Increased Potency of a Novel Complement Factor 5a Receptor Antagonist in a Rat Model of Inflammatory Bowel Disease


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Received March 23, 2005; accepted May 4, 2005

ABSTRACT

We have previously shown that complement factor 5a (C5a) plays a role in the pathogenesis of 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced colitis in rats by using the selective, orally active C5a antagonist AcF-[[OP(D-Cha)WR]. This study tested the efficacy and potency of a new C5a antagonist, hydrocinnamate (HC)-[[OP(D-Cha)WR], which has limited intestinal lumenal metabolism, in this model of colitis. Analogs of AcF-[[OP(D-Cha)WR] were examined for their susceptibility to alimentary metabolism in the rat using intestinal mucosal washings. One metabolically stable analog, HC-[[OP(D-Cha)WR], was then evaluated pharmacokinetically and investigated at a range of doses (0.03–10 mg/kg/day p.o.) in the 8-day rat TNBS-colitis model, against the comparator drug AcF-[[OP(D-Cha)WR]. Using various amino acid substitutions, it was determined that the AcF moiety of AcF-[[OP(D-Cha)WR] was responsible for the metabolic instability of the compound in intestinal mucosal washings. The analog HC-[[OP(D-Cha)WR], equiactive in vitro to AcF-[[OP(D-Cha)WR], was resistant to intestinal metabolism, but it displayed similar oral bioavailability to AcF-[[OP(D-Cha)WR]. However, in the rat TNBS-colitis model, HC-[[OP(D-Cha)WR] was effective at reducing mortality, colon edema, colon macroscopic scores, and increasing food consumption and body weights, at 10- to 30-fold lower oral doses than AcF-[[OP(D-Cha)WR]. These studies suggest that resistance to intestinal metabolism by HC-[[OP(D-Cha)WR] may result in increased local concentrations of the drug in the colon, thus affording efficacy with markedly lower oral doses than AcF-[[OP(D-Cha)WR] against TNBS-colitis. This large increase in potency and high efficacy of this compound makes it a potential candidate for clinical development against intestinal diseases such as inflammatory bowel disease.

Inflammatory bowel disease (IBD) is a chronic, debilitating disorder of the bowel in which there is targeted inflammation at one or more sites of the gastrointestinal tract. Although the precise etiology of IBD is not known, numerous inflammatory mediators have been implicated in the pathogenesis, including arachidonic acid metabolites (prostaglandins, thromboxanes, and leukotrienes), reactive oxygen species, nitric oxide, nuclear factors, growth factors, various cytokines (tumor necrosis factor-α, interleukins, and interferons), and complement system activation products (Nielsen and Rask-Madsen, 1996; Kolios et al., 1998; Papadakis and Targan, 2000). Current therapies for IBD remain inadequate and unsafe in many patients, despite recent advances in therapeutics. Existing treatment strategies include the use of aminosalicylates (sulfasalazine), corticosteroids (prednisolone and budesonide), immunomodulators (azathioprine, 6-mercaptopurine, cyclosporine, and methotrexate), or the new biological agents (antitumor necrosis factor-α or anti-interleukin antibodies), which are targeted against one or more of the above-mentioned inflammatory mediators (Lim and Hanauer, 2004). However, to date, none of these therapies are targeted toward, or have any effects on, the inhibition of complement activation products.

A role for the complement system in the pathogenesis of this disease has been suggested from the observation of increased complement deposition on epithelial cells as well as in the muscularis mucosa and submucosal blood vessels in patients with IBD (Halstensen et al., 1990, 1992; Halstensen and Brandtzaeg, 1991; Ueki et al., 1996). However, the evidence for complement involvement in this disease has been relatively limited to date, due to lack of readily available specific anti-complement agents.

ABBRVIATIONS: IBD, inflammatory bowel disease; TNBS, trinitrobenzene sulfonic acid; C5a, complement factor 5a; PBMC, peripheral blood mononuclear cell; HC, hydrocinnamate; Cin, cinnamate; PMN, polymorphonuclear leukocyte.
We have recently identified a role for the complement activation product C5a, in the pathogenesis of TNBS-induced colitis in rats, by the use of a specific, potent C5a antagonist AcF-[OP(α-Cha)WR], developed in our laboratories (Woodruff et al., 2003). This orally active cyclic peptide has been shown to be efficacious in a variety of inflammatory disease states (Strachan et al., 2000, 2001; Arumugam et al., 2002, 2003, 2004; Woodruff et al., 2002, 2004; Harkin et al., 2004) and is currently undergoing clinical development for use in humans. Given the oral activity and high efficacy of this compound in this model of IBD, and its excellent safety profile in humans when given orally (phase I clinical trials; www.promics.com), it is presently under investigation as a novel therapeutic target for IBD.

One potential drawback to the use of this particular compound in human IBD is the relatively high dose level (10 mg/kg/day) used to treat rats in the TNBS-colitis model (Woodruff et al., 2003). In an attempt to identify more potent analogs of AcF-[OP(α-Cha)WR], we recently synthesized a series of analogs and examined their C5a receptor affinity and activity (March et al., 2004). Although no single compound displayed significantly increased potency over AcF-[OP(α-Cha)WR], several analogs were found to be equally potent in vitro (March et al., 2004).

In the present study, we initially examined the relative alimentary tract metabolism of AcF-[OP(α-Cha)WR] in rats. We found that although it was stable in the stomach, it was readily metabolized by intestinal enzymes, perhaps limiting its therapeutic effects in the TNBS-colitis model. Consequently, we identified a cyclic analog hydrocinnamate (HC)-[OP(α-Cha)WR], which was resistant to intestinal metabolism. When administered orally, this compound was found to be 10- to 30-fold more potent than AcF-[OP(α-Cha)WR] in the TNBS-colitis model, despite displaying equiactive in vitro and pharmacokinetic properties. This compound may be a more suitable clinical candidate for use in intestinal diseases such as inflammatory bowel disease.

Materials and Methods

Antagonist Manufacture and Preparation

Cyclic Peptide Synthesis. All compounds were synthesized as described previously (March et al., 2004) and purified by reverse phase high-performance liquid chromatography. The compounds AcF-[OP(α-Cha)WR] (mol. wt. 955) and HC-[OP(α-Cha)WR] (mol. wt. 892) were further characterized by mass spectrometry and proton NMR spectroscopy.

Preparation of Compounds for in Vitro and in Vivo Studies. The majority of the analogs were dissolved in sterile distilled water before use in all experiments. The anthrily-alanine compounds, however, were poorly soluble in water and consequently were solubilized in 25% ethanol/water. For in vivo experiments, AcF-[OP(α-Cha)WR] and HC-[OP(α-Cha)WR] were dissolved in sterile distilled water at the appropriate concentrations (1 mM/kg) for oral dosing by oral gavage.

In Vitro Studies

Alimentary Tract Metabolism of C5a Antagonist Analogs. Female Wistar rats (210–230 g) were anesthetized (25 mg/kg xylazine, 25 mg/kg tiletamine (Zoletil); Lypppard Pty. Ltd., Brisbane, Australia) and 10 mg/kg xylazine (Lypppard Pty. Ltd.), and the stomach contents and small intestinal (including jejunum, ileum, and duodenum) contents were washed out with 3 ml of 0.1 M phosphate buffer, pH 7. The suspension was centrifuged and then sterile filtered. The remaining small intestine was then homogenized in 3 ml of phosphate buffer and processed in a similar manner. AcF-[OP(α-Cha)WR] or antagonist analogs (12.5 µl of 2.75 mM stock solution) were then added to either stomach contents, intestinal homogenate, or intestinal contents and incubated at 37°C for 1 h. Cold acetonitrile was added; samples were vortexed and centrifuged, and the supernatants were stored at –20°C. Samples were then analyzed on a high-performance liquid chromatograph using standard conditions, and the change in peak size after 1-h incubation was calculated and expressed as a percentage of change.

Receptor Binding Affinity of C5a Antagonist Analogs. The affinity of all C5a antagonist analogs was examined at polymorphonuclear leukocyte (PMN) C5a receptors. Additionally, we investigated the affinity of AcF-[OP(α-Cha)WR] and HC-[OP(α-Cha)WR] at the peripheral blood mononuclear cell (PBMC) C5a receptor.

PMNs were isolated from heparinized human blood using Ficoll-Hypaque density gradient centrifugation (Histopaque-1077; Sigma Chemical Co., St. Louis, MO) as described previously (Woodruff et al., 2001). Similarly, PBMCs were separated from heparinized human blood after density gradient centrifugation (Histopaque 1077; Sigma Chemical Co.) and gentle washing with Hank’s balanced salt solution containing 13 mM sodium citrate (Sigma Chemical Co.). PBMC suspension, obtained with this method, routinely contained numbers greater than 91.8 ± 2.3% PBMCs (n = 4) determined by KwikDiff staining (>200 cells counted per slide). The remaining cells were contaminating PMNs.

The PMN C5a receptor affinity of test analogs was determined by competition with 125I-C5a (50 pM; PerkinElmer Life and Analytical Sciences, Boston, MA) as described previously in detail (Woodruff et al., 2001; March et al., 2004). PBMC C5a receptor affinity was determined in the same manner; however, 5 x 10^5 cells/well were used.

C5a Antagonist Potency of HC-[OP(α-Cha)WR] versus AcF-[OP(α-Cha)WR]. The analog HC-[OP(α-Cha)WR] was examined for antagonist potency using C5a-induced myeloperoxidase release from isolated human PMNs, and β-hexosaminidase release from isolated human PBMCs, and compared with AcF-[OP(α-Cha)WR]. The myeloperoxidase assay has been described in detail previously (March et al., 2004). Suspended PBMCs (5 x 10^6 cells/ml) were incubated (10 min; 37°C) with 10 µg/ml cytochalasin B (Sigma Chemical Co.) and then added (50 µl) to tubes containing release buffer (148 mM NaCl, 5 mM KCl, 9 mM d-sorbitol, 0.34 mM K₂HPO₄, 1 mM KH₂PO₄, 10 mM HEPES, pH 7.4, 1 mM CaCl₂, and 0.1% bovine serum albumin) or increasing concentrations of antagonist. After a second incubation (37°C for 10 min), C5a or agonist peptides were added, and the tubes were incubated (30 min at 37°C) and centrifuged (12,000 rpm; 3 min). Supernatant (100 µl) was added to substrate solution (100 µl, 2 mM hexosaminidase substrate (N-acetyl β-D-glucosaminide diluted in 0.2 M citrate buffer, pH 4.5). After 3 h at 37°C, and after addition of 300 µl of 1 M Tris buffer, pH 9.0, the absorbance was measured at 405 nm. Spontaneous enzyme release was subtracted from all values, and β-hexosaminidase release was calculated as a percentage of the maximal release of 1 µM C5a.

In Vivo Studies

Pharmacokinetic Analysis of HC-[OP(α-Cha)WR] versus AcF-[OP(α-Cha)WR]. For oral pharmacokinetic studies, female Wistar rats (220–280 g) were orally gavaged with AcF-[OP(α-Cha)WR] (n = 9–13/group) or HC-[OP(α-Cha)WR] (n = 9–14/group) at a dose rate of either 10 or 0.3 mg/kg, followed by immediate anesthesia with 25 mg/kg zolazepam, 25 mg/kg tiletamine, and 10 mg/kg xylazine. Blood (250 µl) was then collected from the tail vein at various time points, placed in heparinized tubes, and centrifuged (10,000g; 10 min), and plasma was collected and stored at –20°C for subsequent analysis.

A separate group of rats was examined to determine the absorption of the C5α antagonists directly from the colon, in either normal or TNBS-dosed rats. Rats were administered either saline (n = 3/group) or TNBS (n = 3/group) intracolonically as described below.
After 24 h, rats were anesthetized (25 mg/kg zolazepam, 25 mg/kg tiletamine, and 10 mg/kg xylazine), and the distal colon exposed through the rectum. A 0.3 mg/ml solution containing either AcF-[OP(d-Cha)WR] or HC-[OP(d-Cha)WR] was then injected into the distal colon (~8 cm from anus) at a dose rate of 0.3 mg/kg. Blood was then obtained at various time points for plasma collection as described above.

Plasma levels of AcF-[OP(d-Cha)WR] and HC-[OP(d-Cha)WR] were determined using liquid chromatography-mass spectrometry methods as described previously for AcF-[OP(d-Cha)WR] (Strachan et al., 2001). Antagonist concentrations are expressed as nanograms per milliliter of plasma. Sensitivity limits of this assay were 5 ng/ml rat plasma.

**Model of Inflammatory Bowel Disease.** Studies were approved by The University of Queensland Animal Ethics Committee. Pathogen-free male Wistar rats (Central Animal Breeding House, Pinjarra Hills, Australia), weighing 297 ± 1.4 g (n = 126), were housed in individual cages with free access to food and water and maintained in 12-h light/dark cycles at 23°C, with 60% humidity. Colitis was induced by the administration of TNBS/ethanol into the colons of rats as described previously (Woodruff et al., 2003). Briefly, rats were fasted for 30 h before the intracolonic instillation of a solution containing TNBS (120 mg/kg picrylsulfonic acid; Sigma Chemical Co.) and 100% ethanol (1 ml/kg), under anesthesia (80 mg/kg i.p. ketamine and 8 mg/kg i.p. xylazine; Lyppard Pty. Ltd.). A solution of 100% ethanol (1 ml/kg) and saline was administered to sham-operated animals. Rats were then allowed to recover and observed daily throughout the duration of the project. After 8 days, the amount of food consumed by rats in the previous 24 h was measured, and the change in body weight from colitis induction was recorded. Rats were then deeply anesthetized with 50 mg/kg i.p. zolazapam and 12 mg/kg ketamine, and the distal colon exposed.

**Statistical Analysis.** All results are expressed as mean ± S.E.M. Statistical analysis was performed using GraphPad Prism 4.01 software (GraphPad Software Inc., San Diego, CA), with significance determined as p < 0.05. A one-way analysis of variance with a Dunnett’s post test was used for all analyzed data, except for macroscopic ulceration scores, for which a nonparametric one-way analysis of variance (Kruskal-Wallis test) with Dunn’s post test was performed.

**Results**

**Alimentary Tract Metabolism of C5a Antagonist Analogs.** Initially, we examined the relative metabolism of the lead C5a antagonist AcF-[OP(d-Cha)WR] in rat plasma, gastric contents, intestinal homogenate, and cell-free intestinal mucosal washings. Interestingly, although this compound was found to be stable in plasma, gastric contents, and intestinal homogenate, the compound was readily degraded in intestinal mucosal washings, indicating pH- and/or enzyme-specific degradation (Table 1).

Numerous analogs of the lead C5a antagonist AcF-[OP(d-Cha)WR] were synthesized to determine their relative resistance to degradation in rat intestinal mucosal contents and to identify the site of metabolic instability. The results are presented in Table 2. Substitution with a sterically bulky anthryl-ALA residue at different positions within the cycle of the lead compound did not protect against degradation by intestinal mucosal washings, apart from partial protection from the Ac-PHE-[ORN-anthryl-ALA-dCHA-TRP-ARG] analog (Table 2). However, when this sterically bulky residue was substituted for the extracyclic side chain PHE, the compound was completely resistant to degradation by intestinal mucosal washings, apart from partial protection from the Ac-PHE-[ORN-anthryl-ALA-dCHA-TRP-ARG] analog (Table 2).

**Human C5a Receptor Binding Affinity of C5a Antagonist Analogs.** The C5a antagonist analogs were further analyzed for C5a receptor affinity to isolated human PMNs and compared with AcF-[OP(d-Cha)WR]. Introducing the
bulky anthryl-ALA residue within the cycle, and to a lesser extent, at extracyclic positions, greatly reduced C5a receptor affinity, as expected (Table 2). Substitution of the AcF moiety with a Cin group led to an ~20-fold reduction in affinity; however, substitution at this same position with HC did not have any significant impact on C5a receptor affinity compared with AcF-[OP(D-Cha)WR] (Table 2).

C5a Receptor Affinity and Antagonist Activity of HC-[OP(D-Cha)WR] versus AcF-[OP(D-Cha)WR]. The similarity in the PMN C5a receptor binding affinities of HC-[OP(D-Cha)WR] and AcF-[OP(D-Cha)WR] prompted further investigation of the hydrocinnamate analog in additional in vitro assays. The PBMC C5a receptor affinity was similar for both HC-[OP(D-Cha)WR] and AcF-[OP(D-Cha)WR] (Table 3) and comparable with that determined for both compounds on isolated PMNs. In functional studies, HC-[OP(D-Cha)WR] behaved as an insurmountable antagonist of C5a-mediated degranulation of both PBMCs and PMNs, as we have described previously for AcF-[OP(D-Cha)WR] (Paczkowski et al., 1999). The in vitro antagonist potencies (Kᵦ) of these compounds were the same in both assays (Table 3).

Pharmacokinetic Analysis of HC-[OP(D-Cha)WR] versus AcF-[OP(D-Cha)WR]. After the in vitro studies, we subsequently examined the pharmacokinetic profile of HC-[OP(D-Cha)WR] to determine whether the resistance to intestinal metabolism of this compound resulted in any improvements in oral bioavailability compared with AcF-[OP(D-Cha)WR]. Blood levels for the C5a antagonists after oral administration at a dose rate of 10 mg/kg were similar, with a peak in circulating plasma levels reached 30 min after oral dosing (Fig. 1A). After oral dosing of the C5a antagonists, at the 30-fold lower dose of 0.3 mg/kg, the plasma levels were not detectable (<5 ng/ml) (Fig. 1A). When either antagonist (0.3 mg/kg) was injected directly into the colons of rats that had previously received an enema of saline only, there were detectable (>5 ng/ml) blood levels within 5 min of injection (Fig. 1B). In contrast, when the antagonists (0.3 mg/kg) were injected into the colons of rats with preexisting colon damage (induced 24 h earlier by TNBS), blood levels were not detected (Fig. 1B).

TNBS-Induced Colitis in Rats. Finally, we sought to determine whether there were any improvements in potency of orally administered HC-[OP(D-Cha)WR] over AcF-[OP(D-Cha)WR] when applied to a model of intestinal inflammatory disease.

Mortality. The induction of colitis resulted in a high rate of mortality in untreated rats over the 8-day study period (37.5%; 12 deaths, 20 survivors; Fig. 2). Rats treated with AcF-[OP(D-Cha)WR] at the highest dose rate (10 mg/kg/day) showed a large decrease in mortality (7.7%; one death, 12 survivors; Fig. 2) compared with untreated rats. Decreasing dose rates of this compound (1, 0.3, and 0.1 mg/kg/day) resulted in reduced inhibition of mortality (Fig. 2). Rats treated with HC-[OP(D-Cha)WR] also showed inhibition of mortality at the 10 mg/kg/day dose rate (11.1%; one death, eight survivors); however, treating rats at decreasing dose rates of this HC analog (1, 0.3, 0.1, and 0.03 mg/kg/day), continued to protect against mortality (Fig. 2). The intracolonic instillation of ethanol/saline-alone in sham-operated animals resulted in no mortalities throughout the study (Fig. 2).

Food Consumption. Eight days after colitis induction, rats without drug treatment were consuming significantly less food/day (9.7 ± 2.1 g; p < 0.01; n = 20), compared with ethanol/saline-injected sham-operated animals, who were

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<th>Assay</th>
<th>AcF-[OP(D-Cha)WR]</th>
<th>HC-[OP(D-Cha)WR]</th>
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<td>Human PBMC receptor binding</td>
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<td>n</td>
<td>−log IC₅₀ ± S.E.</td>
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<td>human PBMC β-Hex release</td>
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<tr>
<td>Human PMN MPO release</td>
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<tr>
<td>n</td>
<td>8.24 ± 0.18</td>
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<td>S.E. IC₅₀</td>
<td>0.09 ± 0.06</td>
<td>2.15 nM</td>
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β-Hex, β-hexosaminidase; MPO, myeloperoxidase.

TABLE 3
C5a receptor affinity and antagonist activity of HC-[OP(D-Cha)WR] and AcF-[OP(D-Cha)WR]
consumed 33.2 ± 1.4 g food/day (n = 8; Fig. 3). Rats treated with AcF-[OP(Cha)WR] at the highest dose rate (10 mg/kg/day) showed the largest inhibition of colitis-induced anorexia (p < 0.01), with decreased dose rates of this compound (1, 0.3, and 0.1 mg/kg/day) providing no protection (Fig. 3). Rats treated with HC-[OP(Cha)WR] at dose rates of 1, 0.3, and 0.1 mg/kg/day were consuming significantly higher food than untreated rats (p < 0.05/0.01); however, rats treated at the highest (10 mg/kg/day) and lowest (0.03 mg/kg/day) dose rates did not show any significant improvements (Fig. 3).

**Body Weight Loss.** Eight days after colitis induction, rats without treatment had lost substantial weight, losing an average of 25.3 ± 5.4 g (n = 20) compared with precolitis induction values (Fig. 4). Rats treated with AcF-[OP(Cha)WR] at 10 and 1 mg/kg/day gained weight over the 8 days and were significantly heavier than drug-free colitis rats (p < 0.01), with lower dose rates being ineffective (Fig. 4). Rats treated with HC-[OP(Cha)WR] at the dose rates of 1, 0.3, and 0.1 mg/kg/day gained weight over the 8 days and were significantly heavier than drug-free colitis rats (p < 0.05/0.01; Fig. 4). Rats treated with this analog at the highest (10 mg/kg/day) and lowest (0.03 mg/kg/day) dose rates did not show any significant improvements (Fig. 4). Sham-operated rats (intracolonic ethanol/saline-alone) gained considerable weight over the 8 days (+75.6 ± 3.9 g; n = 8; Fig. 4), in part due to the prior fasting of these rats before experimentation.

**Macroscopic Ulceration Scores.** Eight days after the induction of colitis, the colons from untreated rats showed considerable ulcerative damage and were scored an average of 11.5 ± 0.4 (n = 20; Fig. 6). Rats treated with AcF-[OP(Cha)WR] at 10 and 1 mg/kg/day had significantly reduced colonic edema compared with untreated rats (p < 0.01), with lower dose rates being ineffective (Fig. 5). Rats treated with HC-[OP(Cha)WR] at 10, 1, and 0.3 mg/kg/day also had significantly reduced colonic edema (p < 0.01/0.05), with lower dose rates (0.1 and 0.03 mg/kg/day) producing no significant reduction (Fig. 5).

**Colon Weight.** Despite the significant reduction in edema, the colon weight from untreated rats was significantly higher than that from drug-free colitis rats (p < 0.01; Fig. 6). The lower doses of this compound were not effective at reducing the ulceration of the colon (Fig. 6). Rats treated with HC-[OP(Cha)WR] at the dose rates of 1, 0.3, and 0.1 mg/kg/day had reduced mean colon macroscopic scores, with the 0.3 mg/kg/day dose rate resulting in significant reduction (p < 0.05; Fig. 6). The lower doses of this compound were not effective at reducing damage to the colon (Fig. 6). Sham-operated rats (intracolonic ethanol/saline-alone) showed no observable colon damage after 8 days and were all scored 0 (data not shown).
The complement system is a series of cascading proteins forming part of the innate immune system. C5a is a major product of this pathway activation and is an extremely potent immune and inflammatory mediator (Taylor and Fairlie, 2005). Complement has been implicated in the pathogenesis of IBD with identification of complement deposition in IBD patients (Halstensen et al., 1990, 1992; Halstensen and Brandtzæg, 1991; Ueki et al., 1996; Kolios et al., 1998) and preliminary evidence from a small, uncontrolled clinical study using a nonspecific anticomplement agent (Kitano et al., 1992, 1993). The precise involvement of complement in this disease has been relatively limited to date, due to lack of specific anticomplement agents. Our group has been developing small, constrained, orally active cyclic peptide C5a antagonists, which we and others have shown to display high potency and efficacy in numerous immunoinflammatory models of disease in rodents (for review, see Allegretti et al., 2005). We recently used the lead compound AcF-[OP(D-Cha)WR] in a model of TNBS-induced colitis in rats and provided the first evidence for a role for C5a in the pathogenesis of this disease (Woodruff et al., 2003).

One potential drawback to the use of this compound in the treatment of human IBD is the relatively high doses (10 mg/kg/day) used to treat rats in the TNBS-colitis model and the relatively low (~5%) oral bioavailability of this antagonist (Strachan et al., 2001). These factors in combination present some barriers to clinical development of AcF-[OP(D-Cha)WR]. We have previously developed a comprehensive series of analogs of AcF-[OP(D-Cha)WR] in an attempt to identify more potent compounds (March et al., 2004). Although several compounds were found to display equal C5a receptor affinity and activity as AcF-[OP(D-Cha)WR], no single compound displayed significantly improved receptor affinity or antagonist potency (March et al., 2004).

In this study, we therefore initially sought to examine the alimentary tract metabolism of AcF-[OP(D-Cha)WR] to determine whether this could affect the absorption of the compound. It was found that the antagonist was rapidly metabolized by cell-free intestinal mucosal washings, despite being stable in plasma, in washings of the gastric mucosa, and in intestinal homogenates. We then synthesized a series of analogs in which a sterically bulky anthryl-alanine residue was substituted at various positions within the molecule in an attempt to block enzymatic activity, and we examined the analogs’ susceptibility to degradation in intestinal mucosal washings. This proved a successful method for determining the site of metabolic attack in the compound. From these experiments, the PHE residue was found to be the key site for the intestinal degradation of the compound. Substitution of the PRO residue within the cycle also provided partial protection from degradation, possibly due to the modulation of the cycle conformation. We then examined the Ac-Phe-substituted compounds Cin-[OP(n-Cha)WR] and HC-[OP(n-Cha)WR], which had previously been synthesized as part of our C5a receptor pharmacophore studies (March et al., 2004). Both of these compounds were also found to be resistant to intestinal metabolism. The HC compound was considered to be the best candidate for further examination, since it was the only analog that was resistant to intestinal metabolism that also showed similar affinity on human PMN C5a receptors as AcF-[OP(D-Cha)WR]. We have now determined that the HC-[OP(n-Cha)WR] analog has the same in vitro C5a antagonist potency as AcF-[OP(n-Cha)WR] in isolated human PMNs and PBMCs, and like the parent compound, behaves as an insurmoutable antagonist.

In the next series of experiments, the oral pharmacokinetic profile of HC-[OP(n-Cha)WR] was examined. This compound displayed similar plasma levels after oral absorption to AcF-[OP(n-Cha)WR], demonstrating similar absolute oral bioavailability. This was an interesting finding, given its metabolic stability in the intestinal washings and suggests that these C5a antagonists are not primarily absorbed in the intestine or that intestinal absorption mechanisms become quickly saturated. Further analysis is required to determine...
the reasons bioavailability was unaffected despite the differing metabolic fates in the intestine.

Finally, we examined the efficacy and dose profile (potency) of the HC compound versus AcF-[OP(d-Cha)WR] in a model of IBD. Since the metabolism of HC-[OP(d-Cha)WR] was limited in the intestinal lumen and the bioavailability was no better than AcF-[OP(d-Cha)WR], we hypothesized that a greater proportion of the drug may reach the colon in its pharmacologically active state. This offered the possibility of an effective increase in the potency of the drug when administered orally in the model of colitis. Both compounds reduced the severity of disease when dosed after the induction of colitis, which again highlights a crucial pathogenic role for C5a in this model of IBD, and the ability of a C5a antagonist to reduce the disease process. There were consistent findings for the HC analog to be more potent than AcF-[OP(d-Cha)WR] in each of the disease parameters examined. Both compounds had similar efficacy in each parameter, but in general, the HC compound reduced parameters of disease at 10- to 30-fold lower oral doses than AcF-[OP(d-Cha)WR]. We did, however, find that the HC compound at the highest dose rate (10 mg/kg/day) did not provide significant protection in the model, and this was unexpected. This is in contrast to AcF-[OP(d-Cha)WR], where the 10 mg/kg/day dose was the most effective dose, and confirms the different in vivo properties of these two antagonists. The lack of efficacy for HC-[OP(d-Cha)WR] at this high dose rate could be due to a number of factors, such as local chemical toxicity in the gastrointestinal tract, deleterious effects at unidentified receptors at this high dose, or to other factors not yet recognized. Regardless of the explanation, lower doses of this analog were consistently and considerably more potent than the parent drug, obviating the need for the high dose.

Given that the HC compound is not absorbed at a higher rate than AcF-[OP(d-Cha)WR], it seems unlikely that the reason for the increased potency in this model is due to more favorable plasma pharmacokinetics. Additionally, in vitro, both compounds have the same C5a receptor affinity and C5a antagonistic activity, and both drugs are insurmountable antagonists. Other factors most likely account for the significant increase in potency of the orally administered HC-[OP(d-Cha)WR]. One possibility is that given the intestinal stability of HC-[OP(d-Cha)WR], administration of this drug to the rat may result in an eventual local accumulation of drug at the site of damage in the colon. Inflammatory cell infiltration and activation to the colon is a hallmark mechanism of pathology in this model (Neurath et al., 1995; Abad et al., 2003). The presence of high local concentrations of a C5a antagonist may prevent these C5a receptor-rich cells from being activated by locally produced C5a and also prevent the chemotaxis and recruitment of further inflammatory cells. This is just one of many possibilities and further studies are required to determine the validity of this hypothesis.

In summary, this study has identified an analog, HC-[OP(d-Cha)WR], of the well characterized C5a antagonist AcF-[OP(d-Cha)WR] with similar in vitro antagonist potency, which is resistant to metabolism in the rat intestinal lumen. Although this latter property did not affect the oral bioavailability, it was associated with a markedly increased (10- to 30-fold) oral potency in a model of IBD in the rat. This improved activity makes it potentially a better candidate than AcF-[OP(d-Cha)WR] for use in the clinical treatment of IBD.

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