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Peptidomimetic C5a receptor antagonists with hydrophobic substitutions at the C-terminus: Increased receptor specificity and in vivo activity

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Abstract—A new class of peptidomimetic C5a receptor antagonists characterized by C-terminal amino acids with hydrophobic side chains is presented. Systematic optimization of the first hits led to JPE1375 (**36**), which was intensively characterized in vitro and in vivo. Compound **36** exhibits high microsomal stability and receptor specificity and is highly active in an immune complex mediated peritonitis model (reverse passive Arthus reaction) in mice.

The complement system (CS) is one central arm of the innate immune system. However, besides its well-known role for enhancing B-cell responses,[1] the modulation of T-cell responses has been described recently.[2] Thus, it becomes increasingly evident, that the CS also interacts with the adaptive immune system. One key player at the interface between innate and adaptive immunity is the anaphylatoxin C5a, a 74 amino acid protein, which is generated during activation of the CS. C5a binds to the G protein-coupled receptors CD88 (C5aR) and C5L2[3] of which only CD88 is functionally active. C5a is a potent chemotactic factor for monocytes, macrophages, neutrophils, activated T- and B-lymphocytes and induces a strong pro-inflammatory response.[4]

As C5a has been discussed to be involved in a number of diseases, several peptidic and non-peptidic C5a receptor antagonists (C5aRAs) have been described (reviewed in Ref. 5). Of the peptide derived inhibitors nearly all compounds reported have a positively charged Arg at the C-terminus,[6] as it is also found in C5a itself. Peptides lacking this Arg have significantly lower binding affinity.[7] Here the discovery of a new class of peptidomimetic C5aRAs is reported. Replacement of the C-terminal Arg by hydrophobic amino acids resulted in highly active antagonists with increased microsomal stability and enhanced specificity for CD88.

The first step in a systematic approach towards new C5aRAs was an alanine scan of **1** (PMX53)[6a] (Table 1). All compounds were synthesized by standard solid-phase peptide synthesis, cleavage from the resin and cyclization of the C-terminal carboxylate to the Orn side chain. The antagonistic activity of all compounds was determined using a functional assay system based on the inhibition of C5a induced glucosaminidase release stimulated from CD88 transfected RBL cells (RBL+).[8] It turned out that the replacement of Phe (**2**), cha (**4**), Trp (**5**) and Arg (**6**) by alanine led to a pronounced loss of activity by a factor of >200. Apparently, the side chains of all of these amino acids play an important role in functional activity. Pro could be replaced by Ala without loss of activity. It has been shown that the small ring size of PMX53 furnishes a rigid secondary structure[6a] and this might overrule any effects of Pro on the secondary structure (Fig. 1).

It is interesting to note that compound **6** is distinctly more active than **2**, **4** and **5**, revealing that Arg is the least important of the four critical side chains. Due to this observation, compounds with additional modifications at the Arg position were synthesized (Table 2). The elongation of the Arg side chain by one methylene group led to a 12-fold decrease in antagonistic activity (**7**).

Keywords: C5a receptor; Antagonist.
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Table 1. Alanine scan of **1**

Compound	Sequence[a]	Inhib. GAR[b] IC_{50} (μM)
1 (PMX53)	Ac-Phe-[Orn-Pro-cha-Trp-Arg]	0.029
2	Ac-**Ala**-[Orn-Pro-cha-Trp-Arg]	23
3	Ac-Phe-[Orn-**Ala**-cha-Trp-Arg]	0.025
4	Ac-Phe-[Orn-Pro-**ala**-Trp-Arg]	55
5	Ac-Phe-[Orn-Pro-cha-**Ala**-Arg]	100
6	Ac-Phe-[Orn-Pro-cha-Trp-**Ala**]	6.9

[a] cha, D-cyclohexylalanine; ala, D-Ala.
[b] Inhib. GAR, inhibition of glucosaminidase release.

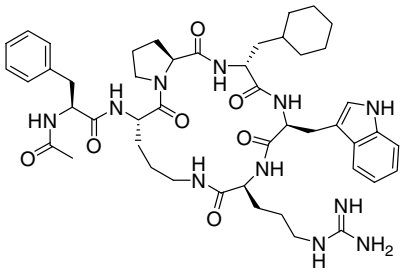

Figure 1. PMX53 (Ac-Phe-[Orn-Pro-cha-Trp-Arg], **1**).

The loss of affinity was even more pronounced when Arg was replaced by Lys (**8**). In contrast to that Orn (**9**) led to an activity similar to Arg. These results suggest that the appropriate orientation of the charged groups is extremely important for a high antagonistic activity.

The replacement of Arg by its bioisostere Cit (**10**) and by Arg(NO_2) (**11**), which are polar but uncharged under physiological conditions, leads to compounds with 17–18 times lower activity compared to **1**. This dramatic loss of activity suggests that the positive charge of Arg is very important for functional activity. In spite of that the charged guanidine group was completely removed by the introduction of Nva leading to a compound with surprisingly high activity (**12**). Compound **12** and additional compounds described below are the first C5aRAs with hydrophobic substitutions at the C-terminus which show activities in the low nanomolar range.

Subsequently, the C-terminal position was analyzed by a variety of hydrophobic amino acid substitutions (**13**–**26**). It turned out that a number of straight chain (**13**), branched (**14**–**16**) and cyclic (**17**–**18**) alkyl groups lead to compounds with high antagonistic activity. Nva and Nle are the best residues tested (Fig. 2). The space for hydrophobic substituents seems to be limited though, since the sterically more demanding cyclohexylethyl (**19**) and *n*-octyl (**20**) led to decreased activity compared to **1**. This was also the case for nle (**21**) and 2Ni (**26**). With small unsaturated or aromatic groups (**22**–**25**) high activity could be achieved as well. The most active compounds contain Eag, Phe or Thi (Fig. 2).

The effect of Arg replacements by amino acids with hydrophobic side chains was also explored for linear peptidomimetics. Two Arg containing compounds derived from **1** by ring opening were synthesized (**27**, **28**, Table 3). It turned out that this modification is not well tolerated and leads to a significant decrease in activity to 0.535 and 0.190 μM, respectively. Surprisingly the carboxamide is more active than the carboxylate, even though C5a has got a C-terminal carboxylate and the group has been proposed to be an essential feature of the pharmacophore of peptidomimetic C5aRAs.[9] Replacement of Arg by Nle (**29**) results in lower activity compared to **28**, suggesting that the SAR of the C-terminus is different in linear peptidomimetics compared to cyclic compounds. However, high activity can be obtained by incorporating aromatic groups like in Phe (**30**, 0.031 μM).

Table 2. Antagonistic activity of Ac-Phe-[Orn-Pro-cha-Trp-Xxx] (**7**–**26**)

Compound	Xxx[a]	Inhib. GAR IC_{50} (μM)	Compound	Xxx[a]	Inhib. GAR IC_{50} (μM)
7	Har	0.360	**17**	Chg	0.038
8	Lys	8.700	**18**	Cha	0.045
9	Orn	0.018	**19**	Hch	0.146
10	Cit	0.490	**20**	Ocg	0.300
11	Arg(NO_2)	0.570	**21**	nle	0.107
12	Nva	0.018	**22**	Eag	0.019
13	Nle	0.025	**23**	Phe	0.026
14	Ile	0.050	**24**	Thi	0.022
15	Leu	0.054	**25**	1Ni	0.051
16	Hle	0.057	**26**	2Ni	0.076

[a] Har, homoarginine; Orn, ornithine; Cit, citrulline; Nva, norvaline; Nle, norleucine; Hle, homoleucine; Chg, cyclohexylglycine; Cha, cyclohexylalanine; Hch, homocyclohexylalanine; Ocg, *n*-octylglycine; nle, D-norleucine; Eag, propargylglycine; Thi, 2-thienylalanine; 1Ni, 1-naphthylalanine; 2Ni, 2-naphthylalanine.

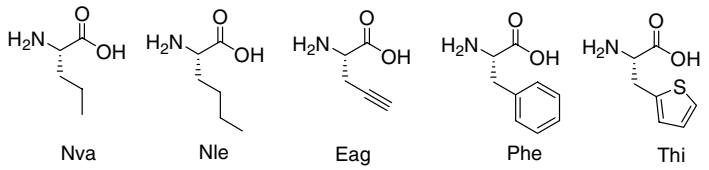

Figure 2. Structures of selected amino acids Xxx in Ac-Phe-[Orn-Pro-cha-Trp-Xxx].

Table 3. Antagonistic activity, stability against degradation by human microsomes and agonistic activity of compounds **27**–**36**

Compound	Sequence[a]	Inhib. GAR IC_{50} (μM)	Stab. micr.[b] (%)	Agonism % at 286 μM
27	Ac-Phe-Orn-Pro-cha-Trp-Arg-OH	0.535	n.d.	n.d.
28	Ac-Phe-Orn-Pro-cha-Trp-Arg-NH_2	0.190	n.d.	n.d.
29	Ac-Phe-Orn-Pro-cha-Trp-**Nle**-NH_2	0.463	n.d.	n.d.
30	Ac-Phe-Orn-Pro-cha-Trp-**Phe**-NH_2	0.031	34	n.d.
31	Ac-Phe-Orn-Pro-**hle**-Trp-Phe-NH_2	0.063	37	0
32	Ac-Phe-Orn-Pro-hle-**Bta**-Phe-NH_2	0.027	1	0
33	Ac-Phe-Orn-Pro-hle-**Dcf**-Phe-NH_2	0.116	49	7.2
34	Ac-Phe-Orn-Pro-hle-**Mcf**-Phe-NH_2	0.060	62	1.2
35	Ac-Phe-Orn-Pro-hle-**Pff**-Phe-NH_2	0.071	65	0
36	**Hoo**-Phe-Orn-Pro-hle-Pff-Phe-NH_2	0.039	80	0

n.d. = not determined.
[a] Nle, norleucine; hle, D-homoleucine; Bta, benzothienylalanine; Dcf, Phe(3,4-Cl); Mcf, Phe(3-Cl); Pff, Phe(4-F); Hoo, L-hydroorotic acid.
[b] Human microsomes, % remaining after 1 h.

The next goal was to optimize the stability in the presence of human liver microsomes. This stability is often used for predicting hepatic clearance. Compound **30** shows a modest stability of 34% (Table 3). The replacement of cha by hle (**31**) leads to a similar stability (37%). The most important determinant for microsomal stability is the Trp position though. The substitution of Trp by Dcf, Mcf or Pff improved stabilities to 49%, 62% and 65%, while retaining functional activity (**33**–**35**). Among the latter compounds **35** is preferred, since undesired agonistic activity has been found for **33** and **34,** which have bulkier side chains. This means that the amino acid at the Trp position determines agonistic or antagonistic activity which is in accordance with results for other C5aRAs[10] and could be a suitable starting point for the development of agonists.

Finally the antagonistic activity of **35** was increased about 2-fold by substitution of Ac for hydroorotic acid (Hoo). This led to JPE1375 (**36**, Table 3, Fig. 3), a new C5a receptor antagonist featuring high in vitro activity (0.039 μM), high stability against human microsomes (80%, 1 h) and complete functional antagonism (0% agonism up to 286 μM).

Compound **36** was further characterized in vitro for activity, stability and specificity (Table 4). Compounds **36** and **1** exhibit similarly high antagonism for human C5aR expressed on RBL cells and human PMNs. The inhibition of C5a binding to C5aR is slightly weaker than the antagonistic potency. This can be explained by binding of the compounds to the transmembrane binding site 2 of the receptor as it was proposed for other C5aRAs.[11] It is expected that binding in this region is sufficient for the inhibition of the agonistic activity of C5a, but the binding of C5a to the extracellular binding site 1 is not effectively inhibited. Compound **36** exhibits high plasma stability (100% after 1 h) and a higher microsomal stability compared to **1** (80% vs 10%). The inhibition of CYP3A4 is negligible (14% at 10 μM) which reduces the risk of undesired interactions with the metabolism of other drugs.

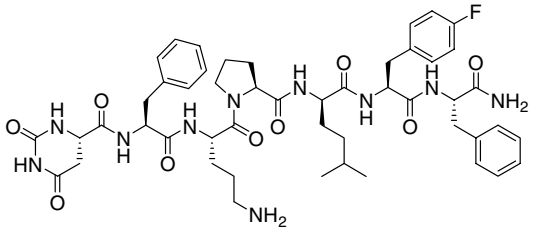

Figure 3. JPE1375 (Hoo-Phe-Orn-Pro-hle-Pff-Phe-NH_2, **36**).

Table 4. In vitro properties of JPE1375 (**36**) and PMX53 (**1**)

Assay	JPE1375	PMX53
Inhib. GAR, RBL+ cells, IC_{50} (μM)	0.039	0.029
Inhib. GAR, hPMN cells, IC_{50} (μM)	0.041	0.031
Binding, HEK293+ cells, IC_{50} (μM)[a]	0.111	0.104
Plasma stability (hum, 1 h, 37 °C) (%)	100	100
Microsomal stability (hum, 1 h, 37 °C) (%)	80	10
CYP3A4 Inhibition at 10 μM (%)[b]	38	77
No. of receptor interactions >50% at 10 μM	2	4
Binding NK2, IC_{50} (μM)	0.71	0.087
Binding MC4, IC_{50} (μM)	13	0.40
Binding V1a, IC_{50} (μM)	0.74	0.74
Binding ORL1, IC_{50} (μM)	> 50	2.20
Chemotaxis, mouse J774A.1 cells, IC_{50} (μM)	0.42	7.1

[a] Inhibition of C5a binding to human C5aR on HEK293 cells.
[b] Inhibition of CYP3A4 activity with BFC as substrate.

For the determination of the receptor specificity a screen with 44 different receptors, four ion channels and two transporter proteins (ExpresSProfile, Cerep, Poitiers, France) was performed at a concentration of 10 μM. For **36** only two receptors with an inhibition of >50% were identified, whereas **1** inhibits four (NK2, MC4, V1a and ORL1). Subsequently, the IC_{50} values for these four receptors were determined (Table 4). Compound **36** had a selectivity of at least 18 (factor between IC_{50} value on NK2 receptor and IC_{50} on C5aR) while for **1** the lowest selectivity factor was 3. Thus, **36** exhibits a markedly improved receptor specificity.

The in vivo efficacy of JPE1375 was tested in a mouse model of the reverse passive Arthus reaction (RPAR), an immune complex (IC) mediated disease which is driven by activation of complement and Fc-receptors. The role of C5a in the RPAR was described by Köhl and Gessner.[12] Here the inhibitory activity of **1** and **36** on the neutrophil influx into the mouse peritoneum after iv challenge with the OVA (chicken ovalbumin) peptide and ip challenge with an anti-OVA-antibody was tested. The compounds (1 mg/kg iv, dissolved 0.15 mg/mL in

PBS buffer) were administered 15 min before the induction of the RPAR. After 6 h, the number of neutrophils in the peritoneal lavage was determined. Results are shown in Figure 4.

Compound **36** is significantly more active in the RPAR model than **1**. This effect can be explained by improved pharmacokinetic properties due to increased microsomal stability, but it is unlikely that this is the only explanation. Therefore, the inhibitory activity of the compounds on the C5a induced chemotaxis of mouse J774A.1 cells was measured (Table 4). Compound **36** (0.42 μM) is 17 times more active on the murine CD88 compared to **1** (7.1 μM). However, the antagonistic activity of the two molecules for human cell lines (Table 4) is similar. This is in accordance with reported binding experiments with **1** to C5aRAs of different species.[13] Due to the activity of **36** on mouse cells this molecule gives a new option for performing in vivo disease models in this species.

In order to elucidate the SAR described, a homology model of the C5a receptor, containing the seven transmembrane helices and the second extracellular loop (EL2), was built on the basis of the X-ray structure of bovine rhodopsin.[14] Compound **1** was constructed within the interior of the receptor binding pocket with various orientations.[15] Subsequently, a multiple simulated annealing protocol was employed to relax the system and to define the best ligand receptor complex. Compound **1** was mutated to the linear antagonist **36** and the complex relaxed again via MD simulations.[16]

One of the main features of the preferred model (Fig. 5) is that Arg of **1** does not penetrate deeply into the binding pocket of the receptor, as it was suggested by Gerber et al. for Me-FKPdChaWdR.[10] Instead, Arg forms a salt-bridge to Asp282 (TM7) as it was described in the closed-loop binding model for **1** by Higginbottom et al.[17] Alternatively, binding of a completely extended Arg6 side chain to Asp37 (TM1) is conceivable. Due to the proximity of Phe93, Val97 (TM2), Val186, Ile187 (EL2) and Val286 (TM7), the same receptor region is also well suited to accommodate a hydrophobic ligand. This perfectly corresponds to our observation that Arg6 can be substituted by hydrophobic amino acids without loss of activity.

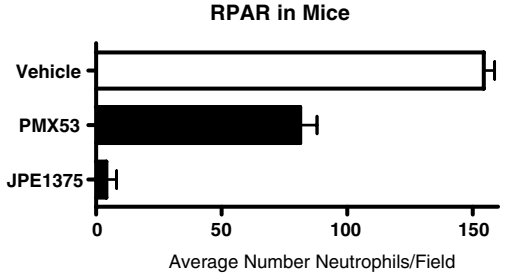

Figure 4. Effect of vehicle ($n = 8$), PMX53 (**1**, $n = 7$) and JPE1375 (**36**, $n = 5$) on the neutrophil influx in the RPAR in mice (+SEM). The inhibition of the neutrophil influx compared to the vehicle is significant ($p < 0.01$) for both compounds.

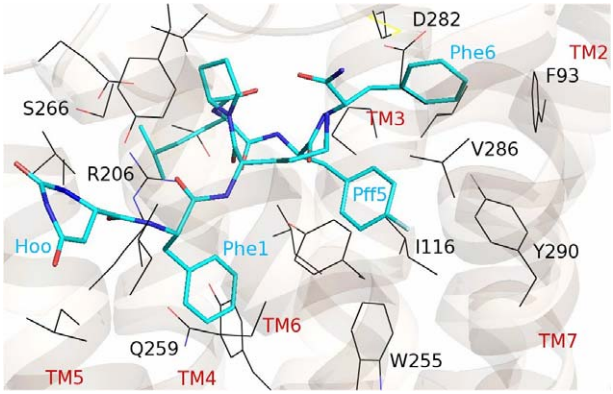

Figure 5. Homology model of CD88 with bound **36**.

Trp (Pff in **36**) is located between Val286 and Ile116 (TM3) as was described before.[10,14] Phe binds close to the position of the retinal head group in the rhodopsin structure. It is surrounded by a number of aromatic and hydrophobic residues. D-Cha (hle in **36**) binds to a patch of aliphatic amino acids from TM3-5 and EL2 (Leu167, Pro170, Val190 and Val202), which explains the importance of this side chain for activity. The increased affinity observed when exchanging Ac by Hoo is due to the interaction with neighbouring polar residues (Ser266, Arg206).

In conclusion, new C5aRAs with hydrophobic substitutions at the C-terminus are presented. Optimization of activity and early ADME parameters led to JPE1375 (**36**), which shows favourable in vitro parameters and high in vivo activity in mice.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2006.07.036.

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