Identification of a Potent and Orally Active Non-peptide C5a Receptor Antagonist*

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The anaphylatoxin C5a is a potent chemotactic factor for neutrophils and other leukocytes, and functions as an important inflammatory mediator. Through a high capacity screening followed by chemical optimization, we identified a novel non-peptide C5a receptor antagonist, N-[(4-dimethylaminophenyl)methyl]-N-(4-isopropylphenyl)-7-methoxy-1,2,3,4-tetrahydronaphthalen-1-carboxamide hydrochloride (W-54011). W-54011 inhibited the binding of 125I-labeled C5a to human neutrophils with a Ki value of 2.2 nM. W-54011 also inhibited C5a-induced intracellular Ca2+ mobilization, chemotaxis, and generation of reactive super oxide species in human neutrophils with IC50 values of 3.1, 2.7, and 1.6 nM, respectively. In C5a-induced intracellular Ca2+ mobilization assay with human neutrophils, W-54011 did not show agonistic activity at up to 10 μM and shifted rightward the concentration-response curves to C5a without depressing the maximal responses. Examination on the species specificity of W-54011 revealed that it was able to inhibit C5a-induced intracellular Ca2+ mobilization in neutrophils of cynomolgus monkeys and gerbils but not mice, rats, guinea pigs, rabbits, and dogs. In gerbils, oral administration of W-54011 (3–30 mg/kg) inhibited C5a-induced neutropenia in a dose-dependent manner. The present report is the first description of an orally active non-peptide C5a receptor antagonist that could contribute to the treatment of inflammatory diseases mediated by C5a.

The complement component C5a is a 74-amino acid peptide generated during the classical, alternative, and lectin pathways of complement activation (1, 2). C5a is a potent chemotactic factor for neutrophils and other leukocytes and is a potent inflammatory mediator. Moreover, C5a causes histamine release from mast cells, smooth muscle contraction, increase in vascular permeability, eliciting of superoxide anion production, enhancement of neutrophil-endothelial cell adhesion, induction of several cytokines (i.e. IL-1, IL-6, IL-8, and TNF-α) from leukocytes, and augmentation of the humoral and cell-mediated immune response. C5a exerts these activities by binding to G-protein-coupled C5a receptor (C5aR)1 on the plasma membrane of target cells (3). These biological activities of C5a are implicated in a variety of diseases such as rheumatoid arthritis (4, 5), systemic lupus erythematosus (6–9), reperfusion injury (10), Alzheimer’s disease (11–15), and sepsis (16, 17). The pathogenic action of C5/C5a is also shown in some animal models. For instance, in murine collagen-induced arthritis, an animal model of rheumatoid arthritis (RA), administration of anti-C5 antibody during the disease induction or after the onset suppresses or ameliorates the disease (18). Anti-C5 antibody is also effective in a new RA model (19), K/BxN mouse model induced by anti-glucose 6-phosphate isomerase (GPI) auto-antibodies, which is detected in 64% of RA patients (20–22). Furthermore, the anti-GPI antibody-induced arthritis does not occur in C5aR-deficient mice, indicating that C5a is important in the development of arthritis rather than C5b, which is another part of C5 and is a component of the membrane attack complex.

Inhibition of C5a function has been attempted with anti-C5a antibodies and C5aR antagonists (for review, see Refs. 23, 24). Anti-C5a antibodies inhibit immune complex-induced inflammation and reperfusion injury and are effective in septic primates and rats (25–27). On the other hand, although C5aR antagonists, including non-peptides, small peptides, C5a mutants, and anti-C5aR antibodies, have been studied for the past 2 decades, only a few candidates have been discovered as non-peptide antagonists. These non-peptide antagonists, however, are not so potent (IC50, more than subhundreds nM in 125I-rhC5a binding assay) and have not been reported on as to their in vivo activities. As a small peptide antagonist, hexapeptide (MeFKP-n-ChaWr, C089) was first reported in 1994 (28). C089 exhibits an IC50 value of 70 nM in 125I-rhC5a binding assay and has been reported recently to inhibit the late airway response in allergic rats and inhibit thrombotic glomerulonephritis in rats (29). The recent reports describe two other types of C5aR antagonists, one of which is a group of F-(OP-D-ChaWr, C089) was first reported in 1994 (28). C089 exhibits an IC50 value of 70 nM in 125I-rh-C5a binding assay and has been reported recently to inhibit the late airway response in allergic rats and inhibit thrombotic glomerulonephritis in rats (29).

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1 The abbreviations used are: C5aR, C5a receptor; RA, rheumatoid arthritis; GPI, glucose 6-phosphate isomerase; rhC5a, recombinant human C5a; FCS, fetal calf serum; HBSS, Hank’s balanced salt solution; ROS, reactive oxygen species; GPCR, G-protein-coupled receptor; gZAS, gerbil zymosan-activated serum.

This paper is available on line at http://www.jbc.org

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A Potent Non-peptide C5a Receptor Antagonist

Materials—Recombinant human C5a (rhC5a) was purchased from Sigma. 125I-labeled recombinant human C5a (125I-rhC5a) was purchased from Amersham Biosciences. Anti-C5aR monoclonal antibody (clone: S5/1) (39) was purchased from Serotec (Oxford, UK). Fresh isolated whole blood of cynomolgus monkeys, anticoagulated with EDTA, was purchased from Shin-nihonkagaku (Kagoshima, Japan). Anti-C5aR monoclonal antibody, or F-[(OP-n-CHAWR)] and specific binding was determined. Data are means ± S.E. of multiple experiments (n = 3).

EXPERIMENTAL PROCEDURES

Materials—Recombinant human C5a (rhC5a) was purchased from Sigma. 125I-labeled recombinant human C5a (125I-rhC5a) was purchased from Amersham Biosciences. Anti-C5aR monoclonal antibody (clone: S5/1) (39) was purchased from Serotec (Oxford, UK). Fresh isolated whole blood of cynomolgus monkeys, anticoagulated with EDTA, was purchased from Shin-nihonkagaku (Kagoshima, Japan).

Animals—BALB/c mice (AnNCrj) and later rats (Cj) were purchased from Charles River Japan (Kanagawa, Japan). Mongolian gerbils (MGSSsa), Hartley guinea pigs (Std), rabbits (KBPJW), and beagle dogs were purchased from Seac Yoshitomi (Fukuoka, Japan), Japan SLC (Shizuoka, Japan), Biotech (Saga, Japan), and Keari (Osaka, Japan), respectively.

Synthesis of Compounds—N-[(4-dimethylamino phenyl)methyl]-N-(4-isopropylphenyl)-7-methoxy-1,2,3,4-tetrahydronaphthalen-1-carboxamide hydrochloride (W-54011) was synthesized as follows: A solution of 7-methoxy-1,2,3,4-tetrahydronaphthalen-1-carboxylic acid (18.3 g, 72.7 mmol) and 4-(4-dimethylaminophenyl)methyl(4-isopropylphenyl) aniline (19.5 g, 72.7 mmol) in CH2Cl2 (200 ml) was stirred overnight at room temperature. The reaction mixture was poured into water and extracted two times with CHCl3. The combined organic layer was washed with brine, dried over anhydrous MgSO4, and concentrated. The residue was chromatographed over silica gel using a mixture of ethyl acetate and hexane (1:4) as eluent to give a yellow oil (29.9 g, 90.1%). To a solution of the oil (29.9 g, 63.5 mmol) in ethanol (300 ml) was added hydrogen chloride (4.0 m solution in 1.4-dioxane, 17.5 ml), and the resulting solid was filtered, recrystallized from ethanol/water to afford W-54011 (24.2 g, 75.8%) as yellow crystals. The melting point was 147°C; mass spectrometry (electrospray ionization) was m/z 457 [M + H]+. The cyclic C5aR antagonist F-[(OP-n-CHAWR)] was synthesized according to the method reported by Finch et al. (31).

Cell Line and Neutrophil Isolation—The human histocytic lymphoma line U-937 was obtained from the American Type Culture Collection. Neutrophils were isolated from whole blood of cynomolgus monkeys (Macaca fascicularis), dogs, and rabbits (Oryctolagus cuniculus), used as neutrophils. Polymorphonuclear cells in this fraction were >99%.

125I-rhC5a Binding Assay—This assay was performed in 96-well filtration plates (MultiScreen MADV NOB, Millipore, Bedford, MA) in a centrifuge tube and then centrifuged at 500 x g for 30 min at room temperature. After centrifugation, the polymorphonuclear cells were washed, resuspended in Hank’s balanced salt solution (HBSS; Invitrogen) containing 1% fetal calf serum (FCS), and used as neutrophils. Polymorphonuclear cells in this fraction were >99%.

Intracellular Ca2+ Mobilization Assay—Neutrophils (5 x 106 cells/ml) were loaded with 5 μM Fura-2 AM (Dojindo, Kumamoto, Japan) for 30 min at room temperature. After centrifugation, the polymorphonuclear cells were washed, resuspended in HBSS containing 1% fetal calf serum (FCS), and used as neutrophils. Polymorphonuclear cells in this fraction were >99%.

Effects of W-54011 and F-[(OP-n-CHAWR)] on rhC5a-induced calcium mobilization, chemotaxis, and ROS release in human neutrophils

The assays were performed as described under “Experimental Procedures.” C5a concentration used in each assay, IC50 values, and the 95% confidential limit of tested compounds are indicated.

<table>
<thead>
<tr>
<th>Assay system</th>
<th>rhC5a added in assay</th>
<th>IC50 (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>W-54011</td>
<td>10 nm</td>
<td>0.1 (2.4–3.9)</td>
</tr>
<tr>
<td>F-[(OP-n-CHAWR)]</td>
<td>10 nm</td>
<td>0.1 (2.7–4.5)</td>
</tr>
</tbody>
</table>

**FIG. 1.** Structure of W-54011

**FIG. 2.** Inhibition of 125I-rhC5a binding to human neutrophils by C5a, W-54011, anti-C5a R antibody and F-[(OP-n-CHAWR)]. Human neutrophils were incubated with 0.2 nm 125I-rhC5a in the absence and presence of a range of concentrations of C5a, W-54011, anti-C5a R antibody, or F-[(OP-n-CHAWR)], and specific binding was determined. Data are means ± S.E. of multiple experiments (n = 3).

**FIG. 3.** Effects of W-54011 on the concentration-dependent curves of rhC5a-induced Ca2+ mobilization in human neutrophils. Ca2+ mobilization in human neutrophils was induced by a range of rhC5a concentrations in the absence and presence of W-54011 at the concentrations indicated. Data are expressed as the maximal changes of the intracellular Ca2+ level. The results shown are representative from three separate studies.

![Hornby, Canada) in a centrifuge tube and then centrifuged at 500 x g for 30 min at room temperature. After centrifugation, the polymorphonuclear cells were washed, resuspended in Hank's balanced salt solution (HBSS; Invitrogen) containing 1% fetal calf serum (FCS), and used as neutrophils. Polymorphonuclear cells in this fraction were >99%.](http://www.jbc.org/)

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change in Ca\textsuperscript{2+} concentration after stimulator addition was quantitated.

Chemotaxis Assay—Neutrophils were resuspended in RPMI 1640 (Invitrogen) containing 25 mM HEPES and 0.1% BSA at a concentration of \(5 \times 10^6\) cells/ml and loaded with 5 \(\mu\)g Calcein-AM (Funakoshi, Tokyo, Japan) for 30 min at 37 °C. After three washes, the cells were resuspended at a concentration of \(1 \times 10^6\) cells/ml in RPMI 1640 containing 0.1% bovine serum albumin. The cells \((1 \times 10^6\) cells/200 \(\mu\)l/well\) and varying concentrations of test compounds (200 \(\mu\)l/well) in RPMI 1640 containing 25 mM HEPES and 0.1% bovine serum albumin were placed into chemotacticells (3-\(\mu\)m pore size, Kurabou, Osaka, Japan) within 24-well plates containing 100 \(\mu\)l rhC5a (300 \(\mu\)l/well). The plates were incubated for 90 min at 37 °C and 5% CO\textsubscript{2}. After removing the chemotacticells, migrated cells were lysed by adding 100 \(\mu\)l/well cell lysis solution (10% SDS, 0.01 N HCl), and the fluorescence was measured using a fluorimeter reader (CytoFlourII, Perseptive Biosystems, Framingham, MA) at excitation wavelength of 485 nm and an emission wavelength of 530 nm.

Reactive Super Oxide Species (ROS) Generation Assay—The studies were conducted by a microtiter plate-based assay using a microplate luminometer (Microtumat Plus LB980V, Berthold, Bad Wildbad, Germany) in white wall 96-well plates (Berthold) in a total volume of 200 \(\mu\)l. The cells \((1 \times 10^6\) cells/150 \(\mu\)l/well\) and varying concentrations of test compounds (25 \(\mu\)l/well) in HBSS containing 1% FCS and 1\% newborn calf serum (Wako, Osaka, Japan) were plated in white wall 96-well plates. After the plates were incubated in a Microtumat at 37 °C for 5 min, rhC5a (25 \(\mu\)l/well) was added at a final concentration of 3 \(\mu\)M, and the luminescence was monitored for 15 min. The maximal change in luminescence was quantitated as ROS generation.

Gerbil Neutropenia Assay—The C5a-induced neutropenia was based on the observation that neutrophils transiently disappear from circulation after systemic infusion of chemotaxant such as C5a or leukotrien B4 (32, 37, 41). Male mongolian gerbils \((6-8)\) were orally treated with W-54011 \((3, 10, 30 \text{ mg/kg})\) suspended in 0.5% carboxypolymethylcellulose 4 h before rhC5a injection. The animals were anesthetized with pentobarbital (Abbott Laboratories, Abbott Park, IL), and the skin was incised to expose the jugular veins for rhC5a injection. Vehicle control was treated with vehicles of W-54011 and rhC5a. Neutropenia control was treated with a vehicle of W-54011 before rhC5a injection. Blood was sampled at \(-1, 1, 3\), and 5 min after rhC5a injection, and neutrophils were counted. Data are expressed as percentages of the neutrophil counts 1 min before rhC5a injection and are shown as means ± S.E. \((n = 6-8)\). * \(p < 0.05\); ** \(p < 0.01\), significantly different from neutropenia control (Dunnett’s multiple comparison test). ### \(p < 0.001\), significantly different from vehicle control (Student’s t test).

RESULTS

To identify a non-peptide C5aR antagonist, a high capacity radioligand screening was configured using \(^{125}\text{I}-\text{rhC5a}\) and C5aR-expressing U-937 cells. In consequence of the followed chemical optimization, we configured a novel non-peptide C5aR antagonist, \(N\)-[4-dimethylaminophenyl]methyl\]-\(N\)-[4-isopro-
maximally effective concentrations of fMLP (1 nM), platelet-activating factor (0.3 nM), and IL-8 (0.1 nM) (data not shown). This result demonstrates that W-54011 is highly specific for C5aR.

The mechanism of the antagonistic effect of W-54011 was examined with human neutrophils by Schild analysis (42). The concentration-response curves for Ca²⁺ mobilization induced by rhC5a were determined in the presence of increasing concentrations of W-54011 (Fig. 3). W-54011 shifted rightward the concentration-response curves to C5a without depressing the maximal responses. This result indicates that W-54011 has a competitive antagonist-like function, but the response curves at low concentrations (0.3–3 nM) of W-54011 were not parallel to those at the other concentrations.

The species selectivity of W-54011 was examined in rhC5a-induced intracellular Ca²⁺ mobilization of neutrophils in various species. The responses of neutrophils to rhC5a were different among these species (Fig. 4A, mice, rats, guinea pigs, rabbits, and dogs; data not shown). The W-54011 was able to inhibit the response in cynomolgus monkeys and gerbils with IC₅₀ values of 1.7 and 3.2 nM, respectively (Fig. 4B) but not in mice, rats, guinea pigs, rabbits, and dogs (data not shown).

The in vivo effect of W-54011 was examined in the C5a-induced neutropenia model of mongolian gerbils. Intravenous injection of 100 µg/kg rhC5a caused a rapid and transient neutropenia, which reached nadir at 1 min after the rhC5a injection and returned to baseline within 5 min. When W-54011 (3–30 mg/kg) was orally administered 4 h before rhC5a injection, it inhibited the neutropenia in a dose-dependent manner (Fig. 5). This inhibition by W-54011 was also observed when it was administered 1 or 8 h before rhC5a injection but not 24 h (data not shown). Because the model described above was based on the reaction between different species, human C5a and gerbil C5aR, it is unclear whether the compound inhibits the interaction between gerbil C5a and C5aR.

In the present report, we represented a potent and orally active non-peptide C5aR antagonist, named W-54011, for the first time. W-54011 inhibited C5a binding and C5a-induced functions in human neutrophils with IC₅₀ values of less than 5 nM and was more potent than F-[OP-n-ChaWR], which has been considered to be the most potent small C5aR antagonist. The C5aR antagonistic activity of W-54011 was species-specific and C5aR-specific. W-54011 also exhibited C5a antagonistic activity in vivo (gerbils).

The binding site of W-54011 is not fully investigated, but we considered that it binds to C5aR by the following three observations. 1) In Ca²⁺ mobilization assay, the inhibitory activity of W-54011 remained after W-54011-treated cells were washed briefly in assay buffer to remove this drug (data not shown). This observation indicates that W-54011 reacts with C5aR-expressing cells, but not C5a. 2) W-54011 does not inhibit IL-8-, PAF-, or fMLP–induced Ca²⁺ mobilization. This observation indicates that W-54011 does not inhibit signal machineries commonly used by GPCRs. 3) The antagonistic action of W-54011 was exhibited only in the neutrophils of human, cynomolgus monkeys, and gerbils, but not in those of other examined species. The homology of C5aRs is more than 90% between human and Rhesus monkeys (43) and is about 60–70% between humans and mice (44), rats (45), rabbits (46), dogs (47), or guinea pigs (48), suggesting that W-54011 binds to a region that is conserved in the C5aRs of humans and cynomolgus monkeys and does not exist in those of other species. Although the C5aR sequence of gerbil has not been defined, it is considered that the binding site of W-54011 is conserved similarly to that of humans. On the other hand, in the Ca²⁺ mobilization assay, W-54011 shifted rightward the concentration-response curves to C5a without depressing the maximal responses. The response curves were not parallel between the low concentrations (0.3–3 nM) of W-54011 and the high concentrations (10–300 nM), although the curves were parallel among the high concentrations (10–300 nM) of W-54011. These results suggest that W-54011 inhibits competitively the action of C5a at high concentrations, but not at low concentrations. However, the binding mechanism of W-54011 remains to be resolved.

Over the past two decades, many efforts have been exerted to
discover C5aR antagonists, then C-terminal mimic peptides of C5a and C5a mutants were identified, but potent and non-peptide antagonists had not been discovered. On this point, Wong (24) and Pellus and Wenneogle (23) review in detail. Briefly, bezodiazepine derivatives and spiroindane-bearing hydantoin derivatives discovered by de Laszlo et al. have affinity to C5aR, but they are partial agonists (49). Lanza et al. also reported substituted 4,6-diaminoquinolines as C5aR antagonists, but their activity is not so potent (IC\textsubscript{50}, 2 μM in C5a binding assay) (50). In addition to these compounds, Astles et al. reported phenylguanidine derivatives as C5aR antagonist with an IC\textsubscript{50} value of 0.8 μM (51), but these compounds seem to be cytotoxic.

Thus, non-peptide C5a binding inhibitors reported previously are not so potent, and some are partial agonists. Moreover, most of these compounds are positively charged. That reason is considered since C5a is a highly cationic polypeptide. C5a binding to its receptor is predominantly through two-site binding of charge-charge interactions (3, 52, 53), one of which is an interaction of the positive charged N-terminal disulfide-linked core of C5a and the negative charged N-terminal domain of its receptor, and the other, which is an interaction of the positive charged C-terminal tail of C5a and the interhelical region of its receptor and which is essential for the functional response to C5a. Based on this information, we dared to pick up an uncharged compound to discover a new type of C5a receptor antagonist after we screened our chemical libraries by binding assay using 125I-labeled C5a according to the similar method for C5a inhibitors described previously. In addition to this point, we used whole cells of stimulated U-937 cells as a source of C5aR in the binding assay instead of the plasma membrane, which have been used in previously reported C5a inhibitors. Moreover, we used two screening systems for chemical optimization, a binding assay system the same as for the first screening and a ROS assay system with human neutrophils. Although it is not clear whether the former contributed to the discovery of potent C5aR antagonists, the latter is thought to be important for this study for the following reasons: structure activity relationships obtained during chemical optimization did not completely coincide between ROS assay using intact human neutrophils and binding assay using human tumor line, U-937 cells (data not shown). Namely, it is thought to be important to use intact human cells.

We discovered a potent and orally active non-peptide C5aR antagonist. Since C5a is implicated in a variety of diseases, it is anticipated that an orally active non-peptide C5aR antagonist may have potential as novel therapeutics.