AF12198, a Novel Low Molecular Weight Antagonist, Selectively Binds the Human Type I Interleukin (IL)-1 Receptor and Blocks in Vivo Responses to IL-1*

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Interleukin-1 (IL-1) α and β are potent regulators of inflammatory responses. The naturally occurring interleukin-1 receptor antagonist (IL-1ra) is effective in vivo and in vitro in modulating biological responses to IL-1. We have previously reported the discovery of IL-1 antagonist peptides from the search of phage display libraries. Further characterization of this group of peptides has led to a 15-mer, AF12198, Ac-FEWTPGW-YQJYALPL-NH₂ (J represents the unnatural amino acid, 2-azetidine-1-carboxylic acid), with both in vitro and in vivo IL-1 antagonist activity. AF12198 selectively binds the human type I IL-1 receptor but not the human type II receptor or the murine type I receptor. In vitro, AF12198 inhibits IL-1-induced IL-8 production by human dermal fibroblasts with a half-maximal inhibition concentration or IC₅₀ of 25 nM and IL-1-induced intercellular adhesion molecule-1 (ICAM-1) expression by endothelial cells with an IC₅₀ of 9 nM. When given as an intravenous infusion to cynomolgus monkeys, AF12198 blocks ex vivo IL-1 induction of IL-6 and down modulates in vivo induction of IL-6. This is the first small molecule to show IL-1 receptor antagonist activity in vivo.

A wide range of immune and inflammatory responses are influenced by the cytokine IL-1α and IL-1β can cause both local and systemic inflammatory responses. Prolonged expression of even low levels of IL-1 can have detrimental consequences in chronic inflammatory diseases such as rheumatoid arthritis (2) and inflammatory bowel disease (3). The naturally occurring antagonist IL-1ra plays an important role in physiological regulation of IL-1-induced activities. The three IL-1 ligands, IL-1α, IL-1β and IL-1ra, bind to the two IL-1 receptors (IL-1R), type I and type II (1). Only the type I receptor induces signaling upon binding of IL-1α or -β. The type II receptor appears to act as a negative regulator of activity by effectively competing for binding of ligand and thus regulating IL-1 concentrations (4). The type II receptor released from cells selectively binds IL-1β or -α over IL-1ra, therefore having little effect on the inhibitory action of the antagonist (5).

IL-1 antagonists, including IL-1ra and soluble IL-1R, have been therapeutically effective in animal models of inflammatory disease and are currently in human trials. Recent studies have shown that IL-1ra can reduce some of the symptoms of established disease in model systems, bacterial cell wall-induced arthritis in rats (6), antigen-induced rheumatoid arthritis in rabbits (7), and lung fibrosis in mice (8). A small, active IL-1 antagonist should have the potential advantages over the recombinant IL-1ra protein including ease of synthesis, the ability to deliver locally in high concentrations, and possible oral delivery. We have screened large libraries of recombinant peptides in search of IL-1 receptor antagonists. The peptide described here, AF12198, is derived from a family of peptide antagonists we recently reported (9). AF12198, Ac-FEWTPGWQFYALPL-NH₂, binds specifically to the human type I IL-1R and blocks both in vitro and in vivo IL-1-induced activities. This peptide is the first low molecular weight IL-1 receptor antagonist to show in vivo activity.

EXPERIMENTAL PROCEDURES

Peptide Synthesis—Routine peptide synthesis was performed on an Advanced Chemtech model ACT350 multiple peptide synthesizer using Fmoc (N-(9-fluorenylethoxycarbonyl) chemistry (10), 2-(1H-benzotriazole-1-yl)-1,3,3-tetramethyluroniumhexafluorophosphate activation, and polystyrene resin derivatization with trifluoroacetic acid-labile Knorr linker. Peptides were cleaved from the resin using a solution of 95:5 trifluoroacetic acid/water and appropriate scavengers for 90 min. The cleavage solution was then filtered through a glass wool plug, concentrated to dryness, and the peptide purified to homogeneity by reverse-phase (C18) high performance liquid chromatography (HPLC) (water/acetonitrile gradient). Peptides prepared in this fashion afforded satisfactory results upon characterization by analytical HPLC, mass spectrometry, and high resolution mass spectrometry.

Binding Analysis—Competition binding analysis was as described (9). The soluble extracellular domain of either the type I or type II human IL-1R was bound to 96-well microtiter plates coated with specific non-blocking antibodies. AF12198 was resuspended at 1 mM in 5% dimethyl sulfoxide in binding buffer (RPMI 1640 (Life Technologies, Inc.), 20 mM HEPES, and 0.1% bovine serum albumin) and diluted to the appropriate concentrations. Peptides and 125I-labeled human IL-1α or -β (Amersham Corp.) at 50 pM were added to the plates in a total volume of 100 μl and incubated for 2 h at 4 °C on a rotating platform. When IC₅₀ values were less than 20 nM, the peptides were assayed with the same procedure using a 24-well format, allowing discrimination of the relative activities (9). Bound IL-1 was released with 1 N NaOH and counted in a gamma counter (Beckman). For binding to the murine cell line EL-4 (American Type Culture Collection), the assay was done in 12 × 75-mm polypropylene tubes using 2.0 × 10⁶ cells, 50 μM 125I-IL-1α, and peptide in a total volume of 0.1 ml. After 2 h incubation with shaking at 4 °C, the cells were collected and washed with a cell harvester (Brandel) and the glass fiber filters counted in a gamma counter. Calculations and binding analyses were performed using InPlot (GraphPad). Nonspecific binding was determined in the presence of excess IL-1ra, 1 μg/0.1 ml.
In Vitro Cell-based Assays—Human dermal fibroblasts, human umbilical vein endothelial cells (HUVEC), and the media used to culture them were from Clonetics. For the IL-8 assay, fibroblasts were plated in 48-well plates (Falcon) and grown to 90% confluence and transferred to serum-free media for overnight incubation. At the beginning of the assay, serum-free media was added along with peptide and recombinant human IL-1β (R&D Systems) at 50 pg/ml in a total volume of 0.5 ml. After 3.5 h incubation, media were collected and stored at −80°C until analysis by ELISA (R&D Systems). Activity was determined by comparison of cultures with peptide to cultures with IL-1β only. Background activation was determined as the IL-1-induced IL-8 in the presence of 1 μg/ml IL-1α.

In the interleukin adhesion molecule-1 (ICAM-1)/E-selectin assay, the HUVEC were cultured in 24-well plates to confluence and transferred to serum-free media the day before the assay. On the day of the assay fresh serum-free media were added, and the cells were stimulated with IL-1β at 100 pg/ml with and without the addition of peptide. After 5 h incubation, the cells were released from the plates with 0.02% EDTA in Dulbecco’s phosphate-buffered saline without Ca2+ or Mg2+. Cells were then stained with fluorescein isothiocyanate-conjugated anti-ICAM-1, anti-E-selectin (AMAC, Inc.) or a control, fluorescein-labeled IgG1. ICAM-1 and E-selectin expression was analyzed on a Coulter EPICS EliteC flow cytometer equipped with an argon laser at 488 nm.

Whole Blood Assay—Blood was drawn into heparinized (10 units/ml) syringes and kept on ice until initiation of the assay, approximately 15 min. Assays were done in 12 × 75-mm polypropylene tubes using 0.2 ml of blood and 0.2 ml of RPMI 1640 containing peptides and IL-1β at a final concentration of 3 ng/ml for human samples and 6 ng/ml for monkey samples. The blood was incubated for 8 h in 5% CO2/95% O2 and the upper plasma-like layer collected and stored at −80°C until analysis by ELISA (R&D Systems).

Analysis in Cynomolgus Monkeys—The ex vivo assay was carried out as described (11), and all procedures were reviewed and approved by the Institutional Care and Use Committee of Hoechst Marion Roussel. Briefly, animals were anesthetized, and a base-line blood sample was drawn into a heparinized syringe. The infusion of AF12198, 8 mg/ml in 5% dimethyl sulfoxide and 5% dextrose in water (McGaw), was initiated at a rate of 3 ml/kg/15 min. After 14 min, while the infusion was still in progress, a second blood sample was drawn. The two blood samples were each split into untreated control and IL-1β-treated groups and assayed as described above. The % activation was determined by the following formula: (pg of IL-6 for infusion sample/pg of IL-6 for preinfusion sample) × 100.

For the in vivo assay, animals were anesthetized with ketamine, intubated, placed on a respirator (isoflurane/O2), and allowed to stabilize (1 h). To maintain fluid volume, a saline infusion of 3 ml/kg/h was administered throughout the experiment. Blood samples were collected at the indicated times into heparinized tubes, plasma isolated by centrifugation, and frozen at −80°C until analysis by ELISA for IL-6. IL-1β (0.3 μg/kg) was administered intravenously in saline as a bolus at zero time. AF12198, 16 mg/kg/h, was infused beginning at −30 min and continuing until +60 min.

Analytical Determination of AF12198 in Plasma—Concentrations of AF12198 in human and monkey plasma were determined by HPLC after solid phase extraction. Plasma aliquots were spiked with an internal standard (WAWF-NH2, 1 nmol) and applied to Bond-Elut C18 extraction cartridge (Varian), preconditioned with methanol followed by 2% acetic acid in water. Following consecutive rinses with water (2 ml) and 10% acetonitrile in water (1 ml), the compounds of interest were eluted with 1 ml of methanol, 2% acetic acid in water, 1.0 M ammonium acetate (80:10:10, v/v/v). Aliquots of the extract (10–15 μl) were chromatographed using a Waters Nova-Pak C8 column (5.9 mm × 15 cm) at a flow rate of 1 ml/min. Separation was achieved using gradient elution (20–80% acetonitrile in water, 0.05% trifluoroacetic acid over 19.5 min). Fluorescence detection was accomplished using a Waters 470 fluorescence detector with excitation wavelength (285 nm) and emission wavelength (345 nm) chosen to allow selective detection of Trp. Linear calibration curves were generated by spiking AF12198 into control plasma over a range of 0.25–10 μM. Plasma concentrations of metabolites were estimated assuming an equivalent quantum yield for each Trp residue and adjusting for the number of Trp residues in each metabolite.

RESULTS

Optimizing Peptide Sequence AF12198—We have previously described the identification of peptides from recombinant peptide display libraries that bind the human type I IL-1R (9). AF11377 (FEWTPGYQYP/QALPL) was found to be a potent competitor of IL-1α binding, IC50 1.9 nM, and IL-1β-induced E-selectin expression by HUVEC, IC50 120 nM. Through analysis of directed peptide libraries and synthesis of specific peptides, we explored the structure-activity relationship of peptides related to AF11377 (Table I). Incorporation of the unnatural amino acid 2-azetidin-2-carboxylic acid (J) for proline in the core sequence QPY in AF11869 increased binding affinity 2-fold. When the carboxyl-terminal ALPL was deleted, AF11486, activity was decreased with the IC50 for binding 3.6 nM. Despite the protection of the carboxyl terminus by amidation, this peptide had an in vitro plasma t1/2 of only 0.5 h. AF11805, with the amino terminus acetylated, had an improved IC50 for binding, 1.4 nM, and a greatly improved in vitro plasma t1/2 of 2.7 h. Inversion of Tyr-7 and Trp-8 in AF11567 again diminished binding affinity but increased in vitro activity as measured by IL-1 induction of IL-8 by human fibroblasts. In this series AF12198, a protected 15-mer, had the best activity in the competition binding assay and the in vitro IL-8 assay and had a reasonable in vitro plasma t1/2 of 2.6 h. Therefore, AF12198 was selected for further in vitro and in vivo characterization.

IL-1-Induced Events Are Blocked in Vitro by AF12198—Many cells in culture are quite responsive to IL-1, and these responses can be used to characterize the in vitro activities of antagonists. We used a variety of assays including IL-1β induction of IL-8 by human dermal fibroblasts and IL-1-induced ICAM-1 and E-selectin expression by HUVEC to test in vitro the functional activity of AF12198 and related peptides. Both cell lines respond to concentrations of IL-1β as low as 10−14 M. Table I shows the inhibition of IL-1β-induced IL-8 in fibroblasts by these peptides. For comparison, IL-1α has an IC50 of 24 pM in this assay. We found that AF12198 had no agonist activity, as fibroblasts treated with AF12198 at concentrations up to 750 nM did not produce IL-8 in the absence of IL-1β.

Cellular adhesion molecules including ICAM-1 and E-selectin are constitutively expressed at low levels on resting endothelial cells but are rapidly induced to high levels of expression by cytokines. As shown in Fig. 1, HUVEC treated for 5 h with IL-1β at 100 pg/ml increased ICAM-1 expression from 2 to 56%
of the cell population (Fig. 1A), whereas E-selectin expression increased from 9 to 80% of the cell population (Fig. 1B). Using maximal expression of ICAM-1 as 56% and endogenous expression as 2%, an IC50 of 9 nM for AF12198 was determined. Similarly, AF12198 blocks E-selectin expression with an IC50 of 24 nM. As with the IL-8 assay, IL-1ra inhibits the IL-1 induction of these adhesion molecules at concentrations 1000-fold lower with an IC50 of 1–20 pm. AF12198 alone failed to induce ICAM-1 or E-selectin expression on HUVEC (data not shown).

**AF12198 Selectively Binds the Human Type I IL-1R**—By competition binding with 125I-IL-1α or -β, we have found that AF12198 is highly selective for the human type I receptor. We compared AF12198 with IL-1ra in binding to the soluble extracellular domains of human type I or type II receptors immobilized on 96-well plates (Fig. 2 and Table II). For the type I receptor, AF12198 competes for binding of 125I-IL-1α with an IC50 of 8.0 nM, nearly equal to that of IL-1ra, IC50 4.0 nM (Fig. 2A). As others have reported (12), soluble type II IL-1R binds radiolabeled IL-1α less well than radiolabeled IL-1β. Therefore, to test the affinity of the peptide and IL-1ra to soluble type II receptor, we used 125I-IL-1β. While IL-1ra competed for binding with an IC50 of 26 nM, AF12198 failed to compete at concentrations up to 6.7 μM (Fig. 2B, Table II).

We used the murine cell lines EL-4 and 3T3 to test for cross-species reactivity of the peptide. Human IL-1ra competes well for binding of human radiolabeled IL-1α to the murine type I IL-1 receptor expressed on EL-4 cells with an IC50 of 1.4 nM (Fig. 2C, Table II). However, AF12198 failed to compete, with the highest peptide concentration tested, 200 μM. The murine fibroblast line 3T3 was used to monitor IL-1β induction of IL-6. While IL-1ra inhibited IL-6 induction, AF12198 had no activity at concentrations up to 10 μM (data not shown). We also tested fibroblast lines from rabbit, dog, and cow and found that these cells neither bound detectable human 125I-IL-1α or -β nor responded to human IL-1β with prostaglandin E2 production.
However, we have previously shown (9) that related peptides will inhibit IL-1-induced prostaglandin E₂ production from CL160 cells derived from Rhesus monkeys. Therefore, it appears that while IL-1 ligands bind receptors from other species, AF12198 recognizes only type I receptor from humans and other primates.

**AF12198 Activity in a Whole Blood Assay**—Whole blood has been used as a model system for analysis of cytokine activity, as some of the cellular interactions important in local inflammation remain intact. We have developed an *in vitro* assay for analysis of IL-1 antagonist peptide activity in human and non-human primate blood, where addition of IL-1β elicits IL-6. Normally, endogenous plasma IL-6 levels are below detection limits (<2 pg/ml). On addition of IL-1β at 3 ng/ml to the blood samples, IL-6 levels increase rapidly, and after 8 h of incubation IL-6 levels are 500 to 5000 pg/ml, depending on the donor. In the experiment with human blood shown in Fig. 3, AF12198 inhibits IL-6 induction with an IC₅₀ of 15 μM whereas IL-1ra inhibits with an IC₅₀ of 2 nM. With blood from cynomolgus monkeys, the IC₅₀ values are 17 μM for the peptide and 30 nM for IL-1ra (data not shown). In the absence of IL-1β, AF12198 did not induce IL-6 in blood from either humans or cynomolgus monkeys.

**In Vivo Analysis of AF12198 Activity**—With the discovery that AF12198 would block IL-1-induced activity in monkey blood, we were able to develop *in vivo* systems for analysis of efficacy. Fischer et al. (13) reported that baboons given human IL-1α have many of the symptoms of endotoxemia, including fever, hypotension, neutrophilia, and induction of various inflammatory mediators including prostaglandin E₂ and IL-6. We have adapted this procedure for cynomolgus monkeys using a suboptimal dose of human IL-1β (0.3 μg/kg). At this IL-1β dose, heart rate increased, blood pressure decreased, and plasma IL-6 levels increased. We present here data for the IL-1β induction of IL-6 as detected in the plasma. The six animals in the study were given IL-1β as a bolus, intravenously (0.3 μg/kg/0.1 ml) at zero time, and plasma was collected at various times between −30 min and +300 min.

### Table II

| IL-1 receptor | Ligand | Antagonist | IC₅₀   |
|---------------|--------|------------|--------|
| Human type I  | hIL-1α | hIL-1α     | 4.4 nM |
| Human type I  | hIL-1α | AF12198    | 8.0 nM |
| Human type II | hIL-1β | hIL-1α     | 26 nM  |
| Human type II | hIL-1β | AF12198    | >6.7 μM|
| Murine type I | hIL-1α | IL-1ra     | 1.4 nM |
| Murine type I | hIL-1α | AF12198    | >200 μM|

AF12198 reduced (75% inhibition) plasma IL-6 levels compared with the response in the previous week (Fig. 4A). The responses of two of the animals (numbers 11 and 37) was not affected by AF12198 infusion. These animals were low responders to IL-1. After a 3-week respite, the response of five of six animals to the IL-1 bolus returned to levels equal to or greater
than seen in week 1.

Analytical determination of plasma concentrations of AF12198 showed that a steady state level of approximately 5 nmol/ml was achieved within 30 min of the initiation of the infusion, Fig. 5. Plasma concentrations of AF12198 decayed rapidly after the infusion ended with levels less than 1 nmol/ml at 90 min. Two major metabolites of AF12198 were detected in plasma at zero time, an 11-mer, Ac-FEWTPGWYQJY-OH, and a 6-mer, Ac-FEWTPG-OH. Both metabolites were present at 30 min after the beginning of the infusion at concentrations equivalent to or greater than that of AF12198, 4.4 nmol/ml for Ac-FEWTPGWYQJY-OH and 9.2 nmol/ml for Ac-FEWTPG-OH. Through the infusion period, the concentration of Ac-FEWTPGWYQJY-OH remained constant while the concentration of Ac-FEWTPG-OH continued to increase, indicating the continued cleavage of AF12198 and likely the 11-mer metabolite as well. Like AF12198, the Ac-FEWTPGWYQJY-OH plasma concentration decreased rapidly when the infusion was ended. The concentration of Ac-FEWTPG-OH, however, declined much more slowly. At the final collection time, 2 h after termination of the infusion, Ac-FEWTPG-OH concentration was 2.7 nmol/ml, while AF12198 and 11-mer were not detected. These truncated metabolites were inactive.

Using the same protocol, we administered an infusion of IL-1ra that resulted in each animal receiving a total dose of 0.3 mg/kg. This dose of IL-1ra, a 1000-fold higher than the amount of IL-1β given as a bolus, blocked IL-6 induction at least 98% (Table III). Fisher et al. (13) report similar results with bavoins, although the IL-1α and IL-1ra doses were higher (10 μg/kg and 10 mg/kg, respectively. In our experiment, plasma IL-1ra levels reached steady state concentrations of 35 pmol/ml within 30 min of the initiation of the infusion (Fig. 5). At the end of the infusion IL-1ra levels declined more slowly than AF12198. At 180 min, 2 h after the infusion ended, IL-1ra plasma levels had declined by only 60% to 14 pmol/ml.

Poor solubility and rapid metabolism seemed to limit the in vivo efficacy of AF12198. Therefore, we developed an ex vivo protocol (11) in which the same AF12198 dose was administered over a shorter infusion period, and consequently higher plasma concentrations of AF12198 were achieved. Furthermore, IL-1 induction of IL-6 was analyzed on ex vivo samples, eliminating some of the influences of rapid in vivo metabolism.

In this system, base-line induction of IL-6 by IL-1 was determined on circulating peptide in blood drawn during the infusion is influ-

**TABLE III**

Animals were treated as described under “Experimental Procedures” and Fig. 5A. Each animal received an infusion of 0.2 mg/kg/h beginning at −30 min and ending at +60 min; total IL-1ra dose 0.3 mg/kg. At 0 time the animals were given an intravenous bolus of IL-1β at 0.3 μg/kg.

| Animal | Plasma IL-6 |
|--------|-------------|
| IL-1β bolus | IL-1β bolus | Activity |
| (pg/ml) | (pg/ml) | (%) |
| 3 | 19,800 ± 1,270 | 325 ± 7 | 2 |
| 11 | 9,300 ± 200 | 26 ± 2 | 0 |
| 36 | 24,000 ± 700 | 160 ± 14 | 0 |
| 29 | 3,100 ± 180 | 48 ± 4 | 1.5 |
| 37 | 6,700 ± 700 | 67 ± 3 | 1 |

**FIG. 5.** Plasma concentrations of AF12198 and two major metabolites. Plasma concentrations of AF12198 (■), Ac-FEWTPGWYQJY-OH (○), and Ac-FEWTPG-OH (●) were determined by HPLC, and fluorescence detection and IL-1ra (□) were determined by ELISA. Infusions of AF12198 at 16 mg/kg/h and IL-1ra at 0.2 μg/kg/h were given. The time of infusion is shown by the arrow.
AF12198 infusion inhibits IL-1-induced IL-6 measured ex vivo

| Infusion | Animal | Sample | IL-6 Activity | Control | +IL-1 |
|----------|--------|--------|---------------|---------|-------|
|          |        |        | pg/ml %       |         |       |
| Vehicle  | 10     | Preinfusion | 0 660 ± 70 100 |         |       |
|          | 10     | Infusion  | 0 780 ± 120 118 |         |       |
|          | 39     | Preinfusion | 0 500 ± 65 100 |         |       |
|          | 39     | Infusion  | 0 490 ± 105 98 |         |       |
| AF12198  | 7      | Preinfusion | 6 ± 1 900 ± 265 100 |         |       |
|          | 7      | Infusion  | 0 0 0 0 |         |       |
|          | 10     | Preinfusion | 5 ± 1 900 ± 105 100 |         |       |
|          | 10     | Infusion  | 0 0 0 0 |         |       |
|          | 27     | Preinfusion | 8 ± 1 1100 ± 135 100 |         |       |
|          | 27     | Infusion  | 0 0 0 0 |         |       |

Animals were treated as described under “Experimental Procedures” for the ex vivo assay. Vehicle was 2% dimethyl sulfoxide in 5% dextrose in water, pH 9. AF12198 was infused at 18 mg/kg over 15 min.

The plasma concentration of AF12198 achieved after 15 min of infusion was determined as 78 nmol/ml, 15-fold higher than that detected in the in vivo protocol. The two major metabolites of AF12198 were again identified as the 11-mer, Ac-FEWTPGWYQJY-OH, and the 6-mer, Ac-FEWTPG-OH. Thus, when plasma concentrations are sufficient AF12198 blocks IL-1R in vivo.

DISCUSSION

In our search for small IL-1 antagonists we have screened diverse recombinant peptide libraries and found a peptide family that specifically binds with high affinity to the human type I IL-1 receptor. We have characterized in vitro and in vivo activities of one of the most potent of these peptides, AF12198, Ac-FEWTPGWYQJY-[NH2]. AF12198 inhibits IL-1-induced in vitro events at an effective molar concentration 1000-fold higher than for IL-1ra. Like IL-1ra, AF12198 has no agonist activity.

This is the first example of a small, low molecular weight IL-1R antagonist with in vivo antagonist activity. When we followed in vivo activity in cynomolgus monkeys, we found that infusion of the peptide did reduce IL-1 induction of IL-6 in four of six animals. However, AF12198 infusion did not affect the response of two of the test animals, both low responders to IL-1.

Several factors seem to be contributing to the marginal in vivo activity of AF12198 in this system. First, rapid metabolism limited the effective plasma concentration of AF12198. Analysis of plasma steady state levels of AF12198 revealed that the peptide was rapidly degraded by endopeptidase activity to two nonactive metabolites, Ac-FEWTPGWYQJY-OH and Ac-FEWTPG-OH. This metabolism was also reflected in the rapid decline in plasma concentrations of AF12198 once infusion was ended. As a consequence of the rapid metabolism, we were able to achieve a plasma concentration of active peptide of only 4 to 5 nmol/ml. When IL-1ra was administered with the same protocol, at a concentration of IL-1ra which completely blocked the response to the IL-1β bolus, steady state plasma concentrations of 35 pmol/ml were attained, and at the end of the infusion IL-1ra levels declined much more slowly than AF12198. If the results from the in vitro cell-based assays are an accurate predictor of in vivo efficacy, we needed to achieve a plasma peptide molar concentration 1000-fold higher than IL-1ra to get comparable activity. Therefore, we would need to achieve a plasma concentration of AF12198 of 35 nmol/ml, 7-fold higher than detected in the in vivo system. Unfortunately, the poor solubility of the peptide limited the dose that could be administered in this model system.

We were able to show that AF12198 does bind IL-1R in vivo, by using an ex vivo format, where cynomolgus monkeys could be treated with an infusion of the same dose of AF12198, but administered over a shorter period. With this protocol, a mean AF12198 plasma concentration of 72 nmol/ml was reached. This concentration completely blocked IL-1-induced activity ex vivo. Again, the two metabolites, 11-mer and 6-mer, were detected in plasma samples at the end of the 15-min infusion. Because we were able to deliver a high volume of AF12198 with a short infusion, the plasma concentration of active peptide was sufficient to block the functional effects of IL-1 binding. We are currently testing AF12198 analogs with modifications designed to limit proteolysis and increase solubility.

IL-1 has shown no species specificity for receptor binding although some differences in induction of biological activity have been reported (14). Human IL-1 clearly binds murine type I IL-1R and induces a variety of biological activities. However, analysis of a series of IL-1β muteins has shown different binding affinities and activation characteristics for human and murine receptors (15). Surface regions involved in binding appear to be distinct, suggesting that the complementary sites on each of the receptors are also distinct. This may explain the receptor selectivity of AF12198. The site where AF12198 binds to the human type I IL-1R is either not present on the human type II and murine type I receptors or is not essential for binding of ligand. That AF12198 does not bind the human type II receptor is a pharmacological advantage. The soluble type II IL-1R binds pro-IL-1β and IL-1β with high affinity and IL-1α with lower affinity but binds IL-1ra with greatly reduced activity (5). This provides a mechanism for blocking activation by limiting circulating levels of IL-1β but not IL-1α. Since AF12198 does not bind the type II receptor, the peptide does not compromise this regulatory interaction.

Recent mutagenesis studies (16, 17) and structural information (18–21) about IL-1 ligands and receptorshaveshownthatligandresidues’keytobindingareindiscontinuous,nonlineararrays. The agonists, IL-1β and -α, possess two main surface sites, A and B, which interact with the receptors (17). It is proposed that site B is essential for agonist activity. IL-1ra has only site A and thus has no agonist activity. Residues within site A identified by Evans et al. (17) as critical for IL-1ra binding are Trp-16, Gln-20, Tyr-34, Gln-36, and Tyr-147. We (20) and others (21) have reported that the crystal structure of IL-1ra like that of IL-1β and IL-1α forms a six-stranded β-barrel which is closed on one side by three β-hairpin loops. In the three-dimensional models of the ligands, the site A residues are clustered at the open end of the β-barrel. We have also been successful in the co-crystallization of IL-1ra and the soluble extracellular domain of the human type I IL-1R (22). Analysis of the model of bound IL-1R derived from the co-crystal show that the Trp-16, Gln-20, Tyr-34, Gln-36, and Tyr-147, identified by Evans et al. (17), are in direct contact with the receptor.

Analyses of peptide mutagenesis libraries designed to determine residues directly involved in AF12198 binding to the type I IL-1R have shown that QPY is essential (9). Furthermore, the
immediate amino-terminal residues of active AF12198 analogs are most frequently aromatics. Computer modeling of the WYQJY core of AF12198 indicates that the peptide can adopt a conformation similar to the A site-critical residues of IL-1ra. The identification of AF12198 from screening large libraries, combined with structural information derived from crystallography and NMR of the human type I receptor and molecular modeling, provides powerful tools for structure-based drug design. We are currently designing small peptidomimetic molecules as potential antagonists based on the model of the bound conformation of AF12198.

With AF12198 we have shown that a small molecule can block in vivo IL-1-induced activity. However, direct therapeutic use of AF12198 or related analogs will be limited due to rapid metabolism and poor pharmacokinetics. The value of AF12198 is as a tool in understanding pharmacophores important for antagonist activity and as a model for rational structure-based design of low molecular weight peptidomimetic antagonists.

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