The peripheral benzodiazepine receptor ligand PK 11195 inhibits arthritis in the MRL-lpr mouse model

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Abstract

Objective. Mice of the MRL-lpr strain develop a severe autoimmune arthritic condition when primed with complete Freund’s adjuvant. The pathology is similar to that seen in human rheumatoid arthritis. We investigated whether PK 11195, a powerful ligand for peripheral benzodiazepine receptors, would have preventative or therapeutic effects in this model.

Methods. MRL-lpr mice were primed with complete Freund’s adjuvant at 13–14 weeks of age. Daily PK 11195 injections were started on the same day as priming to test for preventative effects. Daily PK 11195 injections were started 10 days after priming to test therapeutic effects.

Results. PK 11195 showed both preventative and therapeutic effects. At 1 mg/kg/day, it inhibited disease onset. At 3 mg/kg/day, it inhibited established disease progression.

Conclusion. The evidence suggests that PK 11195 may be the prototype of a new class of anti-inflammatory agents.

Key words: Autoimmune disease, Rheumatoid arthritis, Anti-inflammatory agents, Macrophages.
autoimmune disorder sharing similarities with human rheumatoid arthritis (RA), Sjögren’s syndrome and systemic lupus erythematosus [16–22]. Continuous breeding in some colonies has resulted, for unknown reasons, in a reduction in the severity and frequency of the spontaneous rheumatic disease. As a consequence, arthritis in these mice must be induced for them to have usefulness in assessing the efficacy of various experimental treatments.

We have utilized the injection of complete Freund’s adjuvant (CFA) as a method of enhancing arthritis in these MRL-lpr mice. Seventy-four per cent of mice injected with CFA at 13–14 weeks of age were found to develop clinical signs of arthritis in the subsequent 2–4 weeks. Histopathological analysis demonstrated that the large and small joints of the hind limbs displayed a significant and reproducible articular pathology. The changes included synovial cell proliferation, pannus formation and articular cartilage erosion adjacent to the proliferating synovial cells. These arthritic changes are more severe than those observed in untreated animals at a later age [23]. This model offers advantages over other arthritic models since the disease more closely resembles the pathology seen in human RA, and the early onset gives an opportunity to assess the effects of therapeutic interventions. It is considered to be a particularly demanding one for screening anti-inflammatory agents because of the severity and comprehensiveness of the disease pathology.

Materials and methods

Animals and treatment

MRL-lpr mice were obtained from a breeding colony located in the animal facilities in the Department of Oral Biology, University of British Columbia. This colony was established from stocks originally purchased from the Jackson Laboratories (Bar Harbor, ME, USA). Thirteen- to 14-week-old male and female mice were injected intradermally at two thoracic sites with a priming dose of 0.05 ml CFA supplemented to 10 mg/ml with heat-inactivated Mycobacterium tuberculosis H37 RA (Difco, Detroit, MI, USA). The adjuvant was prepared as a water in oil emulsion and administered with a 27 G needle. Control animals of the same strain and age were injected with phosphate-buffered saline. The mice were subsequently maintained on a standard diet with water ad libitum.

To test the preventative effects of PK 11195, one experimental group was treated with daily injections of 0.1 ml of PK 11195 (Tocris, Baldwin, MO, USA) dissolved in 25% ethanol in distilled water and another with daily injections of 0.1 ml of PK 11195 dissolved in dimethylsulphoxide (DMSO), starting on the day of CFA induction (day 0). Final concentrations of PK 11195 were equivalent to 1 or 3 mg/kg/day. Controls for these groups were injected with either 0.1 ml of 25% ethanol in distilled water or 0.1 ml of DMSO. Injections were given i.p. on a daily basis until killing on day 30. To test the therapeutic effects of PK 11195, the same protocol was followed, except that administration of the drug or DMSO vehicle was not commenced until day 10 after CFA administration. By day 10, most animals display significant joint swelling (average increase of 0.185 mm). The final concentrations of PK 11195 in this series of experiments were equivalent to 0.03, 0.3 and 3 mg/kg/day.

Clinical and histological examination of joints

The presence of clinical disease (visual appearance of arthritis) was evaluated in all groups of mice every 5 days and scored as positive if erythema and swelling of a fore or hind paw was observed. Bimalolar ankle width measurements were also taken in all animals every 5 days (always prior to PK 11195 injection in the treatment group) using a micrometer. Thirty days after injection of adjuvant, all animals were killed using CO2 asphyxiation, and the hind paws removed and placed in buffered formalin. Following removal of skin from the joints, they were next placed for 48 h in 10% formic acid for decalcification, and processed for paraffin embedding. Serial sections were cut to a thickness of 5 μm, and stained with haematoxylin and eosin. Sections were examined and scored by a blinded observer. The following parameters were graded 0–2 as previously reported in detail [23]: (a) subsynovial inflammation (0, normal; 1, focal inflammatory infiltrates; 2, inflammatory infiltrate dominates the cellular histology); (b) synovial hyperplasia (0, normal; 1, a continuous minimum three-layer-thick synovial lining of one joint; 2, minimum three-layer-thick synovial lining detected in several joints); (c) cartilage erosion and pannus formation (0, normal; 1, pannus partially covers cartilage surfaces without evident cartilage loss; 2, pannus connected to evident cartilage loss); (d) bone destruction (0, normal; 1, detectable destruction of bone by pannus or osteoclast activity; 2, pannus or osteoclast activity destroyed a significant part of the bone).

Statistical analyses

Statistical comparison of paired sets of ankle width measurements and histopathological indices between groups was determined using ANOVA followed by Student’s t-tests with Holm’s correction [24] for multiple comparisons.

Results

Examples of the articular pathology developing in mice at day 30 following CFA injection are shown in Fig. 1. Thickening of synovial tissue in a mouse injected only with CFA is illustrated in Fig. 1A and B. Similar synovial thickening with leucocyte infiltration is shown in a mouse receiving CFA plus DMSO vehicle in Fig. 1C and D. Pannus formation in mice receiving CFA plus DMSO vehicle is shown in Fig. 1E and osteoclastic activity in Fig. 1F. By contrast, a normal appearing joint of an animal receiving 3 mg/kg of PK 11195 starting at day 10 after CFA administration

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is shown in Fig. 1G. Figure 1H is from an untreated mouse. Animals of this strain spontaneously develop joint disease starting at 4–5 months, and mild synovial thickening of the joint was observed in this 28-week-old animal. In each case, only one area of the joint is illustrated. Evaluation was based on an assessment of the whole joint.

The preventative effects of PK 11195 are shown in Table 1, which compares inflammatory scores of drug-treated vs vehicle-treated animals. Data for animals treated only with CFA are also given. The table indicates that PK 11195 significantly reduced the severity of arthritis whether in ethanol or DMSO vehicle. The arthritic indices of vehicle-treated animals were highly comparable to each other and to CFA-only-treated animals.

The therapeutic effects of PK 11195 were tested by administering the agent in DMSO starting 10 days after CFA treatment. Three doses were tested: 0.03, 0.3 and 3 mg/kg. There was a trend towards reduced total arthritic scores with increasing doses, with the 3 mg/kg dose reaching statistical significance at the P < 0.001 level for the total score (Table 2).

The therapeutic effect of 3 mg/kg of PK 11195 was also evident through measurements of ankle width taken at 5 day intervals between initiation of treatment at day 10 and killing at day 30. This is illustrated in Fig. 2. The figure shows that ankle swelling had disappeared by day 30 in the mice treated with 3 mg/kg/day of PK 11195, while ankle swelling continued to increase in the DMSO-vehicle-treated animals.

**Discussion**

The data presented here show that PK 11195 at a dose of 1 mg/kg/day significantly inhibits the development of arthritic joint disease in the MRL-lpr mouse model when administered from the time of CFA induction (Table 1). More importantly, PK 11195 significantly inhibits established disease progression when adminis-

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**Table 1. Preventative effects of PK 11195 on MRL-lpr arthritis**

<table>
<thead>
<tr>
<th>Index</th>
<th>DMSO experiment</th>
<th>EtOH experiment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CFA</td>
<td>Vehicle</td>
</tr>
<tr>
<td></td>
<td>n = 10</td>
<td>n = 11</td>
</tr>
<tr>
<td>Synovial inflammation</td>
<td>1.40 ± 0.16</td>
<td>1.18 ± 0.25</td>
</tr>
<tr>
<td>Synovial hyperplasia</td>
<td>1.55 ± 0.16</td>
<td>1.50 ± 0.14</td>
</tr>
<tr>
<td>Bone destruction</td>
<td>0.5 ± 0.13</td>
<td>0.64 ± 0.12</td>
</tr>
<tr>
<td>Cartilage destruction and pannus formation</td>
<td>0.7 ± 0.13</td>
<td>0.64 ± 0.14</td>
</tr>
<tr>
<td>Total</td>
<td>4.15 ± 0.3</td>
<td>3.96 ± 0.48</td>
</tr>
</tbody>
</table>

A significant difference from vehicle treated, as determined by Student’s t-tests, is indicated by *P < 0.05; **P < 0.01. The histopathological score is given as the mean ± s.e.m. for each index. See [23] for rating method.

**Table 2. Therapeutic effect of 0.03, 0.3 or 3 mg/kg/day of PK 11195 in DMSO-MRL-lpr arthritis**

<table>
<thead>
<tr>
<th>Index</th>
<th>DMSO experiment</th>
<th>EtOH experiment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CFA</td>
<td>DMSO</td>
</tr>
<tr>
<td></td>
<td>n = 10</td>
<td>n = 11</td>
</tr>
<tr>
<td>Synovial inflammation</td>
<td>1.43 ± 0.16</td>
<td>1.19 ± 0.28</td>
</tr>
<tr>
<td>Synovial hyperplasia</td>
<td>1.59 ± 0.24</td>
<td>1.51 ± 0.16</td>
</tr>
<tr>
<td>Bone destruction</td>
<td>0.52 ± 0.15</td>
<td>0.63 ± 0.12</td>
</tr>
<tr>
<td>Cartilage destruction and pannus formation</td>
<td>0.71 ± 0.12</td>
<td>0.65 ± 0.14</td>
</tr>
<tr>
<td>Total</td>
<td>4.13 ± 0.31</td>
<td>3.97 ± 0.47</td>
</tr>
</tbody>
</table>

Significant differences between DMSO and PK 11195 groups are indicated by *P < 0.05; **P < 0.01; ***P < 0.001. The histopathological score is given as the mean ± s.e.m. for each index. See [23] for rating method.

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**Fig. 1.** H and E sections of ankle joint tissue in MRL-lpr mice. B, bone; C, cartilage; S, synovial tissue. (A) Lower power photomicrograph of the ankle joint of a mouse injected with CFA alone. The synovial tissue shows grade 2 thickening. (B) Higher power magnification of the area boxed in (A). The arrow points to grade 1 leucocyte infiltration. (C) Lower power photomicrograph of the ankle joint of a mouse treated with DMSO vehicle alone. The joint shows grade 1 synovial thickening. (D) Higher power photomicrograph of the boxed area in (C). The arrow points to grade 1 leucocyte infiltration. (E) High-power photomicrograph of the ankle joint of a mouse treated with DMSO alone. The arrow points to grade 1 pannus formation as the interstitial tissue extends over the cartilage. (F) High-power photomicrograph of the ankle joint of a mouse treated with DMSO vehicle alone, illustrating grade 1 bone osteoclastic activity (arrows). (G) Normal appearing ankle joint of a mouse treated with CFA and, commencing at day 10 post-treatment, 3 mg/kg of PK 11195 in DMSO. All of the above animals were killed at 30 days, i.e. 1 day post last injection. (H) Ankle joint of a 28-week-old mouse demonstrating spontaneous development of the disease. There is very mild hyperplasia of the synovium, indicative of the mild disease which develops endogenously in some older animals. Bars in (A, C, G, H) = 200 μm; bars in (B) and (D) = 100 μm; bars in (E) and (F) = 50 μm.

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**PK 11195 inhibits and treats mouse arthritis**

1071
tered at day 10 after CFA induction (Table 2 and Fig. 2). Thus, PK 11195 has both preventative and therapeutic actions. The mechanism is unknown. The high affinity of PK 11195 for endogenous PBRs ($K_d$ 4.3 nm [2]) suggests that it is more likely to be acting as an antagonist rather than an agonist at its receptor sites, although this remains to be proven.

Three endogenous proteins have been identified to which PBR ligands bind. These proteins might therefore correspond to the endogenous receptors for the ligands. They have molecular weights of 17–18, 30 and 32 kDa. The genes for all three proteins have been cloned and sequenced. The benzodiazepine subclass of PBR ligands, the prototype of which is 4'-chlorodiazepam (Ro 5-4864), binds preferentially to the 32 and 30 kDa proteins, while PK 11195 binds preferentially to the 17–18 kDa protein [25–27].

The physiological functions of PBRs remain a mystery. Evidence has been presented that they are associated with the outer mitochondrial membrane [28, 29], leading to the proposal that they affect mitochondrial respiration. However, there is a poor correlation between receptor density and the ability of ligands to affect respiration [30], weakening this hypothesis.

PBR ligands have been reported to inhibit mitogen-driven T- and B-cell stimulation in vitro, but these properties are shared by a number of central benzodiazepine ligands [31, 32]. PK 11195 has also been reported to stimulate antibody production in mice following immunization with sheep red blood cells [33–35]. However, if this were a mechanism of action, an opposite effect to that seen in this study should have been observed.

One possible explanation is that PK 11195 is blocking a receptor on monocyte-derived cells that is upregulated during inflammation, and, when activated, stimulates the inflammatory process. It is known that monocytes bind PK 11195 and that this binding is enhanced on macrophages and reactive microglia. Such an explanation is consistent with many previous reports showing enhanced PK 11195 binding in such conditions as multiple sclerosis [7], stroke [7, 12, 15] and Alzheimer’s disease [11], where inflammation is known to accompany the lesions. It is also consistent with in vitro results, which we will report separately, showing that PK 11195 inhibits the respiratory burst of activated macrophages and inhibits their ability to secrete neurotoxic substances.

MRL-lpr mice are known to develop lesions that, in addition to RA, are analogous to systemic lupus erythematosus and Sjögren’s syndrome. The CFA induction method employed in this study selectively enhances the rheumatoid arthritic component. Further research should be directed towards defining whether PK 11195 might similarly combat the other components of the spontaneous MRL-lpr disease with a view to the possible application of this agent to a range of human inflammatory disorders.

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PK 11195 inhibits and treats mouse arthritis

1073


