The anti-allergic drug, N-(3′,4′-dimethoxycinnamonomyl) anthranilic acid, exhibits potent anti-inflammatory and analgesic properties in arthritis


Objectives. The degradation of tryptophan by indoleamine 2,3-dioxygenase yields a number of immunomodulatory metabolites, including 3-hydroxyanthranilic acid, 3-hydroxykynurenine acid and quinolinic acid. N-(3′,4′-dimethoxycinnamonomyl) anthranilic acid (3,4-DAA) is a synthetic anthranilic acid derivative that has been used therapeutically in Japan for many years as an anti-allergic drug and has recently been shown to be effective in a murine model of multiple sclerosis.

Methods. In the present study, we tested the efficacy of 3,4-DAA in collagen-induced arthritis, a mouse model of rheumatoid arthritis, and analysed its mechanism of action.

Results. Administration of 3,4-DAA after arthritis onset reduced clinical and histological severity of arthritis and reduced pain. It completely abrogated thermal and mechanical hyperalgesia. 3,4-DAA also suppressed Th1 cell activity in lymph node cell cultures and raised serum levels of IL-10. In vitro, 3,4-DAA suppressed IFNγ production and proliferation of both T and B lymphocytes in a manner comparable with the endogenous tryptophan metabolite, 3-hydroxyanthranilic acid, suggesting similar mechanisms of action.

Conclusion. It is concluded that 3,4-DAA has both anti-inflammatory and analgesic properties, and may therefore be useful in filling an unmet need, in the treatment of rheumatoid and other forms of arthritis, especially in the light of its analgesic properties.

Key words: Rheumatoid Arthritis, T cells, B cells, Rodent.

Introduction

There is now compelling evidence to suggest that degradation of the essential amino acid tryptophan by indoleamine 2,3-dioxygenase (IDO) plays an important role in the regulation of T-cell immunity [1]. IDO acts partly by depriving T cells of the tryptophan required for proliferation and partly by producing tryptophan catabolites (collectively known as kynurenines) which themselves have potent immunomodulatory properties. Kynurenines that have been shown to affect T-cell activity include picolinic acid (PA), quinolinic acid (QA), 3-hydroxyanthranilic acid (3-HAA) and 3-hydroxykynurenine acid (3-HKA) [2].

N-(3′,4′-dimethoxycinnamonomyl) anthranilic acid (3,4-DAA) is a synthetic anthranilic acid derivative that shares the anthranilic acid core with both 3-HAA and 3-HKA. Furthermore, 3,4-DAA has been shown to suppress antigen-specific T-cell proliferation and IFN-γ and tumour necrosis factor-α (TNF-α) production, and to increase production of IL-4 and IL-10 in a manner comparable with the structurally related kynurenines [3]. 3,4-DAA is an orally active anti-allergic drug that is approved for routine use in Japan and has been shown by Platten et al. [3] to reverse paralysis in mice with established experimental autoimmune encephalomyelitis (EAE) at least partly by inhibiting the activation of myelin-specific T cells.

The ability of 3,4-DAA to reverse established EAE prompted us to evaluate its efficacy and mechanism of action in collagen-induced arthritis (CIA), a T-cell mediated disease that displays many pathological, genetic and immunological similarities to human rheumatoid arthritis (RA) [4]. We are able to confirm the findings of Platten et al. [3] by showing that 3,4-DAA suppresses clinical and histological severity of an autoimmune disease, and in this case arthritis, and is able to suppress Th1 cell activity whilst increasing levels of IL-10 in vivo. Furthermore, an analysis of this model for an important symptom, pain, revealed that 3,4-DAA has analgesic properties, augmenting its potential therapeutic ability.

Materials and methods

Reagents

Type II collagen was purified from bovine cartilage, as described [4] and solubilized by stirring overnight at 4°C in acetic acid (0.1M) or Tris buffer (0.05 M Tris, containing 0.2 M NaCl, pH 7.4). 3,4-DAA was synthesised by Pacific Therapeutics. For in vitro studies 3,4-DAA was dissolved at a maximum concentration of 10 mg/ml in 1% sodium bicarbonate by heating for 1 h at 70°C. Upon cooling, an emulsion was formed. For in vivo studies 3,4-DAA was dissolved in dimethyl sulphoxide (DMSO). 3-Hydroxy-anthranilic acid (3-HAA) was purchased from Sigma (Poole, UK) and dissolved in PBS.

Induction and assessment of arthritis

Male DBA/1 mice (8–12 weeks old) were immunized intradermally at the base of the tail with bovine type II collagen (200 μg) emulsified in complete Freund’s adjuvant (CFA; Difco, West Molesey, UK). Arthritis was monitored clinically using the following scoring system: 0 = normal, 1 = slight swelling and/or erythema, and 2 = pronounced oedematous swelling. Each limb was graded, giving a maximum score of 8 per mouse. In addition, paw-swelling was measured using calipers.

Histopathological assessment of arthritis was carried out in a “blinded” fashion on decalcified haematoxylin and eosin-stained sections using a scoring system as follows: 0 = normal; 1, minimal synovitis without cartilage/bone erosion; 2, synovitis with some marginal erosion but joint architecture maintained; 3, severe synovitis and erosion with loss of normal joint architecture.
This research was approved by the local ethical review process committee and by the Home Office of Great Britain.

**Serum anti-collagen antibody levels**

ELISA plates (Nunc, Uxbridge, UK) were coated with 2 μg/ml of bovine CII dissolved overnight in Tris Buffer (0.05 M Tris, containing 0.2 M NaCl, pH 7.4) blocked with 2% bovine serum albumin (BSA) and then incubated with serial dilutions of test sera. A reference sample was included on each plate. Bound total IgG, IgG1 or IgG2a was detected by incubation with HRP-conjugated sheep anti-mouse IgG, IgG1 or IgG2a, followed by TMB substrate. Optical density was measured at 450 nm.

**Analysis of lymph node cell responses**

Inguinal lymph nodes were excised from 3,4-DAA-treated and control mice. Alternatively, inguinal lymph nodes were removed from untreated arthritic mice (day 1–5 of arthritis) and 3,4-DAA was added in vitro. In both cases, a single cell suspension was prepared and cultured in RPMI 1640 containing FCS (10% v/v), 2-mercaptoethanol (20 μM), l-glutamine (1% w/v), penicillin (100 U/ml) and streptomycin (100 μg/ml) in the presence or absence of type II collagen (50 μg/ml). Secreted cytokines (IFN-γ, IL-5 and IL-10) were measured after 72 h by enzyme-linked immunosorbent assay (ELISA). In brief, 96-well ELISA plates were coated with the respective capture antibody, blocked with bovine serum albumin (2% w/v), and then incubated with lymph node culture supernatants overnight at 4°C. After washing, bound cytokines were detected using biotinylated detect antibodies. A standard curve was generated using known concentrations of the appropriate recombinant cytokine and the concentrations of cytokines present in culture supernatants were estimated by reference to the standard curve.

**B- and T-cell purification and activation**

A single-cell suspension was prepared by pushing splenic tissue through a cell strainer, and erythrocytes were lysed using an ammonium chloride solution (Sigma, St Louis, MO, USA). B cells were positively enriched by using anti-IgM microbeads (BD Pharmingen), and T cells were positively enriched using anti-CD4 MACS microbeads, according to the manufacturer’s guidelines (Miltenyi Biotec, Bergisch Gladbach, Germany). Purity was assessed by flow cytometric analysis (B cell >90% CD19+, T cell >90% CD4+). Cells were cultured at 5 × 10^6 cells/ml in 200 μl complete RPMI, as above, in a flat bottom 96-well plate and cultured for 72 h. B cells were stimulated with anti-CD40 monoclonal antibody (10 μg/ml; BD), and T cells were stimulated with 5 μg/ml plate-bound anti-CD3 (ebioscience) plus 5 μg/ml soluble anti-CD28 (ebioscience). 3,4-DAA, 3-HAA, or vehicle (DMSO) was added at graded concentrations immediately prior to stimulation. Forty-eight hours after stimulation, 100 μl culture medium was collected, and cells were pulsed with 1 μCi ^3H thymidine per well for 18 h. Cells were then harvested and plates assessed for thymidine incorporation. Each assay was performed on a minimum of three occasions. IFN-γ levels were assessed in the culture medium by ELISA, as above.

**Hyperalgesia assessment with 3,4-DAA therapy**

The pain thresholds of the mice were assessed prior to immunization (naïve) on the day of onset (day 0) and up to 10 days following therapy with 3,4-DAA (200 mg/kg/day) or vehicle alone (n = 9 per group). The Ugo Basile 37400 Plantar Von-Frey microprocessor controlled unit was used to assess mechanical hyperalgesia and Ugo Basile 7370-6 Plantar Test (Hargreaves test) was used to assess thermal hyperalgesia. Mechanical hyperalgesia was assessed by applying an increasing force to the hind paw at the rate of 3 g/s, and measuring the force required to elicit lifting of the paw. Thermal hyperalgesia was assessed by applying an increasing infrared source (intensity 50), and measuring the time required for lifting of the paw (paw withdrawal latency; PWL).

**Statistical analysis**

Group means were analysed by one-way analysis of variance, followed by the Dunnett Multiple Comparisons test, where appropriate.

**Results**

**Prophylactic administration of 3,4-DAA inhibits the development of CIA**

In order to assess its anti-arthritic potential, 3,4-DAA was injected into DBA/1 mice (200 mg/kg/day) from the day of immunization with type II collagen in CFA. By day 28, five of seven (71%) vehicle-treated mice had developed arthritis of moderate severity (clinical score 2.8 ± 0.6), whilst one of seven (14%) 3,4-DAA-treated mice had developed mild arthritis (clinical score 1). Analysis of the sera of treated and control mice revealed no change in anti-collagen IgG1 or IgG2a levels in 3,4-DAA-treated mice (data not shown).

**3,4-DAA administered after disease onset reduces the severity of arthritis**

We next tested the ability of 3,4-DAA to treat established CIA. Mice were immunized with type II collagen in CFA. On day 1 of clinical arthritis (the day that arthritis was first observed), mice were randomly assigned to different treatment groups and given 3,4-DAA (100 mg/kg/day, 200 mg/kg/day or 400 mg/kg/day) or vehicle alone over a 10-day period. In two separate experiments, a dose-dependent reduction in both clinical scores and paw-swelling was observed in the 3,4-DAA-treated mice (Fig. 1A and B). Significant differences between 3,4-DAA-treated and control mice were observed from day 3 until the end of the treatment period (day 10). On day 10, the mice were killed and the first paw to show clinical evidence of arthritis was processed for histology. Joints were examined ‘blindly’ for severity of inflammation and joint erosion. Again, a clear dose-dependent reduction in histological severity of arthritis was observed in the 3,4-DAA-treated mice (Fig. 1C).

Sera from control and treated mice were analysed for levels of anti-type II collagen IgG1 and IgG2a, but no differences were observed between any of the groups (data not shown). Sera were also analysed for IL-10 production and a dose-dependent increase in circulating IL-10 levels was detected following treatment with 3,4-DAA (Fig. 2).

At the end of the experiment, draining (inguinal) lymph node cultures from control and treated mice were cultured for 72 h in the presence or absence of type II collagen. IFN-γ, IL-5 and IL-10 production was measured by ELISA. IFN-γ production was found to be significantly reduced in the mice given 3,4-DAA at 400 mg/mouse (Fig. 3A). However, on re-stimulation with collagen, differences between the groups were not significant (Fig. 3B), indicating that the ability of the T cells to respond to antigenic stimulation returned to normal once the 3,4-DAA had been removed from the system. IL-5 production was unaffected by treatment with 3,4-DAA with or without the addition of collagen (Fig. 3C and D, respectively).

The above data suggests that on removal of 3,4-DAA, lymph node cell cultures regain the ability to be activated with specific antigen. Therefore, it is clearly of interest to establish what happens in vivo when treatment with 3,4-DAA is stopped. Is there a disease flare and if so, does it occur immediately after cessation of treatment? Hence, a group of arthritic mice were treated from day 1 to day 5 of arthritis with 3,4-DAA (400 mg/kg/day) (Fig. 4). Treatment was then stopped and mice were monitored for a further 7 days. As before, there was a dramatic reduction in
reduced IFN-\(\gamma\) production, both 3,4-DAA and 3-HAA therapy dose-dependently inhibited B- and T-cell proliferation. Inhibition of proliferation was also observed when B cells were stimulated with LPS or anti-IgM (data not shown). In terms of cytokine production, both 3,4-DAA and 3-HAA therapy dose-dependently reduced IFN-\(\gamma\) production by T cells (Fig. 5C). It is of note that arthritis severity during the treatment period. When treatment was stopped on day 5, exacerbation of arthritis was observed from day 9, although the severity of arthritis did not reach that of the control group.

3,4-DAA and 3-HAA show comparable inhibitory effects on B and T cells

Platten et al. [3] showed that 3,4-DAA has similar effects on T-cell activity as naturally occurring tryptophan metabolites, including PA, QA, 3-HAA and 3-HKA. To confirm and extend these findings, we compared the anti-proliferative action of 3,4-DAA with 3-HAA on both B and T cells (Fig. 5). Activation of purified B (Fig. 5A) and T cells (Fig. 5B) was induced by anti-CD40 and anti-CD3/CD28, respectively, and proliferation was assessed by \(^{3}\text{H}\)-thymidine incorporation. Both 3,4-DAA and 3-HAA dose-dependently inhibited B- and T-cell proliferation. Inhibition of proliferation was also observed when B cells were cultured with type II collagen in CFA and monitored for development of arthritis. On day 1 of arthritis, mice were injected intraperitoneally with 3,4-DAA on a daily basis. Paw thickness was measured with calipers. The clinical scoring system was as follows: 0=normal; 1=slight swelling and/or erythema; 2=pronounced oedematous swelling. Each limb was graded, giving a maximum score of 8 per mouse. Histological assessment of arthritis was carried out on haematoxylin and eosin stained sections using a scoring system as follows: 0=normal; 1=slight swelling and/or erythema; 2=pronounced oedematous swelling. There were 14 mice/group (data pooled from two separate experiments). *P < 0.05 (compared with control group).

Fig. 1. Treatment of established CIA with 3,4-DAA. DBA/1 mice were immunized with type II collagen in CFA and monitored for development of arthritis. On day 1 of arthritis, mice were injected intraperitoneally with 3,4-DAA on a daily basis. Paw thickness was measured with calipers. The clinical scoring system was as follows: 0=normal; 1=slight swelling and/or erythema; 2=pronounced oedematous swelling. Each limb was graded, giving a maximum score of 8 per mouse. Histological assessment of arthritis was carried out on haematoxylin and eosin stained sections using a scoring system as follows: 0=normal; 1=slight swelling and/or erythema; 2=pronounced oedematous swelling. There were 14 mice/group (data pooled from two separate experiments). *P < 0.05 (compared with control group).

Fig. 2. Treatment with 3,4-DAA leads to increased IL-10 levels in vivo. Mice with established CIA were treated with 3,4-DAA or vehicle (n = 7) for 10 days (Fig. 1), then bled. IL-10 in the sera was measured by ELISA.

Fig. 3. Mice with established CIA were treated for 10 days with 3,4-DAA or vehicle control. Mice were then killed and draining (inguinal) lymph node cells were cultured for 72 h in the absence or presence of type II collagen. IFN-\(\gamma\) and IL-5 production was measured by ELISA and was found to be significantly reduced in the mice given 3,4-DAA at 400 mg/kg. However, on re-stimulation with collagen, differences between the groups were not significant, indicating that the ability of the T-cells to respond to antigenic stimulation returned to normal in the absence of the drug.

Fig. 4. Relapse of arthritis 4 days after cessation of therapy. Mice with established CIA (n = 6) were treated with 3,4-DAA (400 mg/kg/day) from days 1 to 5 or arthritis and clinical severity of arthritis was monitored up to day 12. Arthritis is seen to relapse at around day 9.
3,4-DAA inhibits B- and T-cell proliferation in vitro. Purified B and T cells were stimulated for 72 h with anti-CD40 (A), or anti-CD3/anti-CD28 (B) respectively, in the presence of varying doses of 3,4-DAA or 3-HAA. Both 3,4-DAA and 3-HAA dose-dependently inhibited B- and T-cell proliferation, assessed by [3H]-thymidine incorporation, and dose-dependently reduced IFN-γ production by T-cells (C). *P < 0.05, **P < 0.01, ***P < 0.001.

3,4-DAA inhibits mechanical and thermal hyperalgesia in arthritic mice. Mechanical (A) and thermal (B) hyperalgesia, were assessed in naïve mice, on the day of arthritis onset, and up to 10 days following therapy with 200 mg/kg 3,4-DAA, or vehicle. 3,4-DAA abolished thermal (A) and mechanical (B) hyperalgesia compared with controls. *P < 0.05, **P < 0.01, ***P < 0.001.

3,4-DAA reduces hyperalgesia in established arthritis

We also addressed the question of whether 3,4-DAA therapy of established arthritis affects inflammatory pain. Thermal and mechanical hyperalgesia was assessed prior to arthritis onset, on the day of arthritis onset, and up to 10 days following therapy with 3,4-DAA or vehicle (Fig. 6). Arthritis induced a 2- and 5-fold decrease in mechanical thresholds on the day of onset, and 5 days post-onset, respectively (Fig. 6A), and a 3.4-fold decrease in thermal thresholds throughout (Fig. 6B). 3,4-DAA abolished mechanical and thermal hyperalgesia, returning thresholds to the levels of non-arthritic animals.

Discussion

3,4-DAA, an analogue of endogenous immunolomodulatory metabolite, is currently used in the treatment of allergic inflammation [3, 6], and has recently been shown to be effective in EAE [3, 6]. The aim of this study was to assess whether 3,4-DAA may be useful for the treatment of RA, using a well-characterized animal model. Initial assessment showed that prophylactic administration of 3,4-DAA interfered with the development of CIA, not unexpected for a drug which mimics an important immunoregulatory pathway. Therapeutically, 3,4-DAA was very effective, and reduced all aspects of the disease, paw swelling, clinical score and histological damage in a dose-dependent fashion and reduced hyperalgesia. This degree of benefit compares well with prior results in this animal model, including with therapeutics that have been highly successful in humans, such as anti-TNF therapy [7]. In prior studies, we have not assessed effects on pain, and so do not have comparative data, but preliminary studies suggest that 3,4-DAA has a greater analgesic effect than dexamethasone at effective anti-inflammatory doses (Inglis et al., unpublished).

A question arises as to whether the doses effective in mice have any relationship to doses which are known to be safe and effective in humans. Analysis of the steady state plasma concentrations in humans following the oral administration of 3,4-DAA at 600 mg/day for the treatment of restenosis were 50–200 μM [8]. In mice, steady-state plasma concentrations of around 44 μM were reported following treatment with 3,4-DAA, at a dose of 100 mg/kg/day and 315 μM at 300 mg/kg/day [9]. This similarity of plasma levels indicates that the range of doses of 3,4-DAA used in the present study (100–400 mg/kg/day) were therapeutically relevant. Side effects of 3,4-DAA therapy, which has been in use in

the IC₅₀ for 3,4-DAA on T- and B-cell proliferation was well below the blood levels that were reached in vivo in human patients treated with the drug [5]. Hence, treatment of patients with 3,4-DAA would have a significant impact on T- and B-cell activity.
Japan for the past 25 years have been reported in a small number of patients, including rashes, nausea, hyperbilirubinaemia and increased hepatic enzymes [8]. However, these symptoms disappear after the administration of 3,4-DAA is discontinued, resulting in no long-term damage. The genetic basis for this effect is understood, and patients can be pre-screened [10].

Of particular interest was the dose-dependent increase in the anti-inflammatory cytokine IL-10 that was observed in serum following 3,4-DAA therapy. Treatment of CIA with IL-10 is efficacious in CIA [11], but IL-10 given systemically in humans with RA was not effective, as it was associated with dose limiting toxicity in humans [12]. IL-10 induction is a potential mechanism of action of 3,4-DAA, but is clearly not the only one. Inguinal lymph nodes from treated arthritic animals displayed reduced spontaneous IFN-γ production. However, short-term culture of lymph node cells with CII increased IFN-γ production to the level of untreated mice. This indicates that the action of 3,4-DAA is rapidly reversible. This is further supported by the observation that upon cessation of therapy of 3,4-DAA, disease severity increased after about 4 days, although it did not reach levels of control mice (Fig. 4). This indicates that continued 3,4-DAA treatment is necessary for therapeutic action, but also suggests that daily therapy may not be necessary.

Another striking observation not reported with other anti-inflammatory reports, was on pain. 3,4-DAA abolished thermal and mechanical hyperalgesia, indicating that the drug is analgesic. However, it is not clear from these studies whether this analgesic effect is secondary to or independent of the anti-inflammatory effect and we are currently addressing this question.

There is increasing evidence that IDO is important in immune regulation, both by depleting tryptophan, and by generating inhibitory tryptophan metabolites [1]. 3,4-DAA is an analogue of the natural tryptophan metabolite 3-HAA, which has inhibitory effects on lymphocytes. Thus, it was proposed that 3,4-DAA and 3-HAA act via similar pathways [3]. To partially address this question, we compared the activity of 3,4-DAA and 3-HAA on B- and T-cell proliferation and IFNγ production. Both 3,4-DAA and 3-HAA inhibited B-cell and T-cell proliferation and inhibited the production of the Th1 cytokine IFN-γ by T-cells. The results presented here are therefore consistent with those of Platten et al. [3] in mice with MBP peptide-induced EAE. Reduced TNF-α expression was also observed in the study of Platten et al. [3] following therapy with 3,4-DAA although we did not analyse levels of this cytokine in our study.

We cannot conclude, however, on the basis of these findings that reduced B-cell activity contributed to the therapeutic effects of 3,4-DAA in this study because levels of anti-collagen IgG were not significantly altered by therapy, although reduced antibody levels would be expected with a longer treatment period. Similarly, although reduced IFNγ production was observed in this study it cannot be concluded that this contributed to the therapeutic effect of 3,4-DAA because a number of studies have shown that IFNγ plays a disease-limiting role in CIA [13–15].

In conclusion, we have shown that 3,4-DAA is a highly effective anti-arthritic drug, both clinically and histologically, at doses leading to plasma concentrations which are safely achievable in humans. We have confirmed that 3,4-DAA inhibits T-cell proliferation, and have shown a novel action in the inhibition of B-cell proliferation. The fact that 3,4-DAA is effective both as an analgesic and anti-inflammatory agent, makes it an excellent candidate for the treatment of RA, as there is a shortage of orally active disease-modifying drugs for arthritis. In light of its established safety profile in allergic patients in Japan, clinical trials to validate this concept are clearly indicated.

**Rheumatology key messages**

- The anti-allergic drug, 3,4-DAA, is effective in experimental arthritis.
- 3,4-DAA appears to act as a mimic of an endogenous tryptophan metabolite.
- The advantages of the use of 3,4-DAA in arthritis is the oral availability, safe therapeutic profile and analgesic actions.

**Acknowledgements**

This study was supported by Pacific Therapeutics Pty. Ltd. The Kennedy Institute of Rheumatology Division is supported by the Arthritis Research Campaign (arc) of Great Britain.

M.S. is a co-founder and a shareholder in Pacific Therapeutics and a patent holder. M.F. is a consultant and one of the scientific founders and a member of the Scientific Advisory Board of Pacific Therapeutics and is a shareholder. R.O.W. and J.J.I. are consultants of Pacific Therapeutics and patent holders.

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