likely acting through A1 and A2 receptors (Zhou et al., 1992; Szabo et al., 1998). As noted above, these data are consistent with the ability of A1- and A2A-selective antagonist to attenuate the acute anti-inflammatory effects of ABT-702 in acute models.

The ability of ABT-702 to block inflammation and joint destruction is likely due to the many documented effects of ADO on inflammatory responses. In addition to the antineutrophil actions, AK inhibitors also inhibit histamine or bradykinin-induced vascular leakage, which contribute to neutrophil-dependent tissue edema formation (Rosengren et al., 1995). Production of proinflammatory cytokines is inhibited by ADO (Bouma et al., 1994). Increased ADO levels significantly inhibit T-cell help through stimulation of the A2a adenosine receptor (Apasov et al., 2000). Since therapy is initiated during the rapid-onset phase of adjuvant arthritis, suppression of Th1 cytokines may contribute substantially to the observed effects. ABT-702 modestly decreased NF-κB binding (densitometry in arbitrary units: vehicle = 161, ABT-702 = 121). n = 8 animals/group.

The chondroprotective effects of ABT-702 are probably related to its ability to regulate MMP expression indirectly. We have previously demonstrated that ADO receptor stimulation inhibits collagenase gene expression in cultured synoviocytes, and this is thought to be mediated through the A3B receptor (Boyle et al., 1996). Methotrexate, which may act through ADO release, suppresses synovial collagenase gene expression in patients with active rheumatoid arthritis (Firestein et al., 1994b). In the current studies, we have shown that the chondroprotective effects of ABT-702 are associated with decreased collagenase and stromelysin gene expression. This action is accompanied by decreased AP-1 binding, which is perhaps the most important regulatory step in MMP transcription (Benbow and Brinckerhoff, 1997). Decreased activation of NF-κB could contribute to the suppression of inflammatory cytokine production, although this was...
not proven since synovial cytokine mRNA levels were below the level of detection in our system (data not shown).

These data indicate that ABT-702, a potent and selective non-nucleoside AK inhibitor, is effective in both acute and chronic models of inflammation. Its efficacy in adjuvant arthritis was associated with both decreased inflammation and joint destruction. This action was likely mediated through an effect of ADO on synovial transcription factor and MMP gene expression. The ability of an agent such as ABT-702 to inhibit inflammatory responses suggests that it might have therapeutic use in chronic inflammatory arthritis.

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