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# Selective Hexapeptide Agonists and Antagonists for Human Complement C3a Receptor

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Human anaphylatoxin C3a, formed through cleavage of complement protein C3, is a potent effector of innate immunity via activation of its G protein coupled receptor, human C3aR. Previously reported short peptide ligands for this receptor either have low potency or lack receptor selectivity. Here we report the first small peptide agonists that are both potent and selective for human C3aR, derived from structure−activity relationships of peptides based on the C-terminus of C3a. Affinity for C3aR was examined by competitive binding with $^{125}$I-labeled C3a to human macrophages, agonist versus antagonist activity measured using fluorescence detection of intracellular calcium, and general selectivity monitored by C3a-induced receptor desensitization. An NMR structure for an agonist in DMSO showed a $\beta$-turn motif that may be important for C3aR binding and activation. Derivatization produced a noncompetitive and insurmountable antagonist of C3aR. Small molecule C3a agonists and antagonists may be valuable probes of immunity and inflammatory diseases.

## Introduction

Human complement is a complex network[1−3] of plasma proteins that initiates inflammatory and cellular immune responses to stimuli like infectious organisms (bacteria/viruses/parasites), chemical/physical injury, radiation, and cancer. Complement activation is an important proteolytic signaling cascade in the immune response to infection and tissue injury, its proteins cooperatively effecting recognition, opsonization, lysis, and removal of pathogens and infected or damaged cells and cellular debris. Among complement activation products are the anaphylatoxins C3a and C5a, which prime and amplify the immune response through chemoattraction of immune cells to inflammatory sites and by promoting secretion of proinflammatory agents, lysosomal enzymes, and reactive oxygen species.[1−3] Unlike C5a, which is almost undetectable in healthy individuals, there is considerable C3a (> 100 nM) present due to continuous degradation of C3. While the pharmacology of C5a and its receptor[4a,b] has been strongly implicated in the pathogenesis of inflammatory diseases through effects of gene deletion, genetic deficiency, antibodies, and a synthetic antagonist, C3a or its receptor C3aR[a] has only more recently been implicated in the pathogenesis and progression of inflammatory diseases such as asthma, allergies, sepsis, lupus erythematosus, diabetes, arthritis, psoriasis, nephropathy, ischemia−reperfusion injury, and others.[5−7] Antimicrobial and antifungal properties have also been reported for C3a derived peptides.[8] The 21-residue C-terminus of C3a and the 20-residue C-terminus of C3adesArg are also antibacterial against *E. faecalis* and *P. aeruginosa*,[9] *Escherichia coli*, and *Staphylococcus aureus*[10] and antifungal against *Candida albicans*.[11] Two nonapeptide derivatives of the C3a C-terminus interfere with intracellular $Ca^{2+}$ release and ERK 1/2 phosphorylation by binding to and promoting phosphorylation of type 1 Fc $\varepsilon$ receptor in mast cells.[12] As more C3a−C3aR pharmacology has become established, interest has been renewed in C3a and there is a realization of the need for synthetic C3a agonists and antagonists for interrogating and potentially treating human C3a-mediated conditions.[5−7]

Human complement component C3a is a 76 residue protein that binds to a single known seven-transmembrane domain receptor C3aR, a rhodopsin-like GPCR that is expressed ubiquitously on the surface of many cells (particularly

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[a]Abbreviations: 3D53, C5aR antagonist cyclo-(2,6)-AcF[OP-(dCha)WR]; Ahx, 6-aminohexanoic acid; Aib, 2-aminoisobutyric acid; $Bt_2$-cAMP, 3′,5′- dibutyryladenosine monophosphate; ATP, adenosine triphosphate; C3aR, C3a receptor; C5aR, C5a receptor; Cha, cyclohexylalanine; dU937, human monocytic cells from histiocytic lymphoma differentiated with $Bt_2$-cAMP; DIPEA, *N*,*N*-diisopropylethylamine; DMF, *N*,*N*-dimethylformamide; $EC_{50}$, molar concentration that produces 50% of the maximum response of an agonist; FBS, fetal bovine serum; Fmoc, fluorenylmethoxycarbonyl; G-protein, guanosine monophosphate protein; GPCR, G-protein-coupled receptor; HATU, 2-(1*H*-7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HBSS, Hank's balanced salt solution; HBTU, *O*-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HEK-293, human embryonic kidney 293 cells; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid); HT-29, human colon adenocarcinoma grade II cell line; hF, homophenylalanine; $^{125}$I, iodine isotope 125; $IC_{50}$, molar concentration of an unlabeled agonist/antagonist that inhibits 50% radioligand binding or molar concentration of antagonist that inhibits 50% of a known concentration of agonist activity; Ig, immunoglobulin; IMDM, Iscove's modified Dulbucco's medium; MBHA, methylbenzhydrylamine; Nal, 1-naphthylalanine; Nle, norleucine; NEAA, nonessential amino acids; NOE, nuclear Overhauser effect; NOESY, nuclear Overhauser effect spectroscopy; Orn, ornithine; Pbf, 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl; PBMC, peripheral blood mononuclear cells; rmsd, root-mean-square deviation; ROE, rotating-frame Overhauser enhancement; ROESY, rotating-frame NOE spectroscopy; rpHPLC, reversed phase high performance liquid chromatography; TFA, trifluoroacetic acid; TIPS, triisopropylsilane; TOCSY, total correlation spectroscopy; U937, human leukemic monocyte lymphoma cell line.

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**Table 1.** Affinity and Potency of Known Truncated Anaphylatoxin Agonists on dU937 Cells

| | | C3a receptor affinity[e] | | | C5a receptor affinity[e] | | | apparent agonist activity[f] | | |
|---|---|---|---|---|---|---|---|---|---|---|
| compd | peptide | *n* | $-\log IC_{50} \pm SE$ | $IC_{50}$[a] (nM) | *n* | $pIC_{50} \pm SE$ | $IC_{50}$[b] (nM) | *n* | $-\log EC_{50} \pm SE$ | $EC_{50}$[c] ($\mu$M) |
| **1** | C3a | 13 | $9.9 \pm 0.07$ | 0.12 | 3 | $\varphi$ | | 10 | $7.3 \pm 0.06$ | 0.052 |
| **2** | C5a | 1 | $\varphi$ | | 4 | $8.9 \pm 0.24$ | 1.4 | 3 | $8.2 \pm 0.09$ | 0.007 |
| **3** | SB290157 | 7 | $6.9 \pm 0.16$ | 140 | 3 | $\varphi$ | | 3 | $\phi$[d] | |
| **4** | 3D53 | 3 | $\varphi$ | | 3 | $7.8 \pm 0.17$ | 15 | 3 | $\phi$ | |
| **5** | WWGKKYRASKLGLAR | 3 | $8.7 \pm 0.20$ | 2.0 | 3 | $\varphi$ | | 5 | $5.9 \pm 0.16$ | 1.3 |
| **6** | $C3a_{63-77}$ | 3 | $6.6 \pm 0.23$ | 250 | 3 | $6.3 \pm 0.33$ | 560 | 4 | $5.8 \pm 0.11$ | 1.8 |
| **7** | $C5a_{60-74}$ | 3 | $5.8 \pm 0.21$ | 1600 | 3 | $5.8 \pm 0.17$ | 1700 | 4 | $4.6 \pm 0.11$ | 24 |
| **8** | YSFKPMPL(Me-a)R | 3 | $7.9 \pm 0.13$ | 13 | 3 | $5.7 \pm 0.14$ | 2000 | 5 | $6.9 \pm 0.06$ | 0.13 |
| **9** | YSFK(Me-D)MPLaR | 3 | $6.1 \pm 0.19$ | 740 | 3 | $4.5 \pm 0.45$ | 29000 | 5 | $5.9 \pm 0.05$ | 1.4 |
| **10** | YSHKPMPLaR | 3 | $\varphi$ | | 3 | $\varphi$ | | 3 | $5.2 \pm 0.03$ | 5.7 |
| **11** | YSFKPMPLaR | 3 | $6.9 \pm 0.10$ | 140 | 3 | $5.3 \pm 0.21$ | 4700 | 2 | $6.7 \pm 0.07$ | 0.22 |
| **12** | HLGLAR | 3 | $5.2 \pm 0.21$ | 5900 | 3 | $\varphi$ | | 3 | $5.4 \pm 0.31$ | 3.8 |
| **13** | HLALAR | 3 | $5.2 \pm 0.29$ | 6300 | 3 | $\varphi$ | | 3 | $\phi$ | |
| **14** | YPLPR | 3 | $\varphi$ | | 3 | $\varphi$ | | 2 | $\phi$ | |
| **15** | WPLPR | 3 | $4.5 \pm 0.50$ | 27000 | 3 | $\varphi$ | | 2 | $\phi$ | |

[a] Concentrations causing 50% of maximum binding of $^{125}$I-C3a to intact human monocytes. [b] Concentration causing 50% inhibition of maximum binding of $^{125}$I-C5a to intact human monocytes. [c] Concentration causing 50% maximum calcium mobilization from $Bt_2$-cAMP differentiated U937 cells. [d] No agonist activity at 1 mM, antagonist with $pIC_{50} = 5.9 \pm 0.1$ ($IC_{50} = 1.3\,\mu M$). [e] $\varphi$: no binding detected at 1 mM. [f] $\phi$: no intracellular calcium release detected at 10 $\mu$M.

immune cells such as macrophages, neutrophils, T cells) and signals through $G_{\alpha i}$ and $G_{\alpha 16}$ proteins. Both crystal[13a] and NMR-derived solution[13b] structures have been reported for human C3a, as well as for complement C3 incorporating C3a.[13c] The crystal structure of C3a (actually C3adesArg) was of low resolution (3.2 Å) but showed the C-terminus of C3a in a turn conformation, while the solution structure showed disorder in the last seven residues. For the C3 structure, the electron density was too diffuse to identify the C-terminal seven residues of the sequence corresponding to C3a. A similar turn structure was, however, also observed for the C-terminus of C5a.[14] Like C5a, it is the C-terminal octapeptide region of C3a that is implicated as the effector domain required for triggering receptor activation,[15] the remaining 69 amino acids at the N-terminus of C3a being a high affinity binding domain. Numerous peptides based on the C-terminus of C3a have been reported to compete with $^{125}$I-C3a for the surfaces of cells. Removal of the C-terminal arginine of C3a abolishes the biological activity of C3a, so all synthetic C3a ligands to date have a C-terminal arginine. Despite three decades of research, endeavors to produce highly potent peptidic C3a ligands of less than eight residues, with agonist activity against human cells at submicromolar concentrations, have been unsuccessful. In fact, there have been relatively few reports of new synthetic C3a ligands since the early 1990s.

Among the most potent reported C3aR binding peptide ligands is a synthetic peptide comprising the C-terminal 21 residues of C3a, which was equipotent with C3a in lung strip and ileal contraction assays but 44% as effective as C3a in suppressing Ig secretion by peripheral blood lymphocytes.[16a] Incorporation of helix-inducing Ala and Aib residues at judicious positions resulted in a 250% enhancement in activity of the peptide, correlating with increased helicity measured by circular dichroism spectra.[16b] Truncation and modification to 15 residues with incorporation of two tryptophans at the N-terminus resulted in a "superagonist" WWGKKYRASKLGLAR that was 15-fold more potent than C3a in a guinea pig platelet aggregation assay.[16c] Numerous other attempts have been made to shorten peptide sequences while retaining either competitive binding with $^{125}$I-C3a or C3a-like function.[17] Comparisons between reported activities of these synthetic C3a agonists are difficult to make because of the variety of assay systems used to evaluate them, ranging from tissue organ preparations for measurement of smooth muscle contraction[16a,b,17b,c,h,k] to mast cell histamine-release[18] assays and ATP-release[1−7,19] assays from guinea pig platelets and affinity measurements on isolated membranes or whole cells. The activities of synthetic ligands are further complicated by the use of different species, especially tissues and cells from guinea pigs that have since been found to have two functional C3a receptors[20] compared with only one receptor in humans. Moreover, GPCRs are notorious for having widely variable species-dependent responses to ligands due to different amino acid polymorphisms in the receptor, C5a being a case in point.[21] This may in part explain why many of these peptides reevaluated by us against intact human cells are either not potent or not selective in their binding to C3aR over C5aR or also bind and activate other receptors. Our experiences have also highlighted some potency and affinity inconsistencies with literature data, warranting reevaluation in more relevant human cells. It is our opinion that there have to date been no truly selective and potent agonists or antagonists reported for probing the pharmacology of human C3a in vivo.

There have been very few reports of small non-peptide ligands for C3aR. The most studied is a hydrophobic capped arginine $N^2$-[(2,2-diphenylethoxy)acetyl]-L-arginine (SB290157, **3**) reported to be a C3a receptor antagonist[22a] but also shown to be an agonist in transfected rat basophilic leukemia cells, Chinese hamster ovary cells, and human U937 cells,[22b,c] as well as acting on other unknown receptors.[22d] Antagonists for C3aR have been reported based on diiminoisoindoline[23] and biphenylimidazole[24] core structures, while more potent partial agonists have been based on arginine derivatives with a 2,5-furyl component ($pIC_{50}$ of 7.1 vs 20 pM $^{125}$I-C3a)[25a] and aminopiperidines without arginine ($pIC_{50} = 7.5$).[25b] One path to rational development of nonpeptidic ligands for C3aR is to first identify potent and selective peptides, which proffer information about side chain fitting to the receptor. This paper identifies some important properties for high affinity ligand binding to human C3aR that can be crucial for future design of druggable compounds.

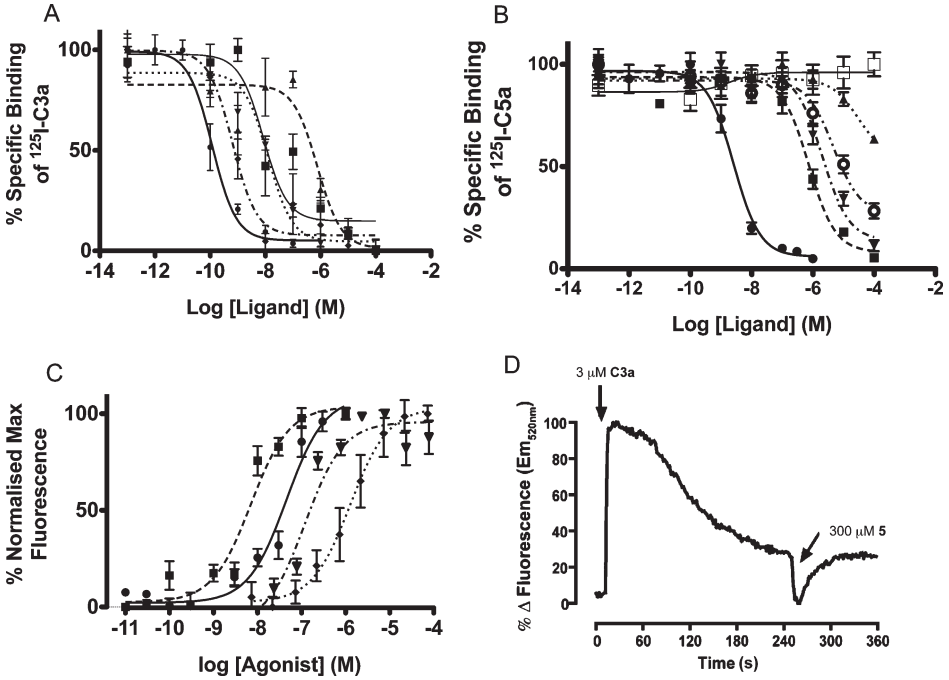


**Figure 1.** Receptor affinity and agonist potency for selected compounds. (A) Affinities for C3a receptor measured by displacement of $^{125}$I-C3a from human PBMCs: **1** (●), **3** (■), **5** (◆), **8** (▼), and **9** (▲). (B) Affinities for C5a receptor measured by displacement of $^{125}$I-C5a on human PBMCs: **2** (●), **4** (■), **5** (□), **8** (▼), **9** (▲), and **10** (○). (C) Activity of **1** (■), **2** (●), **5** (◆), and **8** (▼) in intracellular $Ca^{2+}$ mobilization assay on human dU937 cells. (D) Intracellular calcium release by **5** in C3aR-desensitized human dU937 cells.

## Results

**Activity of Truncated Anaphylatoxins.** N-Terminal truncation of C3a has previously been used to determine what constitutes the active portion of C3a, but past efforts to access short, potent peptides to mimic C3a function have met with limited success. It has been proposed that, as for C5a-C5aR, C3a-C3aR interactions can be described by a two-site model, with the N-terminal helix bundle of C3a providing affinity while the C-terminus carries the receptor-activating motif.[15] We chose to begin addressing binding affinity and agonist induced $Ca^{2+}$ release from the promonocytic cell line U937 differentiated with $Bt_2$-cAMP (dU937). When both binding and functional assays were used, progressive truncation of C3a dramatically reduced both affinity and activity of truncated peptides (Table 1).

C3a (**1**) and C5a (**2**) are selective for their respective receptors on human PBMCs, with no crosstalk between them, as well as potent inducers of intracellular $Ca^{2+}$ release in dU937 cells (Table 1). Similarly, the C3a antagonist **3**[22a] and the C5a antagonist **4**[4a,d−f] also bind specifically to C3aR and C5aR, respectively, on PBMCs, with no cross-reactivity, and neither compound induces $Ca^{2+}$ release in dU937 cells at 1 mM (Table 1). For 2 decades the 15-residue peptide (**5**), reported as a superagonist,[16c] has been used as a benchmark for measuring comparative agonist activity against C3aR. Although it has high affinity for C3aR on PBMCs (Table 1), it had only comparable weak $Ca^{2+}$-inducing activity to that of $C3a_{63-77}$ (**6**) in dU937 cells. Also, although it did not bind to C5a receptors on human PBMCs, **5** was not selective for C3aR because it induced $Ca^{2+}$ release even after C3aR in dU937 cells was first densensitized by human C3a (Figure 1D). Thus, it would appear that future functional studies with **5** could be misleading because of lack of selectivity for human C3aR.

Truncation of C3a to its 15-residue C-terminal native peptide sequence leads to a substantial reduction in functional potency measured by the capacity to induce intracellular calcium release from dU937 cells (Table 1) ($C3a_{63-77}$ **6**, $EC_{50}$ = 1.8 $\mu$M; C3a **1**, $EC_{50}$ = 52 nM; 35-fold), a reduction that is 100 times even more pronounced for C5a ($C5a_{60-74}$ **7**, $EC_{50}$ = 24 $\mu$M; C5a **2**, $EC_{50}$ = 7 nM; 3500-fold). These 15 residue peptides based on the C-terminal native sequences of C3a and C5a were, however, not selective for their receptors (Table 1), $C5a_{60-74}$ **7** having some affinity for C3aR ($IC_{50}$ = 1.6 $\mu$M) and $C3a_{63-77}$ **6** having even greater affinity for C5aR (560 nM) as well as 2000-fold lower affinity than C3a for C3aR. As a result of the nonselectivity of $C5a_{60-74}$ **7**, we decided to also investigate the binding of reported C5a decapeptide agonists (**8**−**11**),[26] which we find have much higher affinity for hC3aR than hC5aR (Table 1). The even shorter hexapeptide analogues (**12**, **13**)[17b] of the C3a C-terminus had marginal affinity for human C3a receptor at micromolar concentrations (Table 1) and were the minimum sequences able to induce any calcium mobilization in human dU937 cells. They were not very active even at 100 $\mu$M, the highest concentration tested, while pentapeptides **14** and **15**[17j] had even weaker or no affinity for C3aR and did not induce $Ca^{2+}$ release.

These results highlight both the difficulty in designing potent and selective small molecules for these receptors, which have high sequence homology in their transmembrane regions, and the need to examine affinity for both C3aR and C5aR when looking for truly potent and selective ligands. On the basis of this and our other unpublished work, we do not consider that there currently exists any short peptide (8 residues or less) that is both potent (at submicromolar concentrations) and selective (≥100-fold) for human C3aR over C5aR or other receptors.

**Structure−Activity Relationships for Agonist Derivatives of FKPLAR.** Our goal was to create short peptides that are both selective for human C3aR over human C5a receptors

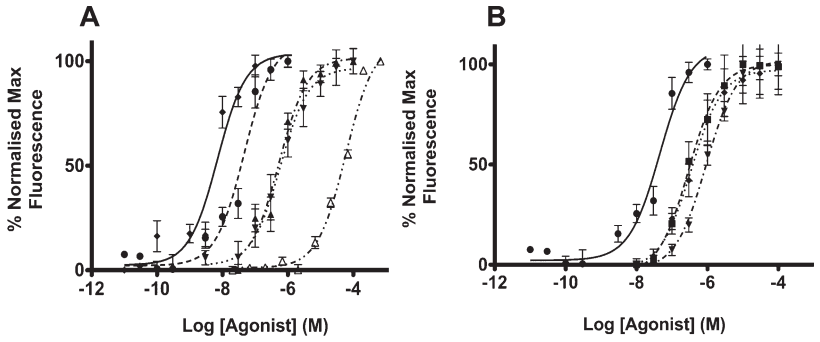


**Figure 2.** Agonist activity for selected peptides in intracellular calcium release in human dU937 cells: (A) **1** (◆), **2** (●), **17** (△), **26** (▲), **31** (▼); (B) **1** (●), **42** (▼), **54** (◆), **55** (■).

**Table 2.** Agonist Activity of FKPLAR (**17**) Analogues on Human dU937 Cells Measured by Calcium Release Assay

| | | | apparent agonist activity[b] | |
|---|---|---|---|---|
| compd | peptide | *n* | $pEC_{50} \pm SE$ | $EC_{50}$[a] ($\mu$M) |
| **16** | FKP(dCha)(Cha)r-$NH_2$ | 2 | $5.7 \pm 0.25$ | 2.2 |
| **17** | FKPLAR | 4 | $4.5 \pm 0.20$ | 33 |
| **18** | HKPLAR | 2 | $\varphi$ | |
| **19** | ChaKPLAR | 3 | $\varphi$ | |
| **20** | WKPLAR | 3 | $\varphi$ | |
| **21** | FOPLAR | 3 | $\varphi$ | |
| **22** | FRPLAR | 4 | $\varphi$ | |
| **23** | FEPLAR | 4 | $\varphi$ | |
| **24** | FIPLAR | 7 | $6.0 \pm 0.08$ | 0.95 |
| **25** | FNlePLAR | 4 | $5.7 \pm 0.14$ | 1.8 |
| **26** | FLPLAR | 3 | $6.4 \pm 0.09$ | 0.42 |
| **27** | FChaPLAR | 3 | $6.1 \pm 0.05$ | 0.78 |
| **28** | FQPLAR | 4 | $5.3 \pm 0.03$ | 5.3 |
| **29** | FFPLAR | 2 | $5.3 \pm 0.09$ | 5.4 |
| **30** | FhFPLAR | 3 | $6.0 \pm 0.06$ | 1.1 |
| **31** | FWPLAR | 8 | $6.2 \pm 0.14$ | 0.58 |
| **32** | FNalPLAR | 3 | $6.1 \pm 0.06$ | 0.72 |
| **33** | FKTLAR | 3 | $5.4 \pm 0.19$ | 3.8 |
| **34** | FKFLAR | 2 | $\varphi$ | |
| **35** | FKPNleAR | 4 | $4.4 \pm 0.20$ | 38 |
| **36** | FKPChaAR | 2 | $\varphi$ | |
| **37** | FKPFAR | 3 | $\varphi$ | |
| **38** | FKPWAR | 2 | $\varphi$ | |
| **39** | FKPLChaR | 3 | $\varphi$ | |
| **40** | FKPLFR | 3 | $\varphi$ | |
| **41** | FKPLWR | 3 | $4.6 \pm 0.34$ | 25 |

[a] Concentration causing 50% maximum calcium mobilization from $Bt_2$-cAMP differentiated human U937 cells. [b] $\varphi$: no calcium release detected at 1 mM.

**Table 3.** Activity of FWPLAR (**31**) and FLPLAR (**26**) Analogues on Human dU937 Cells Measured by Calcium Release Assay

| | | | apparent agonist activity[b] | |
|---|---|---|---|---|
| compd | peptide | *n* | $pEC_{50} \pm SE$ | $EC_{50}$[a] ($\mu$M) |
| **42** | WWTLAR | 3 | $6.04 \pm 0.06$ | 0.91 |
| **43** | hFLTLAR | 2 | $4.96 \pm 0.08$ | 11 |
| **44** | Ac-FLTLAR | 2 | $6.28 \pm 0.15$ | 0.52 |
| **45** | YLTLAR | 2 | $5.61 \pm 0.15$ | 2.5 |
| **46** | RLTLAR | 4 | $\varphi$ | |
| **47** | FOTLAR | 1 | $\varphi$ | |
| **48** | FRTLAR | 1 | $5.17 \pm 0.36$ | 6.8 |
| **49** | FYTLAR | 2 | $5.32 \pm 0.35$ | 4.8 |
| **50** | FNleTLAR | 3 | $6.51 \pm 0.30$ | 0.31 |
| **51** | FWGLAR | 2 | $6.33 \pm 0.09$ | 0.47 |
| **52** | FWALAR | 3 | $5.93 \pm 0.12$ | 1.2 |
| **53** | FWSLAR | 5 | $6.31 \pm 0.09$ | 0.49 |
| **54** | FWTLAR | 6 | $6.43 \pm 0.09$ | 0.37 |
| **55** | FLTLAR | 5 | $6.50 \pm 0.12$ | 0.32 |
| **56** | FLGLAR | 3 | $6.29 \pm 0.33$ | 0.52 |
| **57** | FLALAR | 3 | $5.96 \pm 0.23$ | 1.1 |
| **58** | FLSLAR | 4 | $6.45 \pm 0.19$ | 0.36 |
| **59** | FLTlAR | 2 | $\varphi$ | |
| **60** | FLTNleAR | 1 | $5.58 \pm 0.26$ | 2.7 |
| **61** | FLTChaAR | 4 | $\varphi$ | |
| **62** | FLT(dCha)AR | 2 | $\varphi$ | |
| **63** | FLTSAR | 3 | $\varphi$ | |
| **64** | FLTLLR | 4 | $\varphi$ | |
| **65** | FLTLPR | 1 | $4.54 \pm 0.19$ | 29 |
| **66** | FLTLAr | 2 | $4.24 \pm 0.12$ | 57 |
| **67** | FLTLAR-$NH_2$ | 1 | $4.44 \pm 0.19$ | 37 |

[a] Concentration causing 50% maximum calcium mobilization from $Bt_2$-cAMP differentiated human U937 cells. [b] $\varphi$: no calcium release detected at 1 mM.

and with submicromolar potency as agonists in the intracellular $Ca^{2+}$ release assay. Among the most potent C5aR short peptide agonists is the hexapeptide FKP(dCha)(Cha)r-$NH_2$ (**16**).[27] Given the high sequence homology between C3a and C5a, we decided to start with this hexapeptide to derive structure−activity relationships for C3aR binding peptides using a functional agonist assay in dU937 cells. Since the Leu-Ala-Arg motif is present at the C-terminus of C3a and has been shown to be vital for activity at C3aR,[17b,f] these residues were incorporated as replacements for (dCha)-(Cha)r to produce hexapeptide F6K5P4L3A2R1 (**17**), with $EC_{50} = 33\ \mu M$ in the intracellular calcium mobilization assay for dU937 cells (Figure 2). Table 2 shows effects of substitution in FKPLAR on agonist activity, as measured by intracellular $Ca^{2+}$ release from dU937 cells. When standard numbering[28] was used, Phe6 in **17** was successively replaced with His, Trp, and the unnatural amino acid Cha but none of the resulting peptides (**18**−**20**) were functional agonists at up to 100 $\mu$M, as there was no detectable calcium release (Table 2).

Lys5 in **17** was substituted by nonpolar (Ile, Leu, Cha, Nle), aromatic (Trp, Phe, homoPhe, naphthylalanine), acidic (Glu), neutral and basic (Gln, Orn and Arg) amino acids (Table 2). Changing Lys to an alternative basic residue Orn (**21**) or Arg (**22**) or to an acidic residue Glu (**23**) abolished activity. However, changing to a hydrophobic aliphatic (**24**−**27**) or aromatic (**29**−**32**) residue or to a Gln residue (**28**) generated compounds that stimulated $Ca^{2+}$ release within the $IC_{50}$ range 0.5−6.0 $\mu$M. The two most potent agonists were **26** (Leu) and **31** (Trp) with $EC_{50}$ of ~0.4 and 0.6 $\mu$M, respectively (Figure 2), and they were subjected to a further round of sequence optimization as shown in Table 3.

Table 3 reports some derivatives of FWPLAR (**31**). Replacing Pro4 with residues Gly, Ala, Ser, and Thr

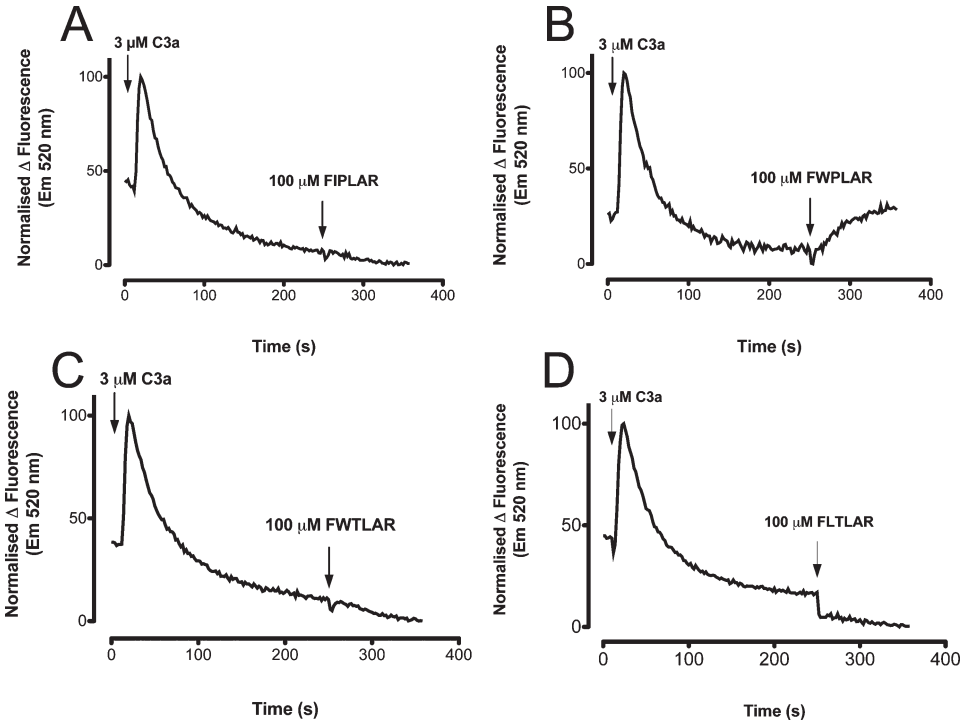


**Figure 3.** C3aR selectivity for peptides **24** (A), **31** (B), **54** (C), and **55** (D). Intracellular calcium release was measured in human dU937 cells desensitized to C3a agonists by C3a. Compounds **24**, **54**, and **55** were selective, while **31** induced calcium release even after the cells were desensitized by C3a, suggesting some activation through a different receptor to C3aR.

(**51**−**54**) enhanced agonist potency except for Ala. The threonine-containing **54** ($EC_{50}$ = 0.37 $\mu$M) was the most potent agonist, but changes from Thr4 to Ser or Gly did not alter agonist potency much. A second family of compounds was based on FLTLAR (**55**) which was slightly more potent than **54**. Changing Phe6 to homophenylalanine or tyrosine or capping the C-terminus with acetyl (**43**−**45**) reduced agonist potency, while an arginine at this position (**46**) abolished activity. Replacing Leu5 with ornithine, arginine, or tyrosine (**47**−**49**) significantly reduced activity, but a change to norleucine (**50**) had little affect. Changing Thr4 to glycine, alanine, or serine (**56**−**58**) also had little effect. Replacing any of Leu3, Ala2, or Arg1 (**59**−**67**) substantially reduced or abolished agonist function of these hexapeptides.

Further investigations were carried out on the tolerance of each position to different residues in order to confirm the tests carried out in the earlier part of the study. As Table 3 shows, all changes to FLTLAR resulted in reduced activity, including addition of an acetyl N-terminal cap (**44**) and changing of the C-terminal acid to an amide (**67**), suggesting that both the free amino group and the carboxylic acid are important for potency. Figure 2 shows agonist responses for potent compounds from Table 2 and 3.

Figure 3 shows receptor desensitization experiments to examine possible selectivity for two of the most active compounds in this series, **24** and **31**. The cells were first exposed to C3a, which triggers phosphorylation of the receptor by tyrosine kinases and desensitization to further addition of C3a or C3aR-selective agonists. Compound **24** does not stimulate calcium release following C3a receptor desensitization, and we conclude that it is selective for C3aR. However, **31** does induce some $Ca^{2+}$ release following desensitization with C3a, indicating that at least some $Ca^{2+}$ release is not mediated through activation of C3aR; thus, **31** is not selective. Compounds **35**−**41** and **59**−**67** represent changes to the L3 and A2 positions, but these reduced agonist potency, suggesting that L3A2R1 at the C-terminus may be optimal among the compounds examined here for agonist activity.

**Table 4.** Affinity of Selected Hexapeptides for C3aR by Competition with Radioligand Binding, Assayed in Human PBMCs

| compd | peptide | $n^a$ | $-\log IC_{50} \pm SE$ | $IC_{50}$ (nM)$^b$ | $IC_{50}$ (nM)$^c$ |
|---|---|---|---|---|---|
| **1** | hC3a | 13 | 9.75 ± 0.12 | 0.18 | $\varphi$ |
| **24** | FIPLAR | 3 | 6.64 ± 0.13 | 230 | $\varphi$ |
| **31** | FWPLAR | 4 | 6.56 ± 0.30 | 274 | $\varphi$ |
| **42** | WWTLAR | 3 | 6.61 ± 0.12 | 244 | $\varphi$ |
| **49** | FYTLAR | 3 | 6.78 ± 0.17 | 166 | $\varphi$ |
| **54** | FWTLAR | 3 | 7.09 ± 0.33 | 82 | $\varphi$ |
| **55** | FLTLAR | 3 | 7.38 ± 0.26 | 42 | $\varphi$ |
| **56** | FLGLAR | 3 | 6.81 ± 0.12 | 157 | $\varphi$ |
| **61** | FLTChaAR | 2 | 6.62 ± 1.59 | 238 | $\varphi$ |
| **63** | FLTLAr | 3 | 5.10 ± 0.50 | 8200 | $\varphi$ |

$^a$ Number of experiments. $^b$ Competitive binding with 80 pM $^{125}$I-C3a. $^c$ Competitive binding with 80 pM $^{125}$I-C5a. $\varphi$: no binding at 1 mM.

**Affinity of C3a Ligands for Human C3aR.** The affinity for C3aR and C5aR of some of the more potent agonists (and one antagonist **61**) is reported in Table 4 and Figure 4. We used competitive radioligand binding experiments using whole PBMCs isolated from human plasma to determine $IC_{50}$ for each ligand (**1**, **24**, **31**, **42**, **49**, **53**, **54**, **55**, **61** and **63**) by displacement of $^{125}$I labeled human C3a or C5a measured with a scintillation proximity assay. The ligands were tested in triplicate on three different occasions to obtain a full dose response curve for each compound. There was a pseudo-linear correlation between C3a-competitive ligand binding affinity and $Ca^{2+}$-inducing agonist potency (Figure 7), and none of the compounds showed any affinity at submillimolar concentrations for human C5a receptors.

**Solution Structure of Agonist 55.** Agonist peptide **55** was examined by 1D and 2D proton NMR spectroscopy in an attempt to identify any propensity for adopting well-defined

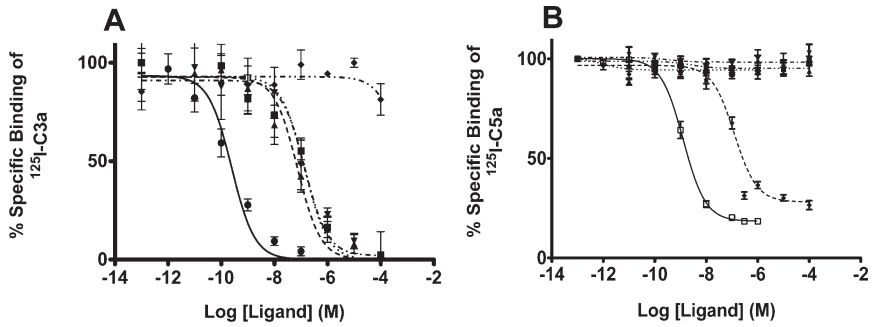


**Figure 4.** Competitive ligand binding affinity for human PBMCs. (A) Affinity of selected peptides for C3aR in competitive radioligand binding vs $^{125}$I-C3a (80 pM): **1** (●), **3** (■), **4** (◆), **54** (▼), **55** (▲). (B) Affinity of selected peptides for C5aR as determined in a radioligand binding assay vs $^{125}$I-C5a (80 pM): **1** (●), **2** (□), **3** (■), **4** (◆), **54** (▼), **55** (▲).

**Table 5.** $^1$H NMR Chemical Shift ($\delta$, ppm) Assignments for **55** in DMSO-$d_6$ at 25 °C

| residue | NH | Hα | Hβ | others |
|---|---|---|---|---|
| F (6) | na | 4.04 | 2.88, 3.09 | Hδ 7.24; Hε 7.30 |
| L (5) | 8.65 | 4.50 | 1.48 | Hγ 1.65; Hδ 0.88 |
| T (4) | 8.05 | 3.97 | 3.97 | Hγ1 4.92; Hγ2 1.04 |
| L (3) | 7.82 | 4.30 | 1.48 | Hδ 0.82 |
| A (2) | 7.96 | 4.26 | 1.19 | |
| R (1) | 8.06 | 4.15 | 1.73, 1.62 | Hγ 1.57; Hδ 3.09; Hε 7.46 |

structure in solution. There was no well ordered structure in water, as expected for short peptides, because water competes strongly for hydrogen bonds and only a random assembly of rapidly interconverting structures is normally observed.[4f] However, in the aprotic solvent DMSO-$d_6$, stable secondary structures can sometimes be observed even for short peptides.[4e,f] Indeed, at 298 K distinct doublet resonances were observed for each of the amide peaks of **55** (Table 5). The phi and psi dihedral angles of the residues were obtained from the Karplus equation using coupling constants displayed for the amides. High (> 8 Hz) or low (< 6 Hz) coupling constants were indicative of secondary structure in solution (Leu5, 8.2 Hz; Thr4, 8.6 Hz; Leu3, 8.3 Hz), whereas a disordered structure has uniform amide coupling constants between 6 and 8 Hz.

Secondary structure in peptides is often stabilized by main chain to main chain or main chain to side chain hydrogen bonds. The low temperature coefficient for the L3 amide proton (3.2 ppb/K) (Supporting Information) suggests that it may be involved in hydrogen bonding. When T4 was replaced by alanine, the L3 amide proton no longer had a low temperature coefficient, indicating that the T4 side chain hydroxyl is the hydrogen bond acceptor. ROESY experiments for **55** showed 19 distinctive short and medium range ROEs, indicating a well-defined structure for **55**. Two weak ROEs $d_{\alpha,N(T4,\ A2)}$ and $d_{\alpha,N(L5,\ A2)}$ are indicative of a $\beta$ turn structure between residues L5 and A2 (Figure 6).

Solution structures were determined using 19 ROE distance constraints and 3 dihedral angle constraints, resulting in a family of 15 lowest energy structures which are displayed in Figure 5 (see Supporting Information for the Ramachandran plot, dihedral angles, C$\alpha(i,i+3)$ distances and H-bond distances). The ROEs (Figure 6), distances separating Cα protons of Leu5 and Ala2 (< 7 Å) are indicative of a $\beta$-turn, the dihedral angles of the two middle residues (T4 and L3) closely mimicking a $\beta$-turn. The rmsd of the peptide backbone within the family of 15 structures is 1.7 Å, which is a reasonable convergence for a short unconstrained peptide in solution.

The peptide turn motif in Figure 5 is similar to what we have reported[4f] for the C5aR specific antagonist FKP-(dCha)Wr in DMSO and appears to be encouraged by a hydrogen bond formed between the hydroxyl side chain of Thr4 and the backbone amide proton of Leu3. Variable temperature NMR data reveal a low temperature coefficient (3.2 ppb/K) for this proton, indicating its possible involvement in a hydrogen bond. A serine at this position, which can also form the same hydrogen bond, resulted in similar agonist potency. Proline- and glycine-containing peptides (**31** and **56**) have similar agonist activity and likely also favor a turn motif but without the need for this hydrogen bond. The amide proton of Leu3 in peptide F6LALAR1 **57** did not exhibit a low temperature coefficient, presumably because it is missing its hydroxyl oxygen bonding partner concomitant with its relatively low activity (1 $\mu$M) in the calcium release assay.

**C3aR Antagonist 61.** Figure 7 shows an almost linear relationship between C3aR affinity (PBMCs) and activity (dU937 cells) for C3a hexapeptides from Table 4, consistent with the expected affinity−activity correlation despite using two different cell types. It was clear that the LAR tripeptide motif comprising the C-terminus of C3a agonists was not amenable to much change without loss of substantial *agonist activity* in a calcium release assay. However, this was not an absolute requirement for tight *binding* to the C3a receptor. For example, FLTChaAR (**61**) was unique among the peptides examined that showed no agonist activity at 100 $\mu$M in the calcium release assay, but it did bind strongly to the receptor ($IC_{50}$ = 240 nM, Table 4).

This indicated that **61** was likely an antagonist, with no agonist activity below 100 $\mu$M. This was subsequently demonstrated through noncompetitive antagonism of $Ca^{2+}$ release in human PBMCs induced by either C3a (Figure 8A) or agonist **54** (Figure 8B). It was not a competitive ligand with either of these two agonists, since depression of the maximal agonist response by varying concentrations of **61** did not change the apparent $EC_{50}$ values of C3a or agonist **54**. Thus, either compound **61** binds to a different site on the C3a receptor or it is an insurmountable antagonist, much like certain hexapeptide antagonists of human C5aR.[7d−f] We favor the latter explanation in view of the similarity of the peptide sequences.

## Discussion

Human C3a or its receptor C3aR is implicated in the pathology of multiple inflammatory and immune conditions, for which either an agonist or an antagonist might have

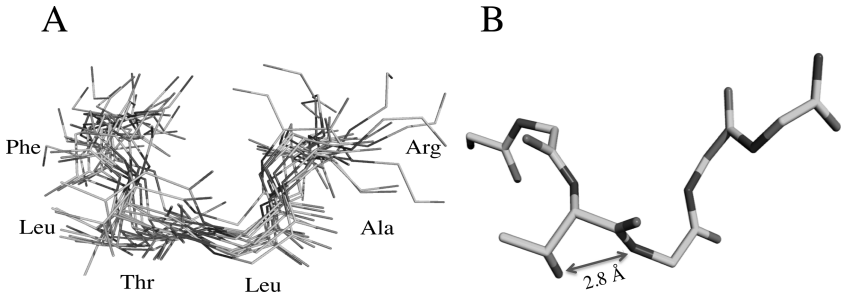


**Figure 5.** NMR-derived solution structure for compound **55** in in DMSO-$d_6$ (298 K). (A) 15 of 20 lowest energy minimized NMR structures showing the backbone of **55**. Residues are labeled with three-letter amino acid codes. (B) Representative NMR structure of **55** showing the H-bond formed between the side chain hydroxyl group of Thr4 and the backbone amide proton of Leu3. A distance of 2.8 Å separates the heavy atoms involved in the bond.

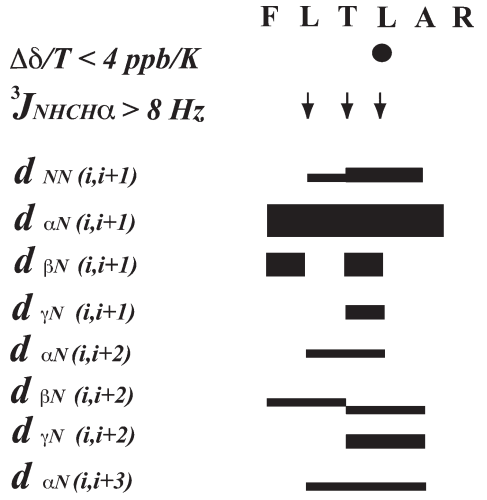


**Figure 6.** NMR summary diagram for **55**. Shown are sequential and medium-range ROEs, the bar thickness being proportional to strong (upper distance constraint 2.7 Å), medium (3.5 Å), weak (5.0 Å), and very weak (6.0 Å) ROE intensities: $^3J_{NHCH} \geq 8$ Hz (↓); amide NH chemical shifts $\Delta\delta \leq 4$ ppb/K (●). The medium range ROEs, especially $d_{\alpha N(i,i+2)}$, $d_{\alpha N(i,i+3)}$ and $d_{NN(i,i+1)}$, are evidence for a $\beta$ turn conformation.

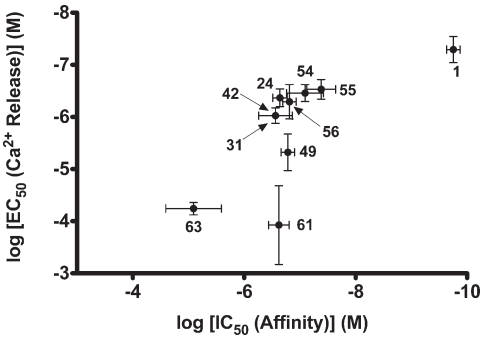


**Figure 7.** Correlation between binding affinity ($IC_{50}$) and agonist activity ($EC_{50}$) for peptides in Table 4. **61** had no agonist activity at 100 $\mu$M; thus, it is given $pEC_{50} \geq 4$.

beneficial effects. However, the ubiquitous expression of C3a and C3aR on many cell types in addition to immune cells may point to additional functions of C3a that await to be discovered. C3a itself induces chemotaxis, secretion of proinflammatory cytokines that define immunostimulatory properties, contraction of smooth muscle, activation of mast cells and eosinophils, and an increase in vascular permeability leading to increased migration of monocytes and neutrophils from the bloodstream into tissue.[1] Furthermore, C3a-desArg, in which the C-terminal arginine is cleaved from C3a by *N*-carboxypeptidase, is known as acylation stimulating protein (ASP) and is implicated in triglyceride synthesis and adipocyte function in metabolic syndrome.[29] Clearly, small molecules that can reproduce the function of C3a and its metabolites can at the very least be useful biological probes of C3a function in vivo, where it is often too expensive and impractical to experiment with the native protein or specific antibodies to it. Small molecule agonists may also have important uses by reproducing beneficial immunological properties of C3a.

The known GPCR activating functions of C3a are localized largely within its C-terminal octapeptide region, and there have been numerous attempts to mimic the functions of C3a by creating short peptides based on its C-terminus. However, most reported small molecule ligands based on C3a have been tested in a diverse array of assays, often in guinea pigs that have more than one C3a receptor, so we needed to reevaluate some of the leading agonists against human C3aR first, before devising new ligands. We found (Table 1, Figure 1) that some reported C5aR ligands actually bound much more tightly to C3aR, while other reported peptide ligands for C3aR were not selective and often bound competitively with $^{125}$I-C5a to cells or induced $Ca^{2+}$ release from cells in which C3aR was first desensitized by C3a. Another compound reported to be a superagonist of C3aR (e.g., **5**) was found to bind tightly to human macrophage C3aR but had only weak agonist activity in the $Ca^{2+}$ release assay and was also not receptor-selective. Consequently, we set out to obtain potent agonists that unambiguously bound selectively and tightly to human C3aR.

From two series of new hexapeptides, we discovered FLPLAR **26** and FIPLAR **24** ($EC_{50}$ of 0.4−0.95 $\mu$M, Table 2) and FWTLAR **54** and FLTLAR **55** ($EC_{50} \approx 0.3$ $\mu$M, Table 3) as the most potent and selective agonists for hC3aR. There was a pseudolinear correlation between agonist potency (measured as release of $Ca^{2+}$ into human dU937 cells) and C3aR binding affinity (measured by competition with $^{125}$I-labeled human C3a on PBMCs). An NMR-derived solution structure for agonist **55** showed significant "turn" or "bend" propensity in the aprotic solvent DMSO, a feature of the C-terminus of C3a itself, hinting at a potential requirement for this structural feature for binding within the transmembrane region of human C3aR. This result is consistent with the findings that over 100 peptide-activated G protein coupled receptors have shown a tendency to recognize protein and peptide ligands with turn structure[30a,b] and that cyclic peptides that mimic turn conformations tend to be potent ligands for GPCRs and other protein targets.[30a−c] Future incorporation of turn-inducing peptidomimetic constraints into such

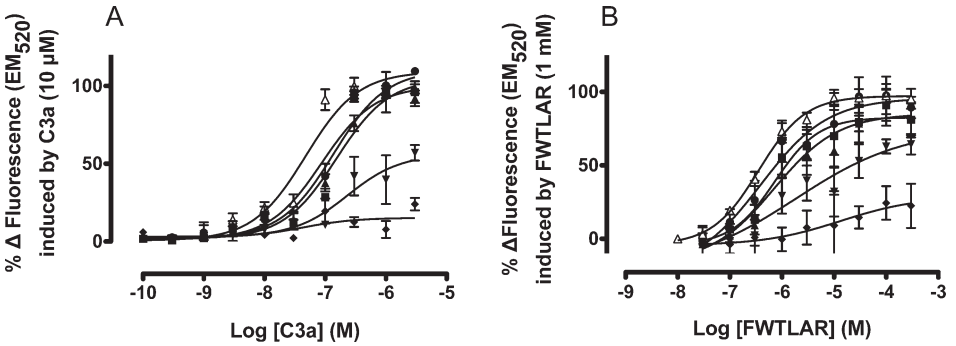


**Figure 8.** Noncompetitive antagonism for **61** on human dU937 cells. (A) Against C3a and (B) against agonist **54**, showing diminished fluorescence of activation by each agonist due to competition from **61** at 0.5 $\mu$M (●), 1 $\mu$M (■), 2 $\mu$M (▲), 4 $\mu$M (▼), and 8 $\mu$M (◆) as measured by intracellular calcium release assay.

peptides, their cyclic or nonpeptidic analogues, could prove fruitful for obtaining even more potent and selective ligands for human C3aR, en route to discovery of candidates suitable for clinical trials.

The reported C3aR antagonist **3**, which does not bind to human C5aR (Table 1), is currently the main antagonist reported in the literature for probing the pharmacology of C3aR in vivo and in vitro. It has been used in animal models for treating asthma, allergy, arthritis, intracerebral hemorrhaging, ischemia/reperfusion injury, among others.[5−7,22] However, it has also been shown to be an agonist in some assays and to induce non-C3a related activities in other assays.[22] Our new antagonist FLTChaAR (**61**) has been found to have comparable binding affinity to **3** for human C3aR on PBMCs ($IC_{50}$ 238 nM (**61**) vs 140 nM (**3**)). Like **3**, it did not bind to hC5aR, and in our study **61** was a more potent antagonist of C3a-induced intracellular $Ca^{2+}$ release than **3** ($IC_{50}$ of 238 nM vs 1.3 $\mu$M). New compound **61** has cyclohexylalanine in place of leucine at position 3 and is the most potent selective antagonist for human C3aR yet reported. Interestingly, this compound was an insurmountable antagonist and may provide a valuable clue to future design of even more potent and selective small molecule C3aR antagonists.

Some conclusions from this study that can be drawn about the requirements of human C3aR for potent and selective hexapeptide ligands are the following: (i) the C-terminal Leu3-Ala2-Arg1 motif is important for agonist activity, with changes to this region leading to significant reductions in agonist potency; (ii) threonine at the fourth position gave the most potent compounds, although other amino acids with small side chains (serine, alanine, proline, glycine) were not that much less potent; (iii) the most potent agonists had tryptophan, norleucine, or leucine at the fifth position; (iv) only ligands with phenylalanine at the sixth or N-terminal position were potent agonists; (v) replacing leucine at the third position of short peptide agonist **55** with a bulkier group leads to compound **61**, which is a functional antagonist of both C3a and the short peptide agonist **54**. These results for ligands with both high affinity and high selectivity for human C3aR finally provide some long awaited clues that have been needed to drive the development of drug leads specific for this important receptor in human immune defense and inflammatory and autoimmune diseases.

## Experimental Methods

**General.** Protected amino acids and resins (Auspep, Parkville, Victoria, Australia; Advanced ChemTech, U.S.; Novabiochem, Laufelfingen, Switzerland), TFA, DIPEA, piperidine and DMF (peptide synthesis grade; Auspep, Parkville, Victoria, Australia), HBTU (IRIS Biotech, Germany) and HATU (Sigma Aldrich) were obtained from commercial sources. Preparative scale reversed-phase HPLC (rpHPLC) separations were performed on Phenomenex C18 columns (100 Å, 250 mm × 20 mm). Analytical rpHPLC were measured on Phenomenex Luna 5 $\mu$m C18 columns [Rt(1)], using gradient mixtures of water/0.1% TFA (solvent system A) and water 10%/acetonitrile 90%/TFA 0.1% (solvent system B). Purity was checked under different gradients to confirm single products. The molecular weight was determined by electrospray mass spectrometry (LCT MICRO-MASS). All compounds were analyzed for purity and molecular weight by rpHPLC and mass spectrometry, respectively, with all compounds having >95% purity. High resolution mass spectrometry was performed on a Bruker micro-TOF by direct infusion in MeCN at 3 $\mu$L/min using sodium formate clusters as an internal calibrant.

**Peptide Synthesis.** Short peptides were assembled by manual stepwise solid phase peptide synthesis using HBTU activation and DIPEA in situ neutralization using Fmoc chemistry. Peptides **16** and **67** were synthesized on Rink amide MBHA resin (Novabiochem, 0.64 mmol/g) to obtain amide functionality at the C-terminus. All other peptides were synthesized on Fmoc-Arg(Pbf)-Wang resin, 0.54 mmol/g substitution (Novabiochem), to obtain peptides with the free C-terminal carboxylic acid group. The peptides were fully deprotected and cleaved from resin by treatment with 9.5 mL of TFA/0.25 mL of TIPS/0.25 mL of water at room temperature for 2 h. After evaporation preparative HPLC was used for peptide purification (gradient 0−100% B over 30 min).

**NMR Spectra Acquisition.** $^1$H NMR spectra were recorded on a Bruker Avance 600 spectrometer on samples containing 1−4 mM peptide in 90% water + 10% $D_2O$ or DMSO-$d_6$. Proton assignments were made using TOCSY (80 ms mixing time), NOESY, and ROESY (350 ms mixing time) spectra according to the sequential assignment method.[31a] Water suppression in 2D experiments was performed using a 3−9−19 Watergate pulse sequence. Variable-temperature 1D $^1$H and TOCSY spectra were typically collected at 5 K increments from 298 to 303 K. For identification of slowly exchanging amides, a series of 1D $^1$H and TOCSY spectra were run immediately after dissolving the peptide (5 mg) in 90% $D_2O/H_2O$ (550 $\mu$L). All spectra were analyzed in TopSpin 1.3 (Bruker, Germany).

**Structure Calculation.** Cross-peaks in ROESY spectra were integrated and calibrated in TopSpin 1.3, and distance constraints from ROE intensities were assigned as strong (2.7 Å upper limit), medium (3.5 Å upper limit), weak (5 Å upper limit), or very weak (6 Å upper limit). Corrections for pseudo-atoms were added to distance constraints where needed. Backbone dihedral angle restraints were inferred from $^3J_{\alpha H\text{-}NH}$ coupling constants in 1D spectra at 298 K. $\phi$ was restrained to $-120 \pm 30°$ for $^3J_{\alpha H\text{-}NH} \geq 8$ Hz. Peptide bond $\omega$ angles were all set to trans,

and structures were calculated without explicit hydrogen bond restraints. Initial structures were generated using XPLOR-NIH 2.19.[31d] Starting structures with randomized $\phi$ and $\psi$ angles and extended side chains were generated using an ab initio simulated annealing protocol.[31b] The calculations were performed using the standard force field parameter set (PARALLHDG.PRO) and topology file (TOPALLHDG.PRO). Refinement of structures was achieved using the conjugate gradient Powell algorithm with 1000 cycles of energy minimization and a refined force field based on the program CHARMm.[31c] Structures were visualized with MacPymol (The PyMOL Molecular Graphics System, Schrödinger, LLC).

**Cell Isolation and Culture.** Human peripheral blood mononuclear cells (PBMC) were isolated from buffy coat (obtained from Australian Red Cross Blood Service, Kelvin Grove) using Ficoll-Paque density centrifugation (GE Healthcare Bio-Science, Uppsala, Sweden) and cultured in complete media, consisting of IMDM with 10% FBS, 10 U/mL penicillin, 10 U/mL streptomycin, and 2 mM L-glutamine (Invitrogen at 37 °C, with 5% $CO_2$). Promonocytic U937 cells (cultured in RPMI media with 10% FBS, L-Glu, PenStrep, and NEAA) were pretreated 72 h prior to assay with membrane-permeable cAMP analogue $Bt_2$-cAMP (0.5 mM), which induces cell differentiation to a monocyte/macrophage- or granulocyte-like phenotype. Cellular differentiation induces increased C3aR and C5aR expression on the cell membrane.[32]

**Receptor Binding.** Receptor binding was performed using $^{125}$I-C3a, 80 pM (2200 Ci/mmol, Perkin-Elmer, Torrance, CA), PBMC cells ($15 \times 10^6$ cells/mL) and in the absence or presence of various concentrations of unlabeled C3a or C3a hexapeptide agonist for 60 min at room temperature with shaking in 50 mM Tris, 3 mM $MgCl_2$, 0.1 mM $CaCl_2$, 0.5% (w/v) bovine serum albumin, pH 7.4. Unbound radioactivity was removed by filtration through glass microfiber filters GF/B (Whatman Iner. Ltd., England) which had been soaked in 0.6% polyethylenimine to reduce nonspecific binding. The filter was washed 3 times with cold buffer (50 mM Tris-HCl), pH 7.4. Bound $^{125}$I-C3a was then assessed by scintillation counting on a $\beta$-counter. Specific $^{125}$I-C3a binding is defined as a difference between total binding and nonspecific binding as determined in the presence of 1 $\mu$M unlabeled C3a. The $IC_{50}$ value is the concentration of antagonist to inhibit the binding of labeled ligand by 50%. Nonlinear regression analysis (GraphPad Prism 5) was performed on concentration−response curves to determine $IC_{50}$ and $-\log IC_{50}$. The $-\log IC_{50}$ for each compound was calculated for separate experiments and expressed as an arithmetic mean standard error (SE). $IC_{50}$ values were expressed as a geometric mean.

**Intracellular Calcium Release Assay.** Pretreated U937 cells and culture medium were centrifuged (25 rpm, 5 min). The supernatant was removed, and the cells were resuspended in 2 mL of wash buffer (1× HBSS, 20 mM HEPES, 2.5 mM probenecid, pH 7.4). Cells were counted on a hemocytometer. One dye-loading buffer (12 mL of wash buffer, 1% FBS, 25 $\mu$L of Fluo-3 (final concentration 4 $\mu$M), 25 $\mu$L 20% pluronic acid) was added per 5−7 million cells. Cells were suspended in dye loading buffer, incubated in a covered culture flask for 1 h at 37 °C, then centrifuged (25 rpm, 5 min). The supernatant was removed, and cells were resuspended in 3 mL of wash buffer and centrifuged (25 rpm, 5 min). The process was repeated for cell density of $2 \times 10^6$ cells/mL (or 100 000 cells per well). Then 50 $\mu$L of test compound or buffer or control/well was plated out on sterile black-wall, clear-bottom 96-well plates (Corning Incorporated, NY). Plate was then loaded into the FLUOstar instrument (BMG LabTechnologies, Offenburg, Germany), where fluorescence was measured over 1 min with excitation at 485 nm and emission at 520 nm of Fluo-3 bound $Ca^{2+}$ complex, at 28 °C. Differentiated U937 cells were administered in situ 10 s into the 1 min reading. Agonist responses were expressed as a percentage of calcimycin (A23187) or control compound activity, measured as the maximum change in fluorescence through emission from the Fluo-3 bound calcium complex.

**Statistical Analysis.** For both receptor binding and agonist assays, nonlinear regression was performed using Prism 5 (GraphPad Software, San Diego, CA).

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**Supporting Information Available:** NMR characterization of agonist **55** including variable temperature NMR, a Ramachandran plot, dihedral angles, and ROE restraints; HPLC and HRMS data for all compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

## References

(1) (a) Haas, P. J.; van Strijp, J. Anaphylatoxins: their role in bacterial infection and inflammation. *Immunol. Res.* **2007**, *37*, 161–175. (b) Gerard, N. P.; Gerard, C. Complement in allergy and asthma. *Curr. Opin. Immunol.* **2002**, *14*, 705–708.

(2) (a) Hugli, T. E. Biochemistry and biology of anaphylatoxins. *Complement* **1986**, *3* (3), 111–127. (b) Hugli, T. E. Structure and function of the anaphylatoxins. *Springer Semin. Immunopathol.* **1984**, *7*, 193–219. (c) Hugli, T. E. The structural basis for anaphylatoxin and chemotactic functions of C3a, C4a, and C5a. *Crit. Rev. Immunol.* **1981**, *1*, 321–366. (d) Hugli, T. E.; Muller-Eberhard, H. J. Anaphylatoxins: C3a and C5a. *Adv. Immunol.* **1978**, *26*, 1–53.

(3) (a) Zipfel, P. F.; Skerka, C. Complement regulators and inhibitory proteins. *Nat. Rev. Immunol.* **2009**, *9*, 729–740. (b) Masters, S. L.; Simon, A.; Aksentijevich, I. Horror autoinflammaticus: the molecular pathophysiology of autoinflammatory disease. *Annu. Rev. Immunol.* **2009**, *27*, 621–668.

(4) (a) Monk, P. N.; Scola, A.-M.; Madala, P. K.; Fairlie, D. P. Function, structure and therapeutic potential of complement C5a receptors. *Br. J. Pharmacol.* **2007**, *152*, 429–448. (b) Allegretti, M.; Moriconi, A.; Beccari, A. R.; Di Bitondo, R.; Bizzarri, C.; Bertini, R.; Colotta, F. Targeting C5a: recent advances in drug discovery. *Curr. Med. Chem.* **2005**, *12*, 217–236. (c) Kohl, J. Drug evaluation: the C5a receptor antagonist PMX-53. *Curr. Opin. Mol. Ther.* **2006**, *8*, 529–538. (d) March, D. R.; Proctor, L. M.; Stoermer, M. J.; Sbaglia, R.; Abbenante, G.; Reid, R. C.; Wadi, K.; Paczkowski, N.; Tyndall, J. D. A.; Taylor, S. M.; Fairlie, D. P. potent cyclic antagonists of the complement C5a receptor on human polymorphonuclear leukocytes. relationships between structures and activity. *Mol. Pharmacol.* **2004**, *65*, 868–879. (e) Finch, A. M.; Wong, A. K.; Wadi, S. K.; Paczkowski, N. J.; Fairlie, D. P.; Taylor, S. M. Low molecular weight peptidic and cyclic antagonists of the receptor for the complement factor C5a. *J. Med. Chem.* **1999**, *42*, 1965–1974. (f) Wong, A. K.; Finch, A. M.; Pierens, G. K.; Craik, D. J.; Taylor, S. M.; Fairlie, D. P. Small molecular probes for G-protein-coupled C5a receptors: conformationally constrained antagonists derived from the C terminus of the human plasma protein C5a. *J. Med. Chem.* **1998**, *41*, 3417–3425.

(5) (a) Kirschfink, M. Targeting complement in therapy. *Immunol. Rev.* **2001**, *180*, 177–189. (b) Drouin, S. M.; Corry, D. B.; Kildsgaard, J.; Wetsel, R. A. Cutting edge: the absence of C3 demonstrates a role for complement in Th2 effector functions in a murine model of pulmonary allergy. *J. Immunol.* **2001**, *167*, 4141–4145. (c) Drouin, S. M.; Corry, D. B.; Hollman, T. J.; Kildsgaard, J.; Wetsel, R. A. Absence of the complement anaphylatoxin C3a receptor suppresses Th2 effector functions in a murine model of pulmonary allergy. *J. Immunol.* **2002**, *169*, 5926–5933. (d) Boos, L.; Campbell, I. L.; Ames, R.; Wetsel, R. A.; Barnum, S. R. Deletion of the complement anaphylatoxin C3a receptor attenuates, whereas ectopic expression of C3a in the brain exacerbates, experimental autoimmune encephalomyelitis. *J. Immunol.* **2004**, *173*, 4708–4714. (e) Boos, L.; Szalai, A. J.; Barnum, S. R. C3a expressed in the central nervous system protects against LPS-induced shock. *Neurosci. Lett.* **2005**, *387*, 68–71. (f) Mueller-Ortiz, S. L.; Hollmann, T. J.; Haviland, D. L.; Wetsel, R. A. Ablation of the complement C3a anaphylatoxin receptor causes enhanced killing of Pseudomonas aeruginosa in a mouse model of pneumonia. *Am. J. Physiol.: Lung Cell. Mol. Physiol.* **2006**, *291*, L157–L165. (g) Rahpeymai, Y.; Hietala, M. A.; Wilhelmsson, U.; Fotheringham, A.; Davies, I.; Nilsson, A. K.; Zwirner, J.; Wetsel, R. A.; Gerard, C.; Pekny, M.; Pekna, M. Complement: a novel factor in basal and ischemia-induced neurogenesis. *EMBO J.* **2006**, *25*, 1364–1374.

(6) (a) Kildsgaard, J.; Hollmann, T. J.; Matthews, K. W.; Bian, K.; Murad, F.; Wetsel, R. A. Cutting edge: targeted disruption of the C3a receptor gene demonstrates a novel protective anti-inflammatory role for C3a in endotoxin-shock. *J. Immunol.* **2000**, *165*, 5406–5409. (b) Kawamoto, S.; Yalcindag, A.; Laouini, D.; Brodeur, S.; Bryce, P.; Lu, B.; Humbles, A. A.; Oettgen, H.; Gerard, C.; Geha, R. S. The anaphylatoxin C3a downregulates the Th2 response to epicutaneously introduced antigen. *J. Clin. Invest.* **2004**, *114*, 399–407. (c) Wenderfer, S. E.; Wang, H.; Ke, B.; Wetsel, R. A.; Braun, M. C. C3a receptor deficiency accelerates the onset of renal injury in the MRL/lpr mouse. *Mol. Immunol.* **2009**, *46*, 1397–1404.

(7) (a) Humbles, A. A.; Lu, B.; Nilsson, C. A.; Lilly, C.; Israel, E.; Fujiwara, Y.; Gerard, N. P.; Gerard, C. A role for the C3a anaphylatoxin receptor in the effector phase of asthma. *Nature* **2000**, *406*, 998–1001. (b) Mocco, J.; Mack, W. J.; Ducruet, A. F.; Sosunov, S. A.; Sughrue, M. E.; Hassid, B. G.; Nair, M. N.; Laufer, I.; Komotar, R. J.; Claire, M.; Holland, H.; Pinsky, D. J.; Connolly, E. S., Jr. Complement component C3 mediates inflammatory injury following focal cerebral ischemia. *Circ. Res.* **2006**, *99*, 209–217. (c) Jacob, A.; Bao, L.; Brorson, J.; Quigg, R. J.; Alexander, J. J. C3aR inhibition reduces neurodegeneration in experimental lupus. *Lupus* **2009**, *19*, 73–82. (d) Mizutani, N.; Nabe, T.; Yoshino, S. Complement C3a regulates late asthmatic response and airway hyperresponsiveness in mice. *J. Immunol.* **2009**, *183*, 4039–4046. (e) Mamane, Y.; Chung Chan, C.; Lavallee, G.; Morin, N.; Xu, L. J.; Huang, J.; Gordon, R.; Thomas, W.; Lamb, J.; Schadt, E. E.; Kennedy, B. P.; Mancini, J. A. The C3a anaphylatoxin receptor is a key mediator of insulin resistance and functions by modulating adipose tissue macrophage infiltration and activation. *Diabetes* **2009**, *58*, 2006–2017. (f) Garrett, M. C.; Otten, M. L.; Starke, R. M.; Komotar, R. J.; Magotti, P.; Lambris, J. D.; Rynkowski, M. A.; Connolly, E. S. Synergistic neuroprotective effects of C3a and C5a receptor blockade following intracerebral hemorrhage. *Brain Res.* **2009**, *1298*, 171–177. (g) Tang, Z.; Lu, B.; Hatch, E.; Sacks, S. H.; Sheerin, N. S. C3a mediates epithelial-to-mesenchymal transition in proteinuric nephropathy. *J. Am. Soc. Nephrol.* **2009**, *20*, 593–603. (h) Rynkowski, M. A.; Kim, G. H.; Garrett, M. C.; Zacharia, B. E.; Otten, M. L.; Sosunov, S. A.; Komotar, R. J.; Hassid, B. G.; Ducruet, A. F.; Lambris, J. D.; Connolly, E. S. C3a receptor antagonist attenuates brain injury after intracerebral hemorrhage. *J. Cereb. Blood Flow Metab.* **2009**, *29*, 98–107. (i) Hutamekalin, P; Takeda, K; Tani, M; Tsuga, Y; Ogawa, N; Mizutani, N; Yoshino, S. Effect of the C3a-receptor antagonist SB 290157 on anti-OVA polyclonal antibody-induced arthritis. *J. Pharmacol. Sci.* **2010**, *112*, 56–63.

(8) Malmsten, M.; Schtmdtchen, A. Antimicrobial C3a. Biology, biophysics, and evolution. *Curr. Top. Innate Immun.* **2007**, *598*, 141–158.

(9) Nordahl, E. A.; Rydengard, V.; Nyberg, P.; Nitsche, D. P.; Morgelin, M.; Malmsten, M.; Bjorck, L.; Schmidtchen, A. Activation of the complement system generates antibacterial peptides. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 16879–16884.

(10) Pasupuleti, M.; Walse, B. r.; Svensson, B.; Malmsten, M.; Schmidtchen, A. Rational design of antimicrobial C3a analogues with enhanced effects against Staphylococci using an integrated structure and function-based approach. *Biochemistry* **2008**, *47*, 9057–9070.

(11) Sonesson, A.; Ringstad, L.; Nordahl, E. A.; Malmsten, M.; Morgelin, M.; Schmidtchen, A. Antifungal activity of C3a and C3a-derived peptides against Candida. *Biochim. Biophys. Acta, Biomembr.* **2007**, *1768*, 346–353.

(12) Peterfy, H.; Toth, G.; Pecht, I.; Erdei, A. C3a-derived peptide binds to the type I Fc epsilon R and inhibits proximal-coupling signal processes and cytokine secretion by mast cells. *Int. Immunol.* **2008**, *20*, 1239–1245.

(13) (a) Huber, R.; Scholze, H.; Paques, E. P.; Deisenhofer, J. Crystal structure analysis and molecular model of human C3a anaphylatoxin. *Hoppe-Seyler's Z. Physiol. Chem.* **1980**, *361*, 1389–1399. (b) Nettesheim, D. G.; Edalji, R. P.; Mollinson, K. W.; Greer, J.; Zuiderweg, E. R. P. Secondary structure of complement component C3a anaphylatoxin in solution as determined by NMR spectroscopy: differences between crystal and solution conformations. *Proc. Natl. Acad. Sci. U.S.A.* **1988**, *85*, 5036–5040. (c) Janssen, B. J. C.; Huizinga, E. G.; Raaijmakers, H. C. A.; Roos, A.; Daha, M. R.; Nilsson-Ekdahl, K.; Nilsson, B.; Gros, P. Structures of complement component C3 provide insights into the function and evolution of immunity. *Nature* **2005**, *437*, 505–511.

(14) (a) Zhang, X.; Boyar, W.; Toth, M. J; Wennogle, L.; Gonnella, N. C. Structural definition of the C5a C terminus by two-dimensional nuclear magnetic resonance spectroscopy. *Proteins* **1997**, *28*, 261–267. (b) Zuiderweg, E. R.; Fesik, S. W. Heteronuclear three-dimensional NMR spectroscopy of the inflammatory protein C5a. *Biochemistry* **1989**, *28*, 2387–2391. (c) Zuiderweg, E. R.; Nettesheim, D. G.; Mollison, K. W.; Carter, G. W. Tertiary structure of human complement component C5a in solution from nuclear magnetic resonance data. *Biochemistry* **1989**, *28*, 172–185.

(15) (a) Hoeprich, P. D.; Hugli, T. E. Helical conformation at the carboxy-terminal portion of C3a is required for full activity. *Biochemistry* **1986**, *25*, 1945. (b) Ember, J. A.; Hugli, T. E. Complement factors and their receptors. *Immunopharmacology* **1997**, *38* (1−2), 3–15.

(16) (a) Huey, R.; Erickson, B. W.; Bloor, C. M.; Hugli, T. E. Contraction of guinea pig lung by synthetic oligopeptides related to human C3a. *Immunopharmacology* **1984**, *8*, 37–45. (b) Hoeprich, P. D., Jr.; Hugli, T. E. Helical conformation at the carboxy-terminal portion of human C3a is required for full activity. *Biochemistry* **1986**, *25*, 1945–1950. (c) Ember, J. A.; Johansen, N. L.; Hugli, T. E. Designing synthetic superagonists of C3a anaphylatoxin. *Biochemistry* **1991**, *3*, 3603–3612.

(17) (a) Hugli, T. E.; Erickson, B. W. Synthetic peptides with the biological activities and specificity of human C3a anaphylatoxin. *Proc. Natl. Acad. Sci. U.S.A.* **1977**, *74*, 1826–1830. (b) Caporale, L. H.; Tippett, P. S.; Erickson, B. W.; Hugli, T. E. The active site of C3a anaphylatoxin. *J. Biol. Chem.* **1980**, *255*, 10758–10763. (c) Unson, C. G.; Erickson, B. W.; Hugli, T. E. Active site of C3a anaphylatoxin: contributions of the lipophilic and orienting residues. *Biochemistry* **1984**, *23*, 585–589. (d) Gerardy-Schahn, R.; Ambrosius, D.; Casaretto, M.; Grotzinger, J.; Saunders, D.; Wollmer, A.; Brandenburg, D.; Bitter-Suermann, D. Design and biological activity of a new generation of synthetic C3a analogues by combination of peptidic and non-peptidic elements. *Biochem. J.* **1988**, *255*, 209–216. (e) Ambrosius, D.; Casaretto, M.; Gerardy-Schahn, R.; Saunders, D.; Brandenburg, D.; Zahn, H. Peptide analogues of the anaphylatoxin C3a; syntheses and properties. *Biol. Chem. Hoppe-Seyler* **1989**, *370*, 217–227. (f) Kohl, J.; Casaretto, M.; Gier, M.; Karwath, G.; Gietz, C.; Bautsch, W.; Saunders, D.; Bitter-Suermann, D. Reevaluation of the C3a active site using short synthetic C3a analogues. *Eur. J. Immunol.* **1990**, *20*, 1463–1468. (g) Kretzschmar, T.; Pohl, M.; Casaretto, M.; Przewosny, M.; Bautsch, W.; Klos, A.; Saunders, D.; Kohl, J. Synthetic peptides as antagonists of the anaphylatoxin C3a. *Eur. J. Biochem.* **1992**, *210*, 185–191. (h) Pohl, M.; Ambrosius, D.; Grotzinger, J.; Kretzschmar, T.; Saunders, D.; Wollmer, A.; Brandenburg, D.; Bitter-Suermann, D.; Hocker, H. Cyclic disulfide analogues of the complement component C3a. Synthesis and conformational investigations. *Int. J. Pept. Protein Res.* **1993**, *41*, 362–375. (i) Takahashi, M.; Moriguchi, S.; Ikeno, M.; Kono, S.; Ohata, K.; Usui, H.; Kurahashi, K.; Sasaki, R.; Yoshikawa, M. Studies on the ileum-contracting mechanisms and identification as a complement C3a receptor agonist of oryzatensin, a bioactive peptide derived from rice albumin. *Peptides* **1996**, *17*, 5–12. (j) Jinsmaa, Y.; Takenaka, Y.; Yoshikawa, M. Designing of an orally active complement C3a agonist peptide with anti-analgesic and anti-amnesic activity. *Peptides* **2001**, *22*, 25–32. (k) Lu, Z. X.; Fok, K. F.; Erickson, B. W.; Hugli, T. E. Conformational analysis of COOH-terminal segments of human C3a. Evidence of ordered conformation in an active 21-residue peptide. *J. Biol. Chem.* **1984**, *259*, 7367–7370.

(18) Mousli, M.; Hugli, T. E.; Landry, Y.; Bronner, C. A mechanism of action for anaphylatoxin C3a stimulation of mast cells. *J. Immunol.* **1992**, *148*, 2456–2461.

(19) Federwisch, M.; Casaretto, M.; Gerardy-Schahn, R.; Bitter-Suermann, D.; Wollmer, A. Enhanced biopotency of synthetic C3a analogues by membrane binding. A fluorescence anisotropy decay study. *Biophys. Chem.* **1992**, *44*, 151–161.

(20) Fukuoka, Y.; Ember, J. A.; Hugli, T. E. Molecular cloning of two isoforms of the guinea pig C3a anaphylatoxin receptor: alternative splicing in the large extracellular loop. *J. Immunol.* **1998**, *161*, 2977–2984.

(21) Woodruff, T. M.; Strachan, A. J.; Sanderson, S.; Monk, P. N.; Wong, A. K.; Fairlie, D. P.; Taylor, S. M. Species dependence for binding of small molecule agonists and antagonists of the C5a receptor on polymorphonuclear leukocytes. *Inflammation* **2001**, *25*, 171–177.

(22) (a) Ames, R. S.; Lee, D.; Foley, J. J.; Jurewicz, A. J.; Tornetta, M. A.; Bautsch, W.; Settmacher, B.; Klos, A.; Erhard, K. F.; Cousins, R. D.; Sulpizio, A. C.; Hieble, J. P.; McCafferty, G.; Ward, K. W.; Adams, J. L.; Bondinell, W. E.; Underwood, D. C.; Osborn, R. R.; Badger, A. M.; Sarau, H. M. Identification of a selective nonpeptide antagonist of the anaphylatoxin C3a receptor that demonstrates antiinflammatory activity in animal models. *J. Immunol.* **2001**, *166*, 6341–6348. (b) Therien, A. G. Agonist activity of the small molecule C3aR ligand SB 290157. *J. Immunol.* **2005**, *174*, 7479–7480. (c) Mathieu, M. C.; Sawyer, N.; Greig, G. M.; Hamel, M.; Kargman, S.; Ducharme, Y.; Lau, C. K.; Friesen, R. W.; O'Neill, G. P.; Gervais, F. G.; Therien, A. G. The C3a receptor antagonist SB 290157 has agonist activity. *Immunol. Lett.* **2005**, *100*, 139–145. (d) Proctor, L. M.; Arumugam, T. V.; Shiels, I.; Reid, R. C.; Fairlie, D. P.; Taylor, S. M. Comparative anti-inflammatory activities of antagonists to C3a

and C5a receptors in a rat model of intestinal ischaemia/reperfusion injury. *Br. J. Pharmacol.* **2004**, *142*, 756–764.
(23) Grant, E. B.; Guiadeen, D.; Singer, M.; Argentieri, D.; Hlasta, D. J.; Wachter, M. Design, synthesis, and biological activity of diiminoisoindolines as complement component 3a antagonists. *Bioorg. Med. Chem. Lett.* **2001**, *11*, 2817–2820.
(24) Claffey, M. M.; Goldstein, S.; Jung, S.; Nagel, A.; Shulze, V. Biphenylimidazole Compounds as C3a Receptor Antagonists. WO 2007034282, 2007.
(25) (a) Denonne, F.; Binet, S.; Burton, M.; Collart, P.; Dipesa, A.; Ganguly, T.; Giannaras, A.; Kumar, S.; Lewis, T.; Maounis, F.; Nicolas, J. M.; Mansley, T.; Pasau, P.; Preda, D.; Stebbins, K.; Volosov, A.; Zou, D. Discovery of new C3aR ligands. Part 1: Arginine derivatives. *Bioorg. Med. Chem. Lett.* **2007**, *17*, 3258–3261. (b) Denonne, F.; Binet, S.; Burton, M.; Collart, P.; Defays, S.; Dipesa, A.; Eckert, M.; Giannaras, A.; Kumar, S.; Levine, B.; Nicolas, J. M.; Pasau, P.; Pegurier, C.; Preda, D.; Van houtvin, N.; Volosov, A.; Zou, D. Discovery of new C3aR ligands. Part 2: Amino-piperidine derivatives. *Bioorg. Med. Chem. Lett.* **2007**, *17*, 3262–3265.
(26) (a) Sanderson, S. D.; Kirnarsky, L.; Sherman, S. A.; Ember, J. A.; Finch, A. M.; Taylor, S. M. Decapeptide agonists of human C5a: the relationship between conformation and spasmogenic and platelet aggregatory activities. *J. Med. Chem.* **1994**, *37*, 3171–3180. (b) Kawatsu, R.; Sanderson, S. D.; Blanco, I.; Kendall, N.; Finch, A. M.; Taylor, S. M.; Colcher, D. Conformationally biased analogs of human C5a mediate changes in vascular permeability. *J. Pharmacol. Exp. Ther.* **1996**, *278*, 432–440. (c) Vogen, S. M.; Paczkowski, N. J.; Kirnarsky, L.; Short, A.; Whitmore, J. B.; Sherman, S. A.; Taylor, S. M.; Sanderson, S. D. Differential activities of decapeptide agonists of human C5a: the conformational effects of backbone N-methylation. *Int. Immunopharmacol.* **2001**, *1*, 2151–2162. (d) Taylor, S. M.; Sherman, S. A.; Kirnarsky, L.; Sanderson, S. D. Development of response-selective agonists of human C5a anaphylatoxin: conformational, biological, and therapeutic considerations. *Curr. Med. Chem.* **2001**, *8*, 675–684.
(27) (a) Kawai, M.; Quincy, D. A.; Lane, B.; Mollison, K. W.; Luly, J. R; Carter, G. W. Identification and synthesis of a receptor binding site of human anaphylatoxin C5a. *J. Med. Chem.* **1991**, *34*, 2068–2071. (b) Kawai, M.; Wiedeman, P. E.; Luly, J. R; Or, Y. S. New Hexa- and Heptapeptides Are Anaphylatoxin Antagonists and Agonists—For Treating Inflammatory and Immunodeficiency Diseases, Cancers and Severe Infections. WO 9212168-A1, 1992 (c) Drapeau, G.; Brochu, S.; Godin, D.; Levesque, L.; Rioux, F.; Marceau, F. Synthetic C5a receptor agonists. Pharmacology, metabolism and in vivo cardiovascular and hematologic effects. *Biochem. Pharmacol.* **1993**, *45*, 1289–1299. (d) Konteatis, Z. D.; Siciliano, S. J.; Van Riper, G.; Molineaux, C. J.; Pandya, S.; Fischer, P.; Rosen, H.; Mumford, R. A.; Springer, M. S. Development of C5a receptor antagonists. Differential loss of functional responses. *J. Immunol.* **1994**, *153*, 4200–4205.
(28) Nomenclature used here for peptides of length *X*: the C-terminal residue is termed P1; the second is P2 up to the N-terminus which is called P*X*.
(29) (a) Baldo, A.; Sniderman, A. D.; Stluce, S.; Avramoglu, R. K.; Maslowska, M.; Hoang, B.; Monge, J. C.; Bell, A.; Mulay, S.; Cianflone, K. The adipsin acylation stimulating protein system and regulation of intracellular triglyceride synthesis. *J. Clin. Invest.* **1993**, *92*, 1543–1547. (b) Maslowska, M.; Cianfione, K.; Rosenbloom, M. Acylation stimulating protein (ASP): role in adipose tissue. *Prog. Obes. Res.* **1999**, 65–70.
(30) (a) Tyndall, J. D.; Pfeiffer, B.; Abbenante, G.; Fairlie, D. P. Over one hundred peptide-activated G protein-coupled receptors recognize ligands with turn structure. *Chem. Rev.* **2005**, *105*, 793–826. (b) Ruiz-Gomez, G.; Tyndall, J. D.; Pfeiffer, B.; Abbenante, G.; Fairlie, D. P. UPDATE 1: Over one hundred peptide-activated G protein-coupled receptors recognize ligands with turn structure. *Chem. Rev.* DOI: 10.1021/cr900344w, in press. (c) Fairlie, D. P.; Abbenate, G.; March, D. R. Macrocyclic peptidomimetics—forcing peptides into bioactive conformations. *Curr. Med.Chem.* **1995**, *2*, 655–686.
(31) (a) Wüthrich, K.; Billeter, M.; Braun, W. *J. Mol. Biol.* **1983**, *169*, 949–961. (b) Nilges, M.; Gronenborn, A. M.; Brünger, A. T.; Clore, G. M. *Protein Eng.* **1988**, *2*, 27–38. (c) Brooks, B. R.; Bruccoleri, R. E.; Olafson, B. D.; States, D. J.; Swaminathan, S.; Karplus, M. *J. Comput. Chem.* **1983**, *4*, 187–217. (d) Schwieters, C. D.; Kuszewski, J. J.; Clore, G. M. Using Xplor-NIH for NMR molecular structure determination. *Prog. Nucl. Magn. Reson. Spectrosc.* **2006**, *48*, 47–62.
(32) (a) Burg, M.; Martin, U.; Bock, D.; Rheinheimer, C.; Kohl, J.; Bautsch, W.; Klos, A. Differential regulation of the C3a and C5a receptors (CD88) by IFN-gamma and PMA in U937 cells and related myeloblastic cell lines. *J. Immunol.* **1996**, *157*, 5574–5581. (b) Klos, A.; Bank, S.; Gietz, C.; Bautsch, W.; Kohl, J.; Burg, M.; Kretzschmar, T. C3a receptor on dibutyryl-cAMP-differentiated U937 cells and human neutrophils: the human C3a receptor characterized by functional responses and $^{125}$I-C3a binding. *Biochemistry* **1992**, *31*, 11274–11282.