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Identification of a new class of small molecule C5a receptor antagonists

Jack J. Chen [a,*], Derek C. Cole [a], Greg Ciszewski [a], Kimberly Crouse [b], John W. Ellingboe [a], Pawel Nowak [a], Gregory J. Tawa [c], Gabriel Berstein [b], Wei Li [b]

[a] Chemical Sciences, Wyeth Research, Pearl River, NY 10956, USA
[b] Inflammation Research, Wyeth Research, Cambridge, MA 02140, USA
[c] Structural Biology, Wyeth Research, Princeton, NJ 08543-8000, USA

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ABSTRACT

C5a is a terminal product of the complement cascade that activates and attracts inflammatory cells including granulocytes, mast cells and macrophages via a specific GPCR, the C5a receptor (C5aR). Inhibition of C5a/C5aR interaction has been shown to be efficacious in several animal models of autoimmune diseases, including RA, SLE and asthma. This account reports the discovery of a new class of C5aR antagonists through high-throughput screening. The lead compounds in this series are selective and block C5a binding, C5a-promoted calcium flux in human neutrophils with nanomolar potency.

The anaphylatoxin C5a is a 74 amino acid glycopeptide produced by complement activation under inflammatory conditions,[1] or via complement cascade-independent mechanisms in certain autoimmune disease models.[2,3] C5a interacts with the C5a receptor (C5aR),[4] a 52 KD cell surface G-protein coupled receptor (GPCR) present in common inflammatory cells to induce a series of innate and adaptive immunological responses including (i) chemotaxis and respiratory burst of neutrophils with up-regulation of CD11b expression; (ii) chemotaxis and degranulation of basophils and mast cells; (iii) production of GMCSF and IL-8 by eosinophils; (iv) chemotaxis of monocytes and macrophages with up-regulation of the activating FcRIII and downregulation of the inhibitory FcRIIB; (v) myeloid and plasmacytoid dendritic cell balance in the lung; and (vi) enhancement of proliferation, differentiation and viability in T-cells.[3,5–10]

The pro-inflammatory nature of C5a/C5aR interaction essentially implicates the pathogenic role of C5aR in acute and chronic inflammatory disorders such as psoriasis, asthma, bullous pemphigoid, and rheumatoid arthritis. In fact, patients with these conditions often have elevated C5aR level in their diseased tissues.[11–13] Pharmacological and genetic studies have also shown that blockade or deficiency of C5aR has protective effects in animal models of systemic lupus erythematosus, rheumatoid arthritis, antiphospholipid syndrome and asthma.[14–19] Thus, there is significant interest in identification of C5aR antagonists as therapeutic agents for these autoimmune diseases, and several peptidomimetic and small molecule antagonists have been reported (Fig. 1).[20–23]

To discover novel C5aR small molecule antagonists, we carried out a high-throughput screen (HTS) using recombinant CHO cells stably transfected with human C5aR and Gα16, and recorded C5a-dependent intracellular calcium mobilization on a fluorescent imaging plate reader (FLIPR). Compounds with over 30% inhibition

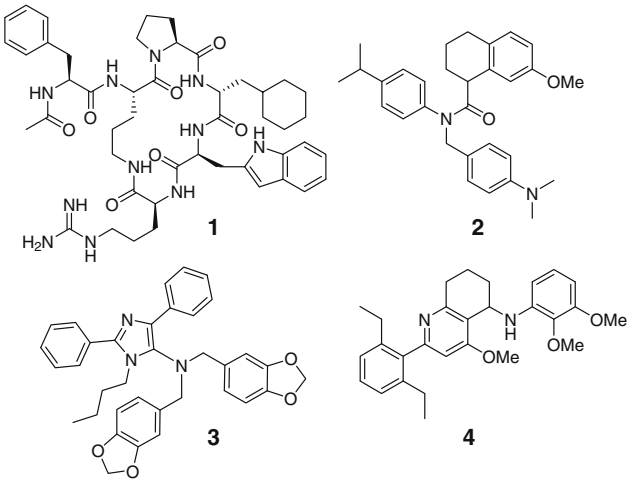

Figure 1. Peptidomimetic and small molecule C5aR antagonists.

* Corresponding author. Tel.: +1 845 602 3470; fax: +1 845 474 4202.
E-mail address: chenj24@wyeth.com (J.J. Chen).

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at 10 μM concentration were structurally prioritized and subjected to IC_{50} determination. The confirmed hits were further characterized in a radioligand binding assay to measure their binding affinity.

This process led to identification of bis-sulfonamide **5** as a potent and selective C5aR antagonist. Substructure and similarity searches were performed to investigate the SAR related to the structure. The results are illustrated in Table 1.

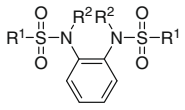
The study revealed constrained SAR around the terminal methyl groups of the tosyl moiety as their removal rendering complete loss of activity (entry **6**). The two *N*-methyl groups could be homologated (entry **7**), but cyclizing them with linkers of various lengths caused complete loss of activity (entries **8** and **9**). Connection of the two tosyl units through piperazine (**10**) or bisphenyl-diaminopropane (**11**) (Fig. 2) was attempted as illustrated by Figure 2, but did not produce active molecules.

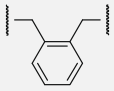
A low energy pharmacophore model (Fig. 3) suggested the possibility of replacing one of the sulfonamide moieties to minimize the symmetric nature of the lead molecule.[26] A series of amide and benzophone sulfonamides were therefore synthesized using the scheme outlined in Scheme 1 (Table 2). Sulfonylation of *N*-methyl anthranilic acid **12** yielded the intermediate **13** which was then converted to the indoline amide **14**, or alternatively benzophone **16** in two steps via the Weinreb amide **15**.

Replacement of the tolyl sulfonamide with an indoline amide (entry **14**) led to loss of binding and FLIPR activity. The activity was restored in thienyl ketone **17**, suggesting the lipophilic preference of pharmacophores in this region. Halogenations of the central phenyl ring resulted in much weaker antagonists (entries **15** and **16**). And substitution of the aryl ketone with acetyl group led to loss of activity (entry **20**).

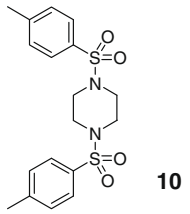
Three lead compounds were further characterized for functional activity in primary cells, mouse C5a receptor cross-reactivity, and selectivity relative to other GPCRs (Table 3). Potency in FLIPR assays correlated well between CHO cells and primary human neutrophils (PMN), indicating that compound activity on recombinant C5aR translated to the naturally expressed receptor. Consistent with this observation, **5** and **7** blocked C5a-promoted chemotaxis

Table 1
Binding affinity and functional activity of bis-sulfonamide analogs[a]



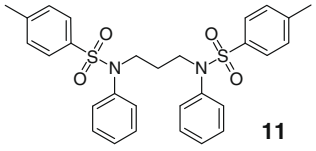
Entry	R^1	R^2	CHO-cell FLIPR IC_{50}[24] (μM)	CHO cell binding IC_{50}[25] (μM)
5	*p*-Tol	Me	0.2	0.2
6	Ph	Me	>60	>60
7	*p*-Tol	CH_2=CH–CH_2–	0.3	0.3
8	*p*-Tol	–CH_2CH_2–	>60	>60
9	*p*-Tol		>60	>60

[a] All active compounds showed full antagonism and were inactive as C5aR agonists with EC_{50} >60 μM.



10

FLIPR IC_{50} (μM) >60



11

FLIPR IC_{50} (μM) >60

Figure 2. Bis-sulfonamides with variations of the benzo backbone.

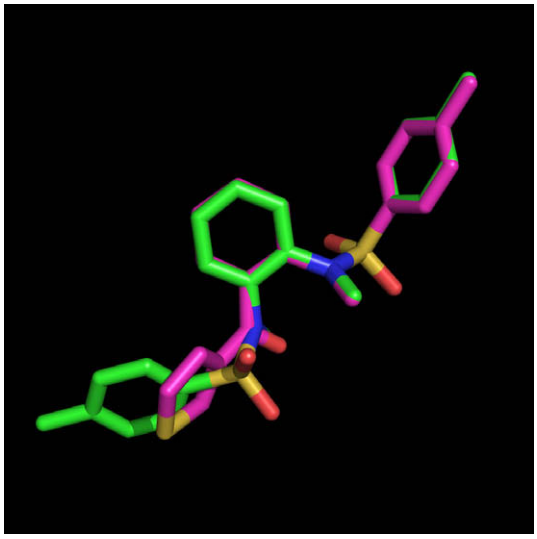
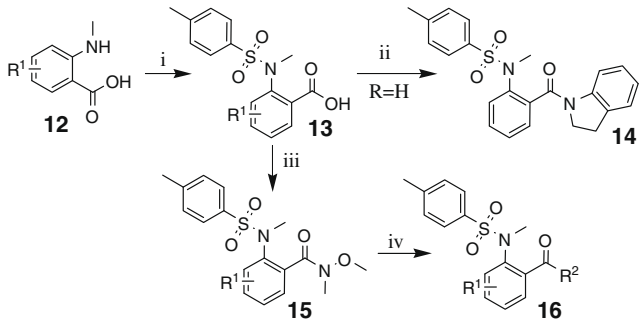
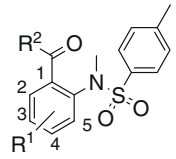


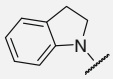
Figure 3. Superimposition of bis-sulfonamide **5** (green) with a benzophone sulfonamide **17** (purple).



Scheme 1. Reagents and conditions: (i) tosyl chloride, Na_2CO_3, water; (ii) oxalyl chloride, indoline; (iii) oxalyl chloride, CH_3NHOCH_3·HCl, TEA, DCM; (iv) aryl bromide, *n*-BuLi, THF, −78 °C.

Table 2
Activity of benzophone sulfonamide analogs[a]



Entry	R^1	R^2	CHO-cell FLIPR IC_{50}[24] (μM)	CHO cell binding IC_{50}[25] (μM)
14	H		38.3	44.5
17	H		0.6	1.7
18	3-Cl	Ph	>60	8.7
19	3-Br	Ph	>60	14.6
20	H	Me	>60	>60

[a] All active compounds showed full antagonism and were inactive as C5aR agonists with EC_{50} >60 μM.

Table 3
Functional activity of lead compounds in various cell lines

Assay	5	7	17
PMN FLIPR IC_{50}[27] (μM)	0.067	0.059	0.4
Human PMN chemotaxis[27] (μM)	5.7	2.5	>60
Human PMN Shape Change, whole blood[28] (μM)	27		>60
RAW cell FLIPR IC_{50}[29] (μM)	>60	>60	3.1
CXCR3 FLIPR IC_{50} (μM)	>60	>60	>60
CXCR4 FLIPR IC_{50} (μM)	>60	>60	>60
P2Y2 FLIPR IC_{50} (μM)	>60	>60	>60

in human PMN. Furthermore, compound **5** also inhibited C5a-mediated human PMN shape change in whole blood. The diminished potency of the compounds in these assays may be due to longer incubation time in the chemotaxis assay and non-specific binding of compounds to serum proteins in the whole blood assay. In contrast to the lack of rodent cross-reactivity reported for other C5aR small molecule antagonists,[20–23] compound **17** showed antagonistic activity for the mouse receptor (RAW cells) which would enable in vivo studies. All three compounds were founded inactive towards other GPCRs, such as human lymphocyte CXCR3 and CXCR4 in human T cells, and P2Y2 in CHO cells, suggesting that these compounds are selective C5aR antagonists.

In summary, a new class of small molecular C5a antagonists has been identified through HTS and a hit-to-lead process. The lead molecules exhibit promising binding and functional activities with human and mouse cell lines. Further optimization of this series will be the subject of future communication.

Acknowledgements

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24. FLIPR assay conditions: CHO-Gα16-hC5aR cells were seeded at 15,000 cells/well in 384 well black clear bottom TC plates (BD 353962) in media (R1 (invitrogen) + 1% FBS + 1 mM L-glutamine) 24 h before the assay. On the day of experiment, cells were washed and loaded with Ca3 dye (molecular devices R8090) for 2 h (1 h 37 °C followed by 1 h at rt). Compounds were added to the cells by FLIPR3 (molecular devices). After 10 min incubation with compound, hC5a was added at the concentration of 2 × EC_{50} (0.1 nM). C5a induced intracellular calcium accumulation was recorded on FLIPR3.
25. *Binding assay conditions*: CHO-Gα16-C5a cells were seeded in growth media (R1 + 10% FBS + 1 mM L-glutamine) at 50,000/well in 96 well white-clear bottom TC plate (Perkin–Elmer 1450-517) the day before the assay. On the day of experiment, culture media was replaced by assay buffer (1 × HANKS + 20 mM HEPES + 0.1% BSA). Compounds were then added to the cells followed by 10 min incubation at rt. ^{125}I-hC5a (0.6 nM) (Perkin–Elmer, NEX250) was then added to the cells. After 1 h incubation at room temp, the cell plate was washed eight times with wash buffer (PBS + 1 mM $CaCl_2$ + 0.1% BSA) on a harvester (Packard). Excess buffer was removed by blotting with paper. 40 ml of Microscint 40 (Perkin–Elmer) was added to each well. The plates were then counted on Trilux (Perkin–Elmer).
26. To create the alignment, each compound was expanded into multiple low conformations using OMEGA (OpenEye Scientific Software, Santa Fe, NM. http://www.eyesopen.com). Then pairwise alignments between the conformations of (**5**) and the conformations of (**17**) were constructed using ROCS (OpenEye Scientific Software). The alignment with the most consistent molecular shape and 3D chemistry orientation was chosen for analysis.
27. *Neutrophil assay conditions*: Human neutrophils (PMN) were purified from human whole blood using a Ficoll gradient protocol. The cells were then re-suspended in 1 × Ca3 dye and seeded at 50,000 cells/well in 384 well black clear bottom TC plates (BD 353962) in assay buffer (1 × HANKS + 20 mM HEPES + 0.1% BSA). After 1 hr incubation at 37 °C, 5% CO_2, Compounds were added to the cells by FLIPR3. 10 nM hC5a was added after 10 min incubation with compound. C5a induced intracellular calcium accumulation was recorded on FLIPR3.
28. *Human PMN shape change assay conditions*: Compounds were added to fresh, EDTA-anticoagulated human whole blood from healthy donors. After 10 min incubation at room temperature, 10 nM C5a was added and the mixture was incubated at 37 °C for 10 min. Then 250 μl ice-cold 1% (v/v) paraformaldehyde was added to fix cells, followed by addition of ice-cold hypotonic buffer and incubation on ice for 40 min to lyse red blood cells. Neutrophils were identified and gated by flow cytometry on FACSCalibur (Becton Dickinson) using forward and side scatter. Increase in neutrophil autofluorescence forward scatter on FL2-H channel, which reflects shape change of neutrophils due to cytoskeletal rearrangement stimulated by the C5a chemoattractant, was measured by Flowjo software.
29. *RAW assay conditions*: RAW264.7 cells were seeded at 50,000 cells/well in 384 well black clear bottom TC plates (BD 353962) in media (DME (invitrogen) + 1% FBS + 2 mM L-glutamine) the day before. On the day of experiment, cells were washed and loaded with Ca3 dye for 1 h at 37 °C. Compounds were added to the cells by FLIPR3. After 10 min incubation with compound, mC5a was added at the concentration of 2 × EC_{50} (10 nM). C5a induced intracellular calcium accumulation was recorded on FLIPR3.