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Potent Thiophene Antagonists of Human Complement C3a Receptor with Anti-Inflammatory Activity

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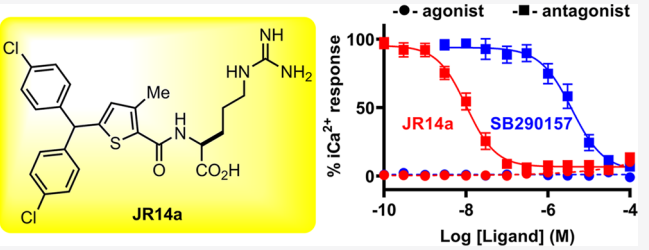
Supporting Information

ABSTRACT: Structure−activity relationships for a series of small-molecule thiophenes resulted in potent and selective antagonism of human Complement C3a receptor. The compounds are about 100-fold more potent than the most reported antagonist SB290157. A new compound JR14a was among the most potent of the new antagonists in vitro, assessed by (a) inhibition of intracellular calcium release (IC_{50} 10 nM) induced in human monocyte-derived macrophages by 100 nM C3a, (b) inhibition of β-hexosaminidase secretion (IC_{50} 8 nM) from human LAD2 mast cells degranulated by 100 nM C3a, and (c) selectivity for human C3aR over C5aR. JR14a was metabolically stable in rat plasma and in rat liver microsomes and efficacious in rats when given orally to suppress rat paw inflammation, macrophage and mast cell activation, and histopathology induced by intraplantar paw administration of a C3aR agonist. Potent C3aR antagonists are now available for interrogating C3a receptor activation and suppressing C3aR-mediated inflammation in mammalian physiology and disease.



INTRODUCTION

Complement is a network of over 40 immunological proteins that supplement the actions of antibodies and immune cells in detecting, lysing, and removing microbes and infected/damaged cells from an organism.[1] The Complement system is activated[1−3] by diverse stimuli, including pathogens, antigen−antibody complexes, carbohydrate-binding lectins, proteolytic enzymes, as well as foreign surfaces, chemical and physical injury, radiation, and neoplasia. These stimuli catalyze complex multipathway cascades involving protein cleavages by serine proteases generated by Complement activation (and by other proteolytic cascades), culminating in inflammation, recruitment of immune cells, tagging of microorganisms and infected or damaged cells, and assembly of a conglomerate of Complement proteins into a pore-forming membrane attack complex (MAC) that induces cell lysis. A pivotal protein in the Complement cascade is C3,[4] an intermediate at the intersection of multiple Complement activation pathways. This protein is cleaved into a small anaphylatoxin C3a and a larger fragment C3b. The human Complement protein C3a causes chemotaxis and degranulation of granulocytes and phagocytes (e.g., neutrophils, monocytes, macrophages, eosinophils, mast cells, and dendritic cells).[5] Human C3a binds to and activates a discrete G-protein-coupled receptor (C3aR) expressed on innate immune and other cell types.[1,2] Activation of this receptor stimulates important immune and metabolic responses and is now believed to play important pathological roles in inflammatory and metabolic diseases[6,7] and cancers.[8] The development of potent, selective, and bioavailable small-molecule ligands for C3aR could lead to a new therapy for treating inflammation-driven diseases, including respiratory, metabolic, cardiovascular, gastrointestinal, central nervous system (CNS), neurodegenerative diseases, and cancers.[9]

We have previously downsized the 77-residue C3a protein to equipotent hexapeptides[10] as well as small-molecule agonists (Figure 1) featuring a heterocyclic constraint, such as oxazole **1** (EC_{50} 7 nM, Ca^{2+}, human monocyte-derived macrophage (HMDM))[5,11] or imidazole **2** (BR103, EC_{50} 15 nM, Ca^{2+}, HMDM).[12] These agonists were equipotent with, and displayed the same functional properties as, human C3a across multiple reporter and signaling cellular assays.[5,13] Unlike C3a, which degrades in a few minutes in vivo, these agonists were stable to proteases and metabolism. The first reported C3aR antagonist, and most important to date, was SB290157 (**3**, Figure 1)[14] that was found to inhibit C3a-induced Ca^{2+} release in human monocyte-derived macrophages with IC_{50} 1.3 μM.[12] A few analogues incorporating a heterocycle, such as furan **4**, oxazole **5**, or thiazole **6**, are reported to be weak antagonists of C3aR with inhibitory activity at micromolar concentrations.[15−17]

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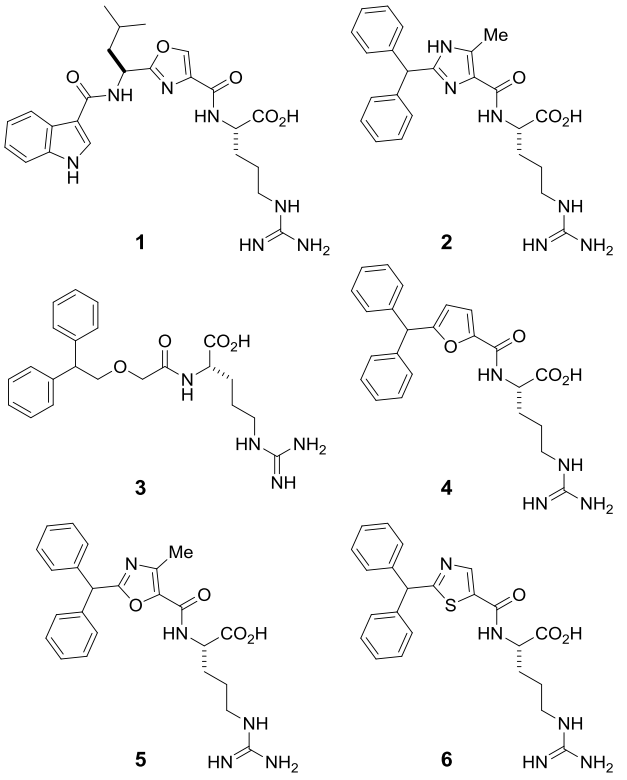

Figure 1. Agonists (**1**, **2**) and antagonists (**3**–**6**) of Complement C3a receptor (C3aR).

RESULTS

The partial charges on the heterocyclic nitrogen atoms of the oxazole in **1** and the imidazole in **2** were calculated using Gaussian[18,19] (Figure 2), with significant partial negative

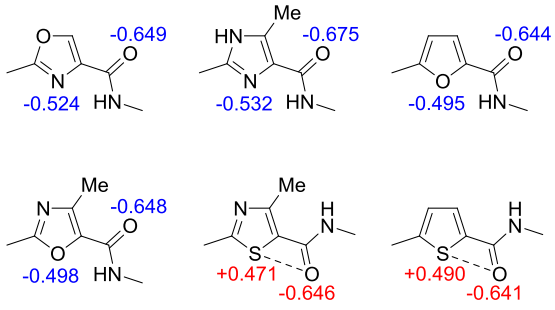

Figure 2. Partial charges calculated (Gaussian 16, NBO 7.0)[18,19] for heteroatoms of heterocycles and an adjacent amide carbonyl oxygen, with expected amide conformation due to electrostatic repulsion (blue) or attraction (red).

electrostatic charge associated with the nitrogen atom common to these two heterocycles. We have previously shown that this nitrogen atom is a hydrogen-bond acceptor that is important for C3aR receptor affinity and agonist activity.[5,12] The furan in **4** and oxazole in **5** also have partial negative charge on the oxygen heteroatom, and both of these oxygens have some, albeit weak, hydrogen-bond-accepting properties.[12,20] In contrast, a thiazole (e.g., **6**) or thiophene heterocycle has a partial positive charge on the sulfur atom (Figure 2) that should prevent it from being a hydrogen-bond acceptor, and thus such compounds are less likely to be C3aR agonists.[21,22] Here, we report the development of a series of thiophene analogues of thiazole **6** as prospective C3aR antagonists and evaluate their function in human innate immune cells (macrophages, mast cells) known to express the C3a receptor, as well as their potential anti-inflammatory activity in a model of C3aR agonist-induced paw inflammation in rats.

Synthesis of Thiophenes and Structure–Activity Relationships. We synthesized two series of thiophene carboxamides **10a–o** and **14a–k** (Scheme 1, Supporting Information), purified by rpHPLC and characterized by ^{1}H and ^{13}C NMR spectroscopy and mass spectrometry (Supporting Information), and evaluated their agonist and antagonist activities on human macrophages (Table 1, Supporting Figures 1–4). Based on our earlier studies[5,12,13] with small-molecule ligands such as **1**–**6**, an arginine residue contributes significant binding affinity for C3aR, and so this residue was not modified in this study. Antagonist **3** also comprises an arginine that is connected to a diphenylmethyl substituent via a very flexible linker. We sought to restrict this flexibility by introducing thiophenes, resulting in C3aR antagonists (**10a–o**). We have disclosed[13] **10a** as an antagonist, measured by Ca^{2+} mobilization in human monocyte-derived macrophages (HMDM; IC_{50} 85 nM against 100 nM C3a). It represented a 15-fold increase in antagonist potency relative to **3** (SB290157) and a 7-fold increase relative to thiazole **6** under the same assay conditions (Table 1). This suggested that the potential hydrogen bond-accepting thiazole nitrogen present in antagonist (**6**) is not required for antagonist activity, and we suspected that it might even be detrimental for antagonist activity. Antagonist **10a** and agonist **2** compete in a concentration-dependent manner with a fluorescent form of the C3a protein,[23] Eu-DTPA-C3a (IC_{50} 2 nM), for binding to HEK293-Gα_{16}-C3aR transfected human cells (Supporting Figure 5), supporting direct interaction with the target receptor human C3aR. Importantly, while **10a** has no agonist activity below 10 μM, we do find that it has some agonist activity at higher μM concentrations tested herein (Supporting Figure 1–4). From this baseline, we now develop structure–activity relationships to identify improved thiophene derivatives as potential C3aR antagonists with minimal or no agonist activity.

We modified the 5-diphenylmethyl moiety in **10a** to explore structure–activity relationships. Compounds (**10a–o**, Scheme 1a) were synthesized via a common route. The 5-position of ethyl 2-thiophenecarboxylate **7a** was deprotonated using lithium diisopropylamide (LDA), then benzophenone derivatives or other ketones were added under cooled, anhydrous conditions. The resulting tertiary alcohols (**8a–o**) were then dehydroxylated under ionic hydrogenation conditions using triethylsilane and trifluoroacetic acid to afford **9a–o**. These reductions proceeded rapidly for diaryl derivatives **8a**, **8h**, and **8l–o** because the reaction proceeds through the stabilized doubly benzylic carbocation intermediate. However, analogues **8b–g** and **8i–k** containing alkyl groups adjacent to the hydroxyl required longer reaction times and, in the case of **8e**, the expected phenyl isopropyl methyl group was not obtained. Instead, elimination gave the stable tetrasubstituted alkene **9e** that was isolated and carried forward. The 2-pyridyl analogue **8g** reacted extremely slowly, presumably because formation of the required carbonium-ion intermediate was impeded by the existing positive charge on the adjacent pyridine nitrogen atom being protonated under the reaction conditions. The ethyl esters of the disubstituted derivatives **9a–o** were hydrolyzed and the resulting carboxylic acids were coupled to L-arginine

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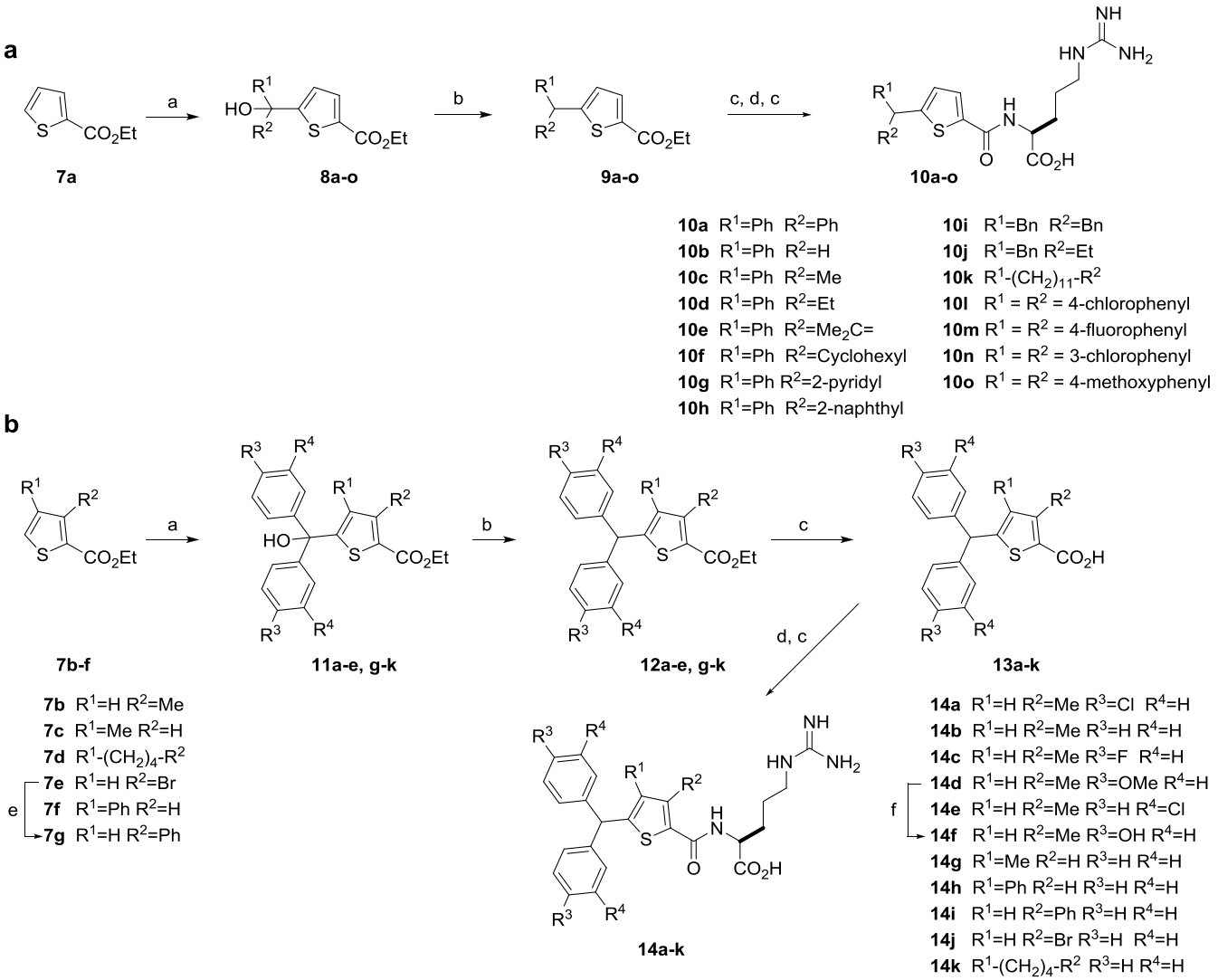
Scheme 1. Synthesis of Thiophene Analogues[a]



[a]Reagents and conditions: (a) lithium diisopropylamide (LDA), tetrahydrofuran (THF), ketones, −78 °C; (b) trifluoroacetyl (TFA), Et_3SiH, room temperature (RT); (c) NaOH, H_2O, MeOH, THF; (d) H-Arg-OMe, *N*,*N*-diisopropylethylamine (DIPEA), BOP, DMF; (e) phenylboronic acid, K_3PO_4, $Pd(PPh_3)_4$, 1,4-dioxane; (f) BBr_3, dichloromethane (DCM). For compounds **8a−o** and **9a−o**, the groups R^1 and R^2 are the same as defined for **10a−o**, except **8e** and **9e**, where R^2 = isopropyl.

methyl ester using BOP. Hydrolysis of the arginine methyl ester gave **10a−o** in moderate to good yields (Scheme 1a).

Relative to **10a**, there was a 100-fold loss in C3aR antagonist activity for **10b** and **10c** (IC_{50} > 10 μM; Table 1), where one of the phenyl groups was replaced with H or Me. Activity was partially recovered when the aliphatic group was lengthened from Me to Et (**10d**, IC_{50} 2.3 μM). The isopropylidene analogue (**10e**) was a 2-fold less active antagonist than the ethyl derivative (**10d**), perhaps suggesting that its 3D shape rather than a flat sp^2 topology is preferred for interaction with the receptor. Changing one of the phenyl groups to a cyclohexyl group seemingly gave a more potent antagonist (**10f**, IC_{50} 4 nM); however, partial agonism up to ∼30% response relative to that of C3a was also observed at higher concentrations (EC_{50} 70 nM), suggesting that receptor desensitization[10,12,24] is the cause of the apparent antagonist activity. Replacing one of the phenyls with a 2-pyridyl group introduced a potential hydrogen-bond acceptor into this region, and this compound became a potent antagonist (**10g**, IC_{50} 40 nM).

To investigate permissible space around the phenyl groups, the larger 2-naphthyl (**10h**) and dibenzyl (**10i**) substituents were introduced, with both showing similar antagonist activity (IC_{50} 0.09 and 0.1 μM, respectively; Table 1). Combining one benzyl group with an ethyl substituent reduced antagonist activity 2-fold (**10j**, IC_{50} 0.2 μM); however, this combination was 12-fold more potent than the phenyl and ethyl combination (**10d**) noted earlier. A single macrocyclic cyclododecanyl ring (**10k**) in place of two separate substituents abolished antagonist activity, suggesting the need for aromaticity in this region or that its flexibility and number of rotatable bonds made it entropically unfavorable for binding. A *para*-chloro atom on each aromatic ring of the diphenylmethyl segment increased antagonist activity 3-fold (**10l**, IC_{50} 0.03 μM), whereas the corresponding bis(4-fluorophenyl) analogue did not increase antagonist activity (**10m**, IC_{50} 0.1 μM). This difference may be due to the greater

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Table 1. Thiophene Derivatives Modulate Ca^{2+} Release in Human Monocyte-Derived Macrophages

	apparent antagonist activity[a]		agonist activity[b]	
compound[c]	pIC_{50} ± SEM	IC_{50} (μM)	pEC_{50} ± SEM	EC_{50} (μM)
3[d]	5.4 ± 0.2	3.8 (1.3[d])	<4	>100
6[13,22]	6.2 ± 0.2	0.60	<4	>100
10a[e] (BR111)[13]	7.1 ± 0.3[e]	0.085	<4	>100
10b	<5	> 10	<4	>100
10c	<5	> 10	<4	>100
10d	5.6 ± 0.3	2.3	<4	>100
10e	5.4 ± 0.3	4.4	<4	>100
10f	8.4 ± 0.2[e]	(0.004)[e]	7.2 ± 0.2	0.07
10g	7.4 ± 0.1[e]	0.04	<4	>100
10h	7.0 ± 0.1	0.09	<4	>100
10i	7.0 ± 0.2	0.10	<4	>100
10j	6.7 ± 0.2	0.20	<4	>100
10k	<4	> 100	<4	>100
10l (JR10)	7.5 ± 0.2	0.03	<4	>100
10m	7.0 ± 0.1	0.1	<5	>10
10n	8.2 ± 0.2[e]	(0.006)[e]	7.3 ± 0.2	0.05
10o	5.2 ± 0.2	6.1	<4	>100
14a (JR14a)	8.0 ± 0.1	0.010	<4	>100
14b (JR14b)	8.2 ± 0.1	0.007	<4	>100
14c	7.5 ± 0.1[e]	0.035	<4	>100
14d	5.7 ± 0.1	2.0	<4	>100
14e	8.5 ± 0.2[e]	(0.003)[e]	7.3 ± 0.2	0.046
14f	6.1 ± 0.6	0.79	<4	>100
14g	6.9 ± 0.3	0.14	<4	>100
14h	6.9 ± 0.2	0.14	<4	>100
14i	<4	> 100	<4	>100
14j	6.3 ± 0.4[e]	0.51	<4	~100
14k	5.6 ± 0.1	2.4	<4	>100

[a]Inhibition of intracellular Ca^{2+} release induced by C3a (100 nM) on HMDM whole cells. All data $n \geq 3$. [b]Concentration to induce 50% maximal Ca^{2+} release in HMDM relative to 100% induced by C3a at 1 μM. [c]Compounds **10c–d**, **10f–h**, and **10j** introduce a new chiral center, but were tested as a mixture of diastereomers. [d]SB290157.[14] [e]30–80% partial agonists at higher concentrations.

steric bulk of chloro versus fluoro substituents. The bis(3-chlorophenyl) analogue resulted in apparently improved antagonist potency (**10n**, IC_{50} 6 nM), but it was also found to have up to 60% partial agonist activity at higher concentrations (EC_{50} 50 nM) consistent with activation and desensitization of the receptor, leading to apparent inhibition. The bulkier 4-methoxy derivative (**10o**, IC_{50} 6 μM) was 60-fold less potent than **10a** and 200-fold less potent than **10l** (JR10) as an antagonist. These data suggest that the *p*-chloro substituent has the ideal size in this series of C3aR antagonists and likely also blocks oxidative metabolism at this position (see ahead).

Next, a series of compounds were synthesized to explore the effect of placing substituents at the 3- and 4-positions of the thiophene ring of compound **10a** to generate **14a–k** (Scheme 1b). Substituted thiophene-2-carboxylic acid ethyl esters **7b–g** were commercially available or were prepared by esterification of the corresponding acids (Supporting Information), and the 3-bromo compound **7e** was converted to the 3-phenyl derivative **7g** by Suzuki–Miyaura coupling with phenylboronic acid. Lithiation at the thiophene 5-position with LDA at −78 °C, followed by addition to benzophenone derivatives, gave the tertiary alcohols **11a–j**. Reduction with Et_3SiH/TFA gave the ethyl 5-diphenylmethylthiophene-2-carboxylates **12a–j**, which were hydrolyzed to the carboxylic acids **13a–j** that were then coupled to L-arginine methyl ester and finally hydrolyzed to give the series of analogues **14a–k** (Scheme 1b).

Methylation at the thiophene-3-position had the most beneficial effect on antagonist potency generally (Table 1), with the 3-methyl derivative **14a** (IC_{50} 10 nM, JR14a) having a 3-fold higher antagonist potency over the bis(4-chlorophenyl) analogue **10l**. Likewise, the 3-methyl analogue **14b** (IC_{50} 7 nM, Table 1) had a 10-fold improved activity over the unsubstituted **10a**, and there was no agonist activity observed at concentrations up to 100 μM. Methylation of the thiophene-3-position of the bis(4-fluorophenyl) compound also improved the antagonist potency by approximately 3-fold (**14c**, IC_{50} 0.035 μM). Methylation of the thiophene-3-position of the bis(4-methoxyphenyl) derivative **14d** resulted in a 3-fold improvement in antagonist potency (**14d**, IC_{50} 2 μM). Thiophene-3-methylation of the bis(*meta*-chlorophenyl) agonist derivative **10n** resulted in **14e**, having an even greater partial agonist response of up to 80% that of C3a (**14e**, EC_{50} 46 nM). The observation of some partial agonist responses, especially at high μM concentrations, for **10f**, **10g**, **10n**, and **14c**, **14e**, **14j** (Supporting Figures 1–4) show how even subtle changes to the structure can influence functional activity, which can also change with different cell types as highlighted by compound **3** having antagonist activity in some cell types but agonist activity in others.[25]

The bis(4-hydroxyphenyl) analogue (**14f**, IC_{50} 0.79 μM) was an almost 3-fold more potent antagonist than the bulkier bis(4-methoxyphenyl) analogue **14d**, but still 80-fold less potent than the similarly sized bis(4-chlorophenyl) analogue **14a** (Table 1). Substituting the thiophene 4-position with a methyl (**14g**, IC_{50} 0.14 μM) or phenyl group (**14h**, IC_{50} 0.14 μM) did not have a large effect on potency, with both being only slightly less potent than the unsubstituted **10a**. However, placing a phenyl in the 3-position inactivated the compound (**14i**, IC_{50} > 100 μM). The addition of a bromo substituent to the 3-position also reduced antagonist potency (**14j**, IC_{50} 0.51 μM). Finally, the 3,4-fused cyclohexyl ring reduced the activity by over 20-fold (**14k**, IC_{50} 2.4 μM), suggesting that a substituent bulkier than a methyl group may not be tolerated in the 3-position. The most promising analogues from this series were the 3-methylthiophenes **14a** (JR14a) and **14b**.

Antagonist Activity, Receptor Specificity, and in Vitro Stability. Compounds **14a** and **14b** were both approximately 10-fold more potent C3aR antagonists than **10a**[13] and 100-fold more potent than SB290157[14] (**3**) in inhibiting intracellular Ca^{2+} (iCa^{2+}) release in this assay after stimulation by human C3a in human macrophages (Figure 3a). They were also selective for the human C3a receptor over the closely related C5a receptor, as they had no effect on the release of iCa^{2+} stimulated in the same cells by C5a (Figure 3b). The more potent C3aR antagonists **10a**, **10l**, **14a**, **14b**, and **14f** were assessed for stability in rat plasma and in the presence of rat liver microsomes. The thiophenes were all stable in rat plasma over 4 h, whereas control tripeptide Boc-LAR-OH was rapidly degraded (Figure 3c) at a rate similar to C3a.[26,27] Both bis(4-cholorophenyl)-substituted compounds **10l** (JR10) and **14a** (JR14a), as well as the putative metabolite bis(4-hydroxyphenyl) analogue **14f**, were metabolically stable to exposure over 1 h to rat liver microsomes (Figure 3d). The unsubstituted diphenylmethyl compounds **14b** and **10a** were

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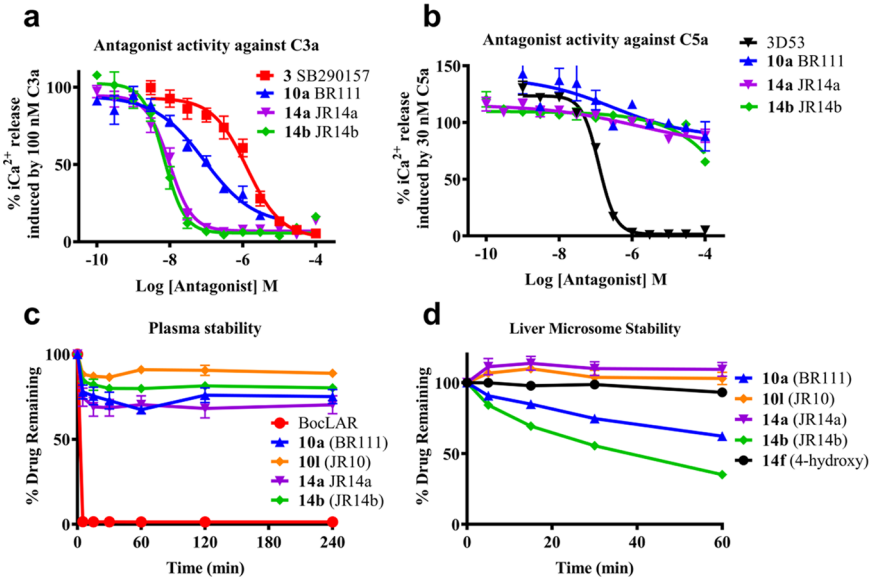

Figure 3. Evaluation of new C3aR antagonists in vitro. (a) C3aR antagonists inhibit iCa^{2+} release stimulated by C3a (100 nM) in HMDM in a concentration-dependent manner. (b) C3a antagonists have no effect on iCa^{2+} release stimulated by C5a (30 nM) in HMDM, in contrast to the known[28,29] C5aR antagonist 3D53, cyclo-(2,6)-AcF[OP(dCha)WR] (black). (c) Potent thiophene antagonists **10a, 10l**, **14a**, and **14b** were stable to proteolytic degradation in rat plasma in vitro, whereas control tripeptide Boc-LAR-OH was rapidly degraded. (d) In the presence of rat liver microsomes, the bis(4-chlorophenyl) analogues **10l** and **14a** showed minimal metabolism in vitro over 60 min, as did bis(4-hydroxyphenyl) **14f** (a potential metabolite of **14b**; data not shown). Unsubstituted analogues **10a** and **14b** were approximately 40 and 60% degraded after 60 min. Data are presented as mean ± standard error of the mean (SEM) (n = 3). Note: all points have error bars, but some are smaller than the data symbol.

	β-hexosaminidase	
Compound	**pIC_{50}** ± SEM	**IC_{50}**[a] nM
3 (SB290157)	6.3 ± 0.2	540
10a (BR111)	8.4 ± 0.2	4
14a (JR14a)	8.1 ± 0.2	8
14b (JR14b)	8.7 ± 0.2	2

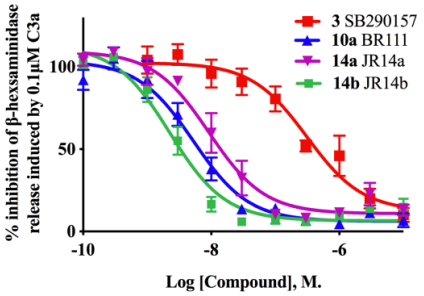

Figure 4. Antagonism of β-hexosaminidase release induced by C3a in LAD2 mast cells in a concentration-dependent manner by: **3**, **10a**, **14a**, and **14b**. [a] Concentration (IC_{50}) and -Log(IC_{50}) of ligand required to inhibit 50% of β-hexosaminidase release induced by C3a (100 nM) in human LAD2 mast cells ($n \geq 3$).

Table 2. Pharmacokinetic Properties of C3aR Antagonists in Wistar Rats[a]

cmpd	dose (mg/kg; route)	AUC (0−8 h, ng h/mL)	V_d (L)	elimination half-life (min)	clearance (mL/(min kg))	T_{max} (min)	C_{max} (ng/mL)
10a	0.5 i.v.	811	0.4	32	10.3		
	10 p.o.	436	N/A	N/A	N/A	210	91
10l	0.5 i.v.	1802	0.3	61	4.6		
	10 p.o.	814	N/A	N/A	N/A	240	143
14a	1 i.v.	3795	0.1	191	4.4		
	10 p.o.	478	N/A	N/A	N/A	300	88
14b	1 i.v.	779	0.7	45	18.0		
	10 p.o.	90	N/A	N/A	N/A	240	23

[a] n = 3 for each route and compound; time course, 0−8 h.

the least stable to rat liver microsomes, showing 40 and 60% degradation, respectively, over 60 min (Figure 3d). In summary, **14a** (JR14a) was the most promising stable C3aR antagonist of these series, containing a 3-methyl substituent on the thiophene ring as well as a *para*-chloro substituent on both aromatic rings of the diphenylmethyl group that may help suppress P450 oxidative metabolism.

Activity in Human Mast Cells. Mast cells are key immune cells that play major roles in regulating immunity, defense, and disease.[30] Activation of mast cells results in rapid degranulation and release of various preformed inflammatory triggers (e.g., histamine, tryptase, β-hexosaminidase, lipid mediators, cytokines, chemokines), and dysregulated activation of these cells can lead to a range of acute and chronic human disease states, including allergies, asthma, atopic dermatitis, arthritis, and

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many others.[31] Here, we show that compounds **10a**, **14a**, and **14b** were potent inhibitors of mast cell degranulation in vitro, as measured by release of the lysosomal enzyme β-hexosaminidase induced by hC3a (100 nM) in human LAD2 mast cells (Figure 4). By this measure, all three compounds were around 100-fold more potent inhibitors of mast cell degranulation than **3** (Figure 4).

Anti-Inflammatory Activity in Vivo. As a prelude to investigating anti-inflammatory activity for C3aR antagonists, the compounds **10a**, **10l**, **14a**, and **14b** were examined for some pharmacokinetic parameters in male Wistar rats (Table 2). Compounds **10a**, **10l**, and **14a** were detectable at higher concentrations than **14b** in rat plasma after oral administration, **10l** and **14a** had the lowest clearance rates in vivo (<5 mL/(min kg)), but **14a** had a longer elimination half-life (>3 h). This supported advancement of **14a** as an antagonist for further studies in vivo.

A single intraplantar injection of the potent and selective C3aR agonist **2** (BR103, 350 μg/paw in saline) into the hind paws of Wistar rats caused an acute edema manifested by paw swelling that peaked after 0.5 h and subsided within 4 h (Figure 5a). C3aR antagonist compounds **10a**, **10l**, **14a**, and **14b** (10 mg/kg p.o. 2 h prior) were evaluated for anti-inflammatory activity in this acute rat paw model of inflammation and edema (Figure 5a). Following oral administration of C3aR antagonists via oral gavage in olive oil, paw swelling was induced 2 h later by the C3a agonist **2** and monitored over a further period of 2 h. Compounds **10a**, **10l**, **14a**, and **14b** reduced paw swelling by 20, 45, 65, and 49%, respectively, over control at 30 min after agonist injection (Figure 5a). In this model, compound **3** does not show measurable anti-inflammatory activity below 30 mg/kg p.o. The lower efficacy in vivo for **14b** and **10a** relative to **14a** was expected based on lower stability to P450 metabolism in vitro in liver microsomes (Figure 3d). The improved efficacy of **14a** in vivo may be due to its dual advantages over **10a** in being both 10-fold more potent as a C3aR antagonist in vitro and also having almost 6-fold greater metabolic stability in liver microsomes.

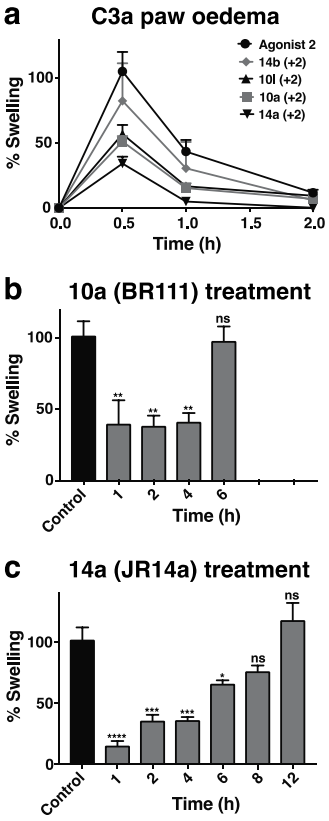

Figure 5. Efficacy and duration of anti-inflammatory activity against agonist-induced paw edema in male Wistar rats. (a) Paw edema (swelling) induced by intraplantar injection of C3aR agonist BR103 (**2**, black circle) is suppressed by 10 mg/kg C3aR antagonists **10a** (gray square), **10l** (black triangle), **14a** (black inverse triangle), and **14b** (gray diamond) given orally 2 h prior to agonist. Duration of effect of (b) antagonist **10a** (BR111) or (c) antagonist **14a** (JR14a) administered orally at 30 mg/kg, prior to challenge with an agonist **2** (BR103) injected intraplantar to induce paw edema in groups of rats after different exposure times to antagonist (1−12 h). $n = 3$ per group. Data are mean ± SEM. Analysis by one-way analysis of variance (ANOVA), $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

Since **14a** (JR14a) had improved metabolic stability over **10a** (BR111), we hypothesized that it may also be more efficacious over a longer period of time. To investigate this, the efficacy of JR14a was monitored in the paw edema model over a period of 12 h and compared to **10a** (Figure 5b vs 5c). Both ligands were administered orally (p.o.) at 30 mg/kg to maximize antagonism for an extended timeframe and paw swelling was induced by agonist **2** (BR103) (350 μg/paw in saline with 5% dimethyl sulfoxide (DMSO), 100 μL, i.pl.). Thiophene antagonist **10a** (Figure 5b) reduced paw swelling by up to 60% for a period of up to 4 h following administration. Thiophene **14a** (Figure 5c) reduced paw swelling by a greater margin (85% reduction) and maintained a significant reduction in paw size for 6−8 h, an improvement in both the duration of efficacy and potency over **10a**.

Next, we examined **14a** (JR14a) for a dose−response relationship and found that, when given orally 2 h prior to BR103 challenge, this antagonist had a ceiling effect at 10 mg/kg when monitored 30 min after agonist injection, with no increase in anti-inflammatory activity in this assay when the oral dose was elevated from 10 to 20 or 30 mg/kg (Figure 6a,b). At 10 mg/kg p.o., JR14a reduced paw swelling even more effectively when given 4 h before i.pl. agonist administration (Figure 6c). The antagonist also inhibited BR103-induced expression (over 30 min) of a range of pro-inflammatory rat cytokines, including *Tnf*, *Il1b*, *Il6*, *Il10*, *Ccl2*, and *Cd244* (Figure 6d−i).

Rat paw tissues were histologically analyzed after 30 min of exposure to agonist (BR103) when paw inflammation had maximized, and this coincided with activation and degranulation of mast cells. Paw tissues of BR103-treated animals had decreased numbers of intact mast cells (Figure 7a,d) and tryptase-positive cells (Figure 7b,e), consistent with increased histamine release (Figure 7f) compared to saline-treated animals. This indicates agonist-induced activation/degranulation of mast cells. There were also increased numbers of ED1 +ve macrophages (Figure 7c,g). Pretreatment with JR14a by oral gavage (10 mg/kg) 2 h prior to intraplantar agonist injection prevented these histological indications of rat paw inflammation, by stabilizing mast cells and inhibiting their degranulation/activation (Figure 7a,b,d,e,f) as well as suppressing numbers of ED1 +ve macrophages (Figure 7c,g) in these tissues.

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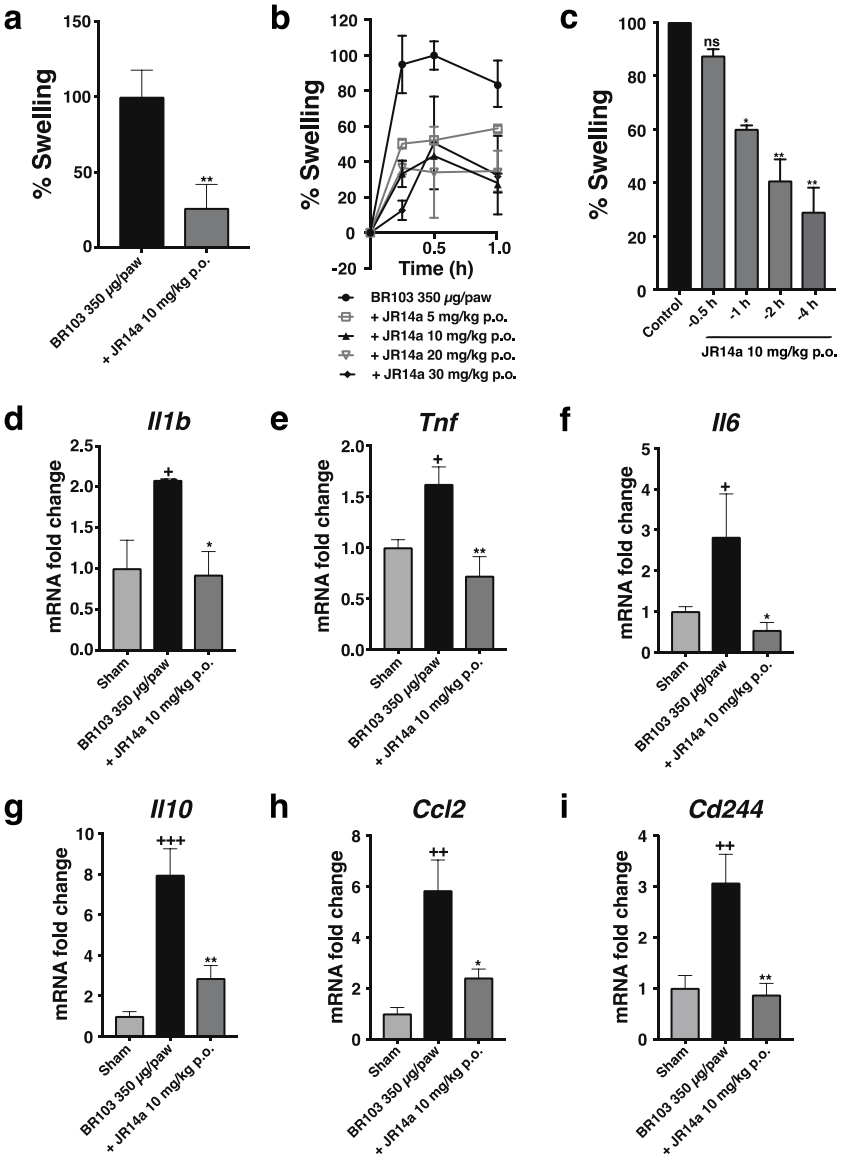

Figure 6. JR14a reduces rat paw inflammation induced by a C3aR agonist. (a) Oral pretreatment with JR14a (10 mg/kg p.o., 2 h prior) significantly reduces paw swelling measured 30 min after intraplantar injection of C3aR agonist BR103 (350 μg/paw) into rat hind paw. (b) When given orally 2 h prior to BR103 (**2**) challenge, JR14a has a ceiling effect at 10 mg/kg. At higher doses (20 or 30 mg/kg p.o.), there is no additional reduction in paw swelling after 30 min. (c) JR14a (10 mg/kg p.o.) maximally reduces paw swelling when given 4 h before i.pl. agonist administration, compared to the BR103 control at 30 min (first bar). (a–c) All measurements are normalized to sham. (d–i) BR103 increases expression of inflammatory genes at 30 min in the rat paw, including *Il1b* (d), *Tnf* (e), *Il6* (f), *Il*10 (g), *Ccl*2 (h), and *Cd*244 (i). Pretreatment with JR14a (2 h before agonist) attenuates increased expression of these genes. Target genes were normalized to housekeeping gene *Hprt*. Error bars = mean ± SEM. n = 3–5 male Wistar rats (7–8 weeks old) per group. Analysis by two-tailed student's t-test (A) or one-way ANOVA (B–G). *p < 0.05, **p < 0.01, ***p < 0.0001 compared to control; +p < 0.05, ++p < 0.01, +++p < 0.001 compared to sham.

DISCUSSION AND CONCLUSIONS

Complement protein C3 is synthesized by a range of cells[4,32] and is a central component of the classical, alternative, lectin, and possibly extrinsic protease-mediated pathways of Complement activation.[9] It is released into the circulation, is proteolytically cleaved at the membrane of microbial and host innate immune cells by Complement convertases, and deposits C3b that contributes to and amplifies the Complement cascade leading to lysis of microorganisms and infected or damaged cells.[9] C3 cleavage to C3b also produces the byproduct C3a, and this Complement protein is known to act independently on a unique G-protein-coupled receptor C3aR expressed on many human cells, including innate immune cells like macrophages, neutrophils, and mast cells.[33] C3a is known to cause degranulation and chemotaxis in vitro, but the physiological properties of C3a itself in vivo remain clouded due to mis-information arising from studies involving administration of, metabolically unstable, C3a to mammals and the unavailability of potent and metabolically stable agonists and antagonists. For example, it does not seem to be commonly known that C3a undergoes extremely rapid proteolytic degradation to products that do not bind to C3aR.[13] Recent studies have identified potent and selective small-molecule agonists,[5,11,12] that have the same physiological and molecular profiles as C3a in vitro but are much more stable in vivo. However, no very potent small-molecule antagonist of human C3aR has yet been reported.

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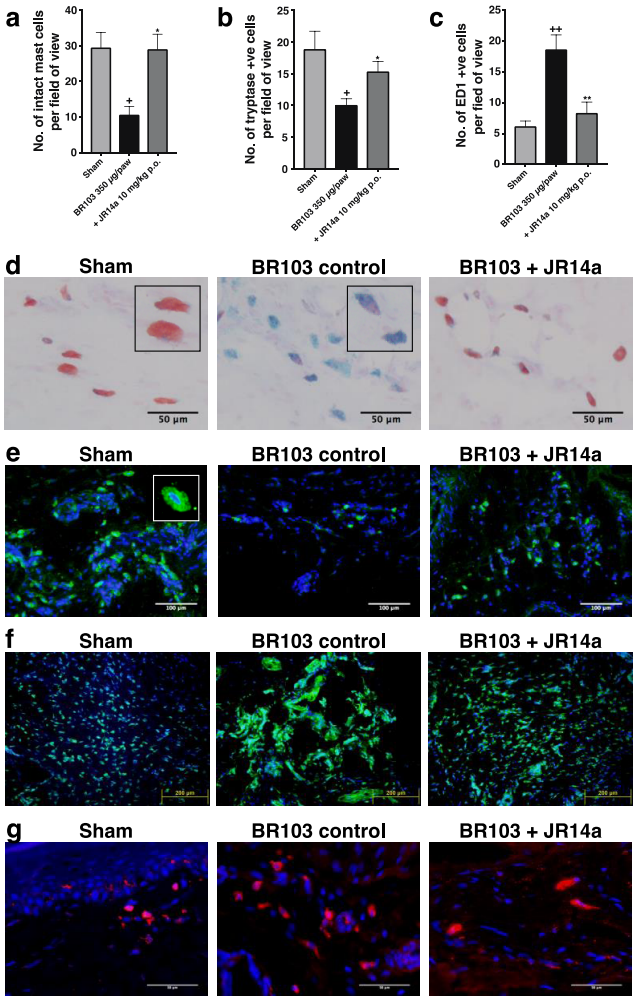

Figure 7. JR14a reduces mast cell degranulation and macrophage infiltration in BR103-induced rat paw edema. (a, d) Relative to sham animals, intact/inactive mast cells are decreased 30 min after intraplantar injection of agonist BR103 (350 μg/paw, i.pl.) into rat paws. Oral pretreatment with JR14a (10 mg/kg, 2 h prior) prevents this decrease. (d) Inactive mast cells (red) stained by safranin O. Degranulated mast cells (blue) stained by alcian blue. Scale bar = 50 μm. (b, e) Tryptase-positive cells (green) stained by immunohistochemistry are significantly reduced in control group compared to sham, indicating activation/degranulation of mast cells (extracellular tryptase undetectable by immunohistochemistry). This decrease is inhibited by oral pretreatment with JR14a. Blue dye stains for cell nuclei. Scale bar = 100 μm. (f) Immunohistochemistry staining of histamine (green), cell nuclei stained blue. Sham paw is dominated by intact/inactive histamine-containing mast cells. BR103 control paw shows diffused extracellular histamine staining, indicating mast cell degranulation and histamine release. JR14a pretreated paw contains inactive mast cells with minimal mast cell activation and histamine release. Scale bar = 200 μm. (c, g) ED1-positive macrophages (stained red by immunohistochemistry) are significantly increased 30 min after BR103 injection. This is prevented by oral pretreatment with JR14a. Blue dye stains for cell nuclei. Scale bar = 50 μm. Error bars = mean ± SEM, n = 5 male Wistar rats (7–8 weeks old) per group. Analysis by one-way ANOVA. *p < 0.05, **p < 0.01 compared to control; +p < 0.05, ++p < 0.01 compared to sham.

Thiophenes are frequently occurring scaffolds in heterocyclic drugs,[34−38] for example, in the antidepressant Duloxetine, antihypertensive Eprosartan, oral anticoagulant Rivaroxban, antipsychotic Olanzapine, antiplatelet agents Clopidogrel, Preasugrel, and Ticlopidine, and there are many other thiophene-containing compounds with demonstrated efficacy in vitro or in animal models of diseases.[34−38] The present study has described structure−activity relationships centered around thiophene-based scaffolds with diverse substitution that has indicated some structural requirements for potent C3aR antagonism. In vitro, thiophene compounds potently inhibited intracellular Ca^{2+} release in human macrophages (e.g., **10a**, IC_{50} 85 nM; **10l**, 30 nM; **14a**, 10 nM; **14b**, 7 nM), without inhibiting the agonist effects in the same concentration range as the related Complement protein C5a. Among key findings from structure−activity relationships in cells were: (i) for Ca^{2+} mobilization in human macrophages, a thiophene confers a 10- to 15-fold increase in antagonist potency over a thiazole or no heterocycle (e.g., **10** vs **6** or **3**), indicating that a sulfur-containing heterocycle is important for conferring antagonism and that the hydrogen bond-accepting nitrogen of thiazole may be detrimental for antagonist potency; (ii) in the same assay, a chlorine at the para position of both aromatic rings of the benzhydryl group confers a further 10-fold increase in antagonist potency for thiophenes (e.g., **14a** vs **10a**); (iii) for LAD2 human mast cells, the thiophene analogues were similarly 100-fold more potent than **3** that has no heterocycle, as antagonists of C3a-induced degranulation measured by secretion of β-hexosaminidase; (iv) for rat liver microsomes in vitro, there was a significant increase in metabolic stability for **14a** and some analogues over other thiophenes (e.g., **10a**, **10l**, **14b**).

Several of the thiophene derivatives from these series were found to have anti-inflammatory activity in vivo, when administered by oral gavage to rats subsequently injected with a C3aR agonist into the paw to induce an acute local inflammation. Among the compounds examined in vivo, orally administered thiophene **14a** (10 mg/kg) had the lowest clearance from rats, consistent with the findings of its stability to rat liver microsomes in vitro and its longer elimination half-life in vivo after oral delivery. This increased metabolic stability coupled with greater C3aR antagonist potency contributed to superior anti-inflammatory activity and a longer duration of action as assessed by C3aR agonist challenge to induce acute rat paw edema. Compound **14a** (JR14a) was the most potent and effective C3aR antagonist from this study.

There is a great need to develop new tactical approaches to develop new modulators for the hundreds of membrane-spanning G-protein-coupled receptors that mediate human physiology.[39,40] Many of the most interesting GPCR targets for prospective drugs are expressed on innate immune cells, for which we are still uncovering relationships between extracellular signals, intracellular signaling pathways, and physiological responses in vivo. Studying the important interplay between innate immune cells and their environment can benefit from the availability of potent new compounds that can modulate specific receptors and immune cell function. Here, we present a new class of small-molecule modulators for the human Complement protein C3a, an initiator of acute inflammatory responses that need to be controlled in a diverse range of disease indications. By virtue of their potency, metabolic stability, and efficacy against different innate immune cells, compounds reported here could prove to be valuable new probes for investigating the detailed pharmacology of C3a and its associated GPCR in vitro and in vivo in mammalian physiology and disease.

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EXPERIMENTAL SECTION

General. All reagent chemicals were purchased from Sigma-Aldrich, Combi-Blocks Inc., Fluorochem Ltd., or Chem-Impex International Inc. All final compounds were analyzed by NMR, UPLC-MS, and HRMS, and the purity was determined to be >98%. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker Avance III HD 600 spectrometer with cryoprobe at 298 K in the deuterated solvents indicated. ^{1}H NMR spectra were referenced to the residual ^{1}H signal DMSO-d_5 2.49 ppm, except $CDCl_3$ solutions were referenced to internal TMS. ^{13}C NMR resonances were referenced to the solvent peak (DMSO-d_6 39.51, $CDCl_3$ 77.0 ppm). The exact concentration of the compounds was determined by the quantitative NMR integration "PULCON" experiment.[41] Thin-layer chromatography was performed on Merck silica gel 60 TLC plates. Preparative-scale rpHPLC separations were performed on a Phenomenex Luna C18 10 μm, 250 × 21.2 mm^2 column. Standard conditions were used for elution of all compounds unless otherwise indicated at a flow rate of 20 mL/min: 100% A to 100% B linear gradient over 15 min followed by a further 10 min at 100% B where solvent B was 90% MeCN, 10% H_2O + 0.1% TFA and solvent A was H_2O + 0.1% TFA. Detection was by UV, and pure fractions were lyophilized. Analytical UPLC-MS was used to assess compound purity using a Zorbax Eclipse plus C18 column (1.8 μm, 90 Å, 2.1 × 100 mm^2) at 0.5 mL/min with detection at three different wavelengths (λ 214, 230, and 254 nm) and electrospray ionization-mass spectrometry (ESI-MS). High-resolution mass spectra (ESI-HRMS) measurements were obtained on a Bruker microTOF mass spectrometer equipped with a Dionex LC system (Chromeleon) in positive-ion mode by direct infusion in MeCN at 100 μL/h using sodium formate clusters as an internal calibrant. Data were processed using Bruker Daltonics DataAnalysis 3.4 software. Mass accuracy was consistently better than 1 ppm error. All final compounds were checked for PAINS and aggregators at http://zinc15.docking.org/patterns/home, and no alerts were found. Full experimental details and spectroscopic data including intermediates are reported in the Supporting Information.

Chemical Synthesis. Human C3a was chemically synthesized, purified, and characterized as described.[42] This material has been shown to have the identical structure, affinity, and functional properties as recombinant C3a.[42] The agonist compound **2** (BR103)[12] and antagonist compound **3** (SB290157)[14] were synthesized, purified by RP-HPLC, characterized by NMR and MS, and functionally profiled for affinity and functions, as described elsewhere.[12,13] The syntheses and characterization of most compounds are reported in the Supporting Information.

*(5-(Bis(4-chlorophenyl)methyl)thiophene-2-carbonyl)-L-arginine **10l** (JR10).* Compound **9l** (1.14 g, 2.92 mmol) was hydrolyzed to produce the carboxylic acid (939 mg, 2.59 mmol, 89%) as a white solid, then an aliquot (200 mg, 0.55 mmol) was converted to (5-(bis(4-chlorophenyl)methyl)thiophene-2-carbonyl)-L-arginine **10l** (TFA salt, 133 mg, 34% over three steps) using a procedure analogous to that for the preparation of **14a** shown below. ^{1}H NMR (600 MHz, DMSO-d_6): δ 1.47−1.57 (m, 2H, $NHCH_2\underline{CH_2}CH_2CH$), 1.66−1.72 (m, 1H, $NHCH_2CH_2\underline{CH_2}CH$), 1.80−1.86 (m, 1H, $NHCH_2CH_2\underline{CH_2}CH$), 3.07−3.11 (m, 2H, $NH\underline{CH_2}CH_2CH_2CH$), 4.29−4.33 (m, 1H, α-CH), 5.92 (s, 1H, $Ph_2\underline{CH}$-thiophene), 6.78 (dd, 1H, J = 3.7 Hz, J = 0.7 Hz, C4 thiophene), 7.23 (d, 4H, J = 8.50 Hz, *o*-Ph), 7.41 (d, 4H, J = 8.4 Hz, *m*-Ph), 7.53 (t, 1H, J = 5.6 Hz, $\underline{NH}CH_2$), 7.73 (d, 1H, J = 3.90 Hz, C3 thiophene), 8.62 (d, 1H, J = 8.1 Hz, NH). ^{13}C NMR (150 MHz, DMSO-d_6): δ 25.5, 27.7, 40.3, 49.5, 52.0, 127.3, 128.67, 128.74, 130.3, 131.7, 138.2, 141.9, 151.8, 156.6, 161.2, 173.4. HRMS: $C_{24}H_{25}Cl_2N_4O_3S^+$ expected 519.1019 found 519.1019.

*Ethyl 5-(Bis(4-chlorophenyl)(hydroxy)methyl)-3-methylthiophene-2-carboxylate **11a**.* A solution of ethyl 3-methylthiophene-2-carboxylate **7b** (7.74 g, 45.5 mmol) in dry THF (75 mL) was stirred at −78 °C under N_2, then a solution of LDA (2 M, 25 mL, 50 mmol, 1.1 equiv) was added over 5 min. After a further 10 min, a solution of 4,4′-dichlorobenzophenone (11.50 g, 45.8 mmol) in THF (100 mL) was added over 10 min at −78 °C. The solution was stirred at −78 °C for 1 h, then at room temperature for 3 h. THF was evaporated and the residue was dissolved in EtOAc and washed with 2 M HCl, 10% $NaHCO_3$, brine, and dried over $MgSO_4$. The solvent was evaporated and the solid residue was triturated with light petroleum ether and collected on a filter giving **11a** as a white solid (18.20 g, 95%). Recrystallization from EtOAc/petroleum ether (1:1, 6 mL/g) gave the pure product (15.7 g, 82%). R_f 0.29 (10% EtOAc/petroleum ether). ^{1}H NMR (600 MHz, $CDCl_3$): δ 1.33 (t, 3H, J = 7.1 Hz, $\underline{CH_3}CH_2O$), 2.47 (s, 3H, CH_3), 2.94 (bs, 1H, OH), 4.29 (q, 2H, J = 7.1 Hz, $CH_3\underline{CH_2}O$), 6.59 (s, 1H, C4 thiophene), 7.28−7.32 (m, 8H, Ph). ^{13}C NMR (150 MHz, $CDCl_3$): δ 14.3, 16.1, 60.8, 79.3, 126.9, 128.4, 128.6, 131.1, 134.1, 143.8, 145.9, 155.1, 162.7. LC/MS m/z: 403.0 (M^+ − OH).

*Ethyl 5-(Bis(4-chlorophenyl)methyl)-3-methylthiophene-2-carboxylate **12a**.* A mixture of the tertiary alcohol **11a** (2.27 g, 5.39 mmol), DCM (10 mL), and Et_3SiH (1 mL, 6.25 mmol, 1.2 equiv) was stirred at room temperature, then TFA (1 mL, 13 mmol) was added. The solution became warm and refluxed spontaneously. After stirring for a further 30 min, the mixture was diluted with DCM, washed with excess 10% $NaHCO_3$ and evaporated to a yellow oil. Purification by flash chromatography (silica gel, eluting with 1−10% EtOAc/petroleum ether) gave **12a** as a colorless oil (2.11 g, 96%). R_f 0.52 (10% EtOAc/petroleum ether). ^{1}H NMR (600 MHz, $CDCl_3$): δ 1.32 (t, 3H, J = 7.1 Hz, $\underline{CH_3}CH_2O$), 2.47 (s, 3H, CH_3), 4.27 (q, 2H, J = 7.1 Hz, $CH_3\underline{CH_2}O$), 5.54 (s, 1H, $Ph_2\underline{CH}$-thiophene), 6.52 (s, 1H, C4 thiophene), 7.11 (m, 4H, Ph), 7.29 (m, 4H, Ph). ^{13}C NMR (150 MHz, $CDCl_3$): δ 14.3, 16.0, 51.0, 60.6, 126.1, 128.8, 130.0, 131.3, 133.1, 140.8, 146.1, 151.3, 162.6. LC/MS m/z: 446.1 (MH^+ + CH_3CN).

*5-(Bis(4-chlorophenyl)methyl)-3-methylthiophene-2-carboxylic Acid **13a**.* The ester **12a** (2.00 g, 4.93 mmol) was dissolved in THF (25 mL) and MeOH (25 mL), then a solution of NaOH (1 g in 25 mL of water) was added. The solution was stirred at room temperature for 3 h, then diluted with water and washed with diethyl ether/petroleum ether 1:1. The aqueous layer was acidified with HCl, and the precipitate was extracted with ether. The ether extracts were washed with brine, dried over $MgSO_4$ and evaporated to give the acid **13a** as a white solid (1.85 g, 99%). ^{1}H NMR (600 MHz, $CDCl_3$): δ 2.47 (s, 3H, Me), 5.55 (s, 1H, $Ph_2\underline{CH}$), 6.56 (s, 1H, C4 thiophene), 7.11 (m, 4H, *o*-Ph), 7.29 (m, 4H, *m*-Ph). ^{13}C NMR (150 MHz, $CDCl_3$): δ 16.2, 51.1, 125.4, 128.9, 130.0, 131.7, 133.2, 140.6, 148.0, 153.4, 167.9. LCMS m/z: 377.1 (MH^+).

*(5-(Bis(4-chlorophenyl)methyl)-3-methylthiophene-2-carbonyl)-L-arginine Hydrochloride **14a** (JR14a).* The acid **13a** (1.85 g, 4.91 mmol) was dissolved in DMF (20 mL) with stirring, then EDC (1.00 g, 5.21 mmol), L-arginine methyl ester dihydrochloride (1.93 g, 7.39 mmol, 1.5 equiv), and DIPEA (2.5 mL, 14.4 mmol, 3 equiv) were added. The mixture was stirred at room temperature for 17 h then the DMF was removed on a rotary evaporator at 0.1 mbar. The residue was dissolved in MeOH (40 mL), then a solution of NaOH (2 g in water 20 mL) was added. The solution was stirred at room temperature for 30 min, then 1-butanol (200 mL) was added and the solution was washed with 1 M HCl (100 mL, pH 1) and evaporated to dryness. The residue was dissolved in 30% MeCN 70% water and lyophilized to give the crude product as a white powder. Further purification was achieved by preparative reversed-phase HPLC, and the product was lyophilized as the TFA salt (3.20 g). Finally, the TFA salt was dissolved in 30% MeCN and 70% water (50 mL) and treated with Dowex-1 × 8 chloride in 200−400 mesh (5 g) for 30 min. The resin was filtered off and washed with 30% MeCN/water (20 mL), and the filtrate was lyophilized to give **14a** as the hydrochloride salt (2.80 g, 78%). ^{1}H NMR (600 MHz, DMSO-d_6): δ 1.57−1.43 (m, 2H), 1.75−1.65 (m, 1H), 1.86−1.76 (m, 1H), 2.33 (s, 3H), 3.14−3.02 (m, 2H), 5.86 (s, 1H), 4.26 (m, 1H), 6.62 (s, 1H), 7.26−7.22 (m, 4H), 7.43−7.39 (m, 4H), 7.70 (t, J = 5.5 Hz, 1H), 8.03 (d, J = 7.7 Hz, 1H). ^{13}C NMR (150 MHz, DMSO-d_6): δ 15.4, 25.4, 27.6, 40.3, 49.3, 52.2, 128.6, 130.1, 130.3, 130.8, 131.7, 140.4, 141.8, 147.8, 156.8, 162.3, 173.3. HRMS m/z calcd for $C_{25}H_{27}Cl_2N_4O_3S^+$ 533.1175 found 533.1175.

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(5-Benzhydryl-3-methylthiophene-2-carbonyl)-L-arginine ***14b*** *(JR14b).* Compound **12b** (228 mg, 0.68 mmol) was hydrolyzed to produce the carboxylic acid (153 mg, 0.50 mmol, 74%) as a white solid, then an aliquot (80 mg, 0.26 mmol) was converted to (5-benzhydryl-3-methylthiophene-2-carbonyl)-L-arginine TFA salt **14b** (114 mg, 0.20 mmol, 77%) using a procedure analogous to that used for the preparation of **14a** (57% over three steps). ^{1}H NMR (600 MHz, DMSO-d_6): δ 1.47−1.53 (m, 2H, NHCH$_2$CH$_2$CH$_2$CH), 1.65−1.72 (m, 1H, NHCH$_2$CH$_2$CH$_2$CH), 1.77−1.83 (m, 1H, NHCH$_2$CH$_2$CH$_2$CH), 2.33 (s, 3H, CH$_3$), 3.06−3.09 (m, 2H, NHCH$_2$CH$_2$CH$_2$CH), 4.25−4.29 (m, 1H, α-CH), 5.77 (s, 1H, Ph$_2$CH-thiophene), 6.62 (s, 1H, C4 thiophene), 7.24−7.26 (m, 6H, Ph), 7.32−7.35 (m, 4H, Ph), 7.52 (t, 1H, J = 5.5 Hz, NHCH$_2$), 8.04 (d, 1H, J = 8.1 Hz, NH). ^{13}C NMR (150 MHz, DMSO-d_6): δ 15.4, 25.5, 27.6, 40.3, 51.0, 52.1, 126.9, 128.5, 128.6, 129.7, 130.5, 140.3, 143.2/143.2, 149.0, 156.6, 162.5, 173.4. HRMS: $C_{25}H_{29}N_4O_3S^+$ expected 465.1955 found 465.1955.

Isolation of Primary Human Monocyte-Derived Macrophages. HMDMs were harvested from buffy coat (of anonymous human donors) provided by the Australian Red Cross Blood Service, Brisbane. Monocytes were isolated using Ficoll-Paque Premium density centrifugation (GE Healthcare Bio-Science, Uppsala, Sweden) and repeatedly washed with ice-cold water to remove erythrocytes, and CD14$^+$ monocytes were then positively selected using human CD14 MicroBeads and LS Columns (Miltenyi Biotec, Auburn). CD14$^+$ monocytes were then differentiated to HMDMs using 100 ng/mL recombinant human macrophage colony-stimulating factor (PeptroTech, Rocky Hill) for 7 days in Iscove's modified Dulbecco's media supplemented with 10% fetal bovine serum, penicillin (50 U/mL), streptomycin (50 μg/mL), and GlutaMAX (2 mM). HMDMs were resupplemented after 5 days with fresh medium containing 10 ng/mL recombinant human macrophage colony-stimulating factor and collected by gentle scraping on day 7.

Intracellular Calcium Release Assay. Intracellular calcium release was monitored in real time at 1 s intervals using a fluorescence imaging plate reader (FLIPR, Molecular Devices, CA) for at least 200 s (excitation 495 nm, emission 520 nm). HMDMs were seeded overnight in 96-well clear-bottom black-wall plates at a density of 5 × 10^4 cells/well. Prior to assay, the cells were incubated with dye-loading buffer (Hank's balance salt solution (HBSS) buffer, 4 μM Fluo-3 AM, 0.04% Pluronic acid F-127, and 1% fetal bovine serum) for 1 h at 37 °C. The cells were then washed once with assay buffer (HBSS supplemented with 2.5 mM probenecid and 20 mM *N*-(2-hydroxyethyl)piperazine-*N*′-ethanesulfonic acid (HEPES), pH 7.4). For agonist measurements, HBSS (50 μL/well) was added first into the assay plate and agonist was dispensed (50 μL/well 2× concentration) using FLIPR. For antagonist measurements, antagonist (50 μL/well 2× concentration) was preincubated for 30 min at 37 °C followed by human full-length synthetic 100 nM C3a (50 μL/well 2× concentration). It was important not to reuse and just wash FLIPR tips to prevent carryover or cross-contamination of compounds in the calcium release assay. The percent response was plotted against log concentration of test compound.

LAD2 Mast Cell Culture. LAD2 human mast cells (kindly provided by Dr. Dean Metcalfe, National Institute of Allergy and Infectious Diseases, National Institutes of Health) were cultured in StemPro-34 Serum Free Media (Invitrogen, Australia) supplemented with StemPro-34 nutrient, 100 U/mL penicillin, 100 μg/mL streptomycin, 2 mM GlutaMAX, and 100 ng/mL human stem cell factor (SCF). Cells were hemidepleted weekly and were not allowed to grow beyond a density of 5 × 10^5/mL. This cell line was authenticated by expression of tryptase and histamine granules.

β-Hexosaminidase Release Assay. LAD2 human mast cells were seeded overnight in 96-well plates at a density of 1 × 10^4 cells/well, and a β-hexosaminidase release assay was performed as previously described.[43] Briefly, LAD2 cells were presensitized with IgE (100 ng/mL) overnight before experiment. In the antagonist assay, LAD2 cells were preincubated with compounds **3**, **10a**, **14a**, or **14b** for 30 min at 37 °C in 0.1 M HEPES buffer (pH 7.4) before the addition of C3a (1 μM), and release of β-hexosaminidase was measured at 405 nm using FLUOstar Optima (BMG LabTechnologies). Responses to 100 nM C3a were comparable to degranulation induced by 10 μg/mL substance P or 30 μg/mL 48/80 in LAD2 mast cells.

Plasma and Metabolic Stability. Compounds (10 μL, 1 mM in DMSO) were added to rat plasma (190 μL) and incubated at 37 °C in a water bath. Aliquots (20 μL) were collected at different time points (0, 2, 5, and 10 min for Boc-LAR-OH; 0, 5, 15, 30, 60, 120, and 240 min for other compounds) and added directly to MeCN/H$_2$O (80 μL, 9:1). Samples were centrifuged (13 000*g*, 3 min) before isolating supernatants and analyzing for compound degradation via LC-MS. Commercially available rat liver microsomes (Life Technologies, GIBCO) stored at −80 °C were slowly defrosted on ice. Test compounds (183 μL, 1 μM in 100 mM phosphate buffer pH 7.4 containing 0.1% DMSO) were added to microsomes (5 μL) and NADPH (2 μL, 20 mM) and incubated (37 °C, 5 min) before adding cofactor NADPH (10 μL, 20 mM). Aliquots (20 μL) at time points 0, 5, 15, 30, and 60 min after adding NADPH were added to MeCN/H$_2$O (80 μL, 9:1), centrifuged (13 000*g*, 3 min), and supernatants were analyzed by LC-MSMS. Concentrations were plotted as a function of time on a semilogarithmic graph. Rat plasma and microsomal activity was confirmed by rapid degradation of SLIGRL-NH$_2$ ($t_{1/2}$ < 5 min) in a parallel experiment.

Animals. All in vivo experimental procedures and animal handling were conducted with approval approval from the Animal Ethics Committee at the University of Queensland (UQ), and adhere to The Australian Code of Practice for Use Of Animals for Scientific Purposes (2013) and reported in accordance with the ARRIVE guidelines.[44] Male Wister rats (8 weeks old, 250−300 g) were bred at the Animal Resources Centre (Canning Vale, WA) and air-transported to the University of Queensland Biological Resources Animal Facility (UQBR) at the Institute for Bioengineering and Nanotechnology (AIBN). Animals were housed at room temperature and kept in 12 h light/dark cycles. Standard chow and water were provided ad libitum. The rats were acclimatized for 48 h before experiments. Experiments were terminated by CO_2 inhalation as stipulated by approved ethical agreements.

Pharmacokinetic Studies. Male Wistar rats were surgically implanted with a jugular vein catheter and fasted overnight, as described.[45,46] Blood samples (200 μL) were collected from the indwelling catheter of an unanesthetized unrestrained rat, 5 min prior to compound administration (10 or 8 mg/kg p.o. in olive oil, or 1 or 0.5 mg/kg i.v. in 50% DMSO) by oral gavage (75 mm soft-tipped polyethylene 18G) or i.v. (directly via the indwelling catheter using a 250 μL glass Hamilton syringe). Further blood samples were collected 5, 15, and 30 min and at given time points from 1 to 8 h post administration. Volumes collected were replaced with sterile heparinized saline (20 U/mL). Blood samples were centrifuged (8000 rpm, 5 min), plasma-extracted, and snap-frozen on dry ice. The rats were fed after the 4 h sample was collected. Compounds were quantitated as described in the Supporting Information.

Rat Paw Edema. Hind paw swelling was induced by i.pl. injection of BR103 (350 μg in 100 μL of 5% DMSO). Vehicle only was given to sham paws. Treated animals were given an oral dose of a C3aR antagonist at 10 mg/kg dissolved in olive oil 2 h prior to agonist challenge. Sham and control rats received olive oil only. Paw thickness and width were measured using digital calipers (WPI) at 0 (baseline), 0.5, 1, and 2 h after agonist BR103 administration. In all experiments, hind paw swelling was expressed as % change in cross-sectional area from baseline, normalized to sham against maximal swelling induced by agonist alone. The magnitude of paw swelling and inflammatory responses induced by the C3aR agonist BR103 were comparable to responses induced by both a PAR2 agonist[47] and L-carrageenan (Supporting Figure 3).

For duration of action experiments, a separate group of rats received 30 mg/kg of either BR111 or JR14a dissolved in olive oil via oral gavage at 1, 2, 4, 6, 8, and 12 h prior to i.pl. injection of agonist BR103 into the hind paws. Control rats received olive oil 2 h prior to agonist challenge. Paw thickness and width were measured using digital calipers at 0 and 0.5 h after BR103 injection. To further probe

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the molecular changes that occur after JR14a administration, a group of sham, BR103 control, and JR14a-treated (10 mg/kg) rats were terminated at 0.5 h after agonist challenge, and their paw tissues were collected for further analyses (see next sections). To further assess the anti-inflammatory property of JR14a, two separate experiments were performed. In the first experiment, JR14a was administered to a group of rats at four different doses (5, 10, 20, and 30 mg/kg) dissolved in olive oil 2 h prior to i.pl. BR103 challenge. Sham and control rats received olive oil only. Paw thickness and width were measured using digital calipers at 0, 0.25, 0.5, and 1 h after BR103 injection. In the second experiment, another group of rats received JR14a at 10 mg/kg dissolved in olive oil at 0.5, 1, 2, and 4 h prior to i.pl. agonist challenge. Again, sham and control rats received olive oil only. Paw thickness and width were measured using digital calipers at 0 and 0.5 h after BR103 injection.

Histopathology and Immunohistochemistry. After euthanasia of the rats by CO_2 inhalation, plantar soft tissues of the left paw were dissected out with a scalpel blade and placed in 10% neutral-buffered formalin for 2 h at 4 °C for fixing, before being transferred to 25% sucrose solution in PBS overnight at 4°C. Tissues were then embedded with Optimal Cutting Temperature compound (OCT, Sakura Finetek, CA). Frozen sections of 5 μm were cut using a cryostat (Leica Biosystems, Germany). Tissue sections were briefly washed with distilled water before staining with 1% alcian blue (Sigma-Aldrich, pH 2.5) for 30 min and 0.5% safranin O (Sigma-Aldrich) for 5 min. They were then dehydrated and mounted in dibutylphthalate polystyrene xylene (DPX, Sigma-Aldrich).

For immunohistochemistry, paw tissue sections were briefly rinsed with PBS to remove the OCT. Except for tryptase staining, antigen retrieval was performed by incubating tissue sections in citrate buffer (0.01 M, pH 6) at 95 °C for 20 min. They were then cooled down briefly and incubated with blocking medium (PBS, 0.1% triton X-100, 10% horse serum) for 1 h at room temperature in a humidity chamber. Samples were then incubated with the respective primary antibodies (PBS, 0.1% Triton X-100, 4% horse serum; 1:100 primary antibody, including histamine, neutrophil elastase, or ED1 antibodies) overnight at 4 °C. The next day, the samples were washed with PBS and incubated with the secondary antibody (PBS, 0.1% Triton X-100, 4% horse serum, detection antibody 1:200) for 2 h at room temperature. For tryptase staining, tissue sections were incubated with primary antibody (as above with tryptase antibody at 1:100 dilution) for 4 h, then secondary antibody (at 1:200 dilution) for 2 h. Finally, tissue sections were dried, counterstained with 4′,6-diamidino-2-phenylindole (DAPI, Invitrogen, Australia), and sealed with clear nail polish. Primary antibodies for tryptase (AA1, ab2378, Abcam, Australia), histamine (H7403, Sigma-Aldrich), neutrophil elastase (ab21595, Abcam, Australia), and ED1 (ab31630, Abcam, Australia) were purchased from commercial sources. Secondary detection antibody was purchased from Abcam, Australia. All microscopic images were obtained using an Olympus BX-51 upright microscope with Olympus DP-71 12Mp color camera, utilizing DP Capture and DP Manager software packages (Olympus, Tokyo, Japan). Trypatse-, neutrophil elastase-, and ED1-positive cells were quantified using FIJI/ImageJ 1.42q software.

RNA Isolation and Gene Analysis. Following euthanasia of the rats, plantar soft tissues from their right hind paws were dissected out with a scalpel blade, snap-frozen with liquid nitrogen, and kept at −80 °C until RNA extraction was performed. Paw tissues were homogenized in TRIsure (Bioline, Australia, 1 mL per sample) using a Bullet Blender tissue homogenizer (Next Advance, NY) at 4 °C. Afterward, 200 μL of chloroform was added and the mixture was shaken vigorously. Samples were incubated at RT for 5 min before centrifugation (11 000g, 15 min, 4 °C). The clear supernatant from each sample was collected for RNA isolation using ISOLATE II RNA Mini Kit (Bioline, Australia) according to manufacturer's instructions. RNA (500 ng) was converted to cDNA using SuperScript III Reverse Transcriptase (Invitrogen, Australia) and Oligo(dT) 12−18 primer (Invitrogen, Australia) in a Veriti 96-well thermal cycler (Applied Biosystems). Real-time polymerase chain reaction (PCR) was conducted using SYBR Green (Life Technologies, Australia) in a ViiA 7 Real-Time PCR System (Applied Biosystems). All samples were duplicates, and target genes were normalized to housekeeping gene *Hprt*. Fold change was calculated relative to sham. Sequences of primers (Sigma-Aldrich) are listed in Supporting Table 1.

Statistical Analysis. Experimental results were expressed as mean ± standard error (SEM). Graphs were plotted and data were analyzed using GraphPad Prism7. Statistical differences were assessed using Student's t-tests (two-tailed) for data sets of two, one-way ANOVA for data sets of three or more with Bonferroni post-hoc tests, or two-way repeated measures ANOVA with Tukey post-hoc tests for temporal data sets, as appropriate. Significance was set at $+p < 0.05$, $++p < 0.01$, $+++p < 0.001$, $++++p < 0.0001$ compared to sham, and $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$ compared to disease control.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jmedchem.9b00927.

Experimental descriptions for synthesis and characterization (^{1}H and ^{13}C NMR, LCMS, HRMS) of compounds **7a−e**, **8a−o**, **9a−o**, **10a−o**, **11a−j**, **12a−j**, and **14a−k**; 1D ^{1}H NMR spectra, HPLC traces, and concentration−response curves for intracellular calcium release in HMDM for **10a−o** and **14a−k**; competitive binding curves for human C3a, BR103 (**2**), and BR111 (**10a**) against Eu-DTPA-C3a on HEK293-Gα_{16}-C3aR cells; and pharmacokinetics description and primer sequences for PCR (PDF)

Molecular formula strings (CSV)

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Notes

The authors declare the following competing financial interest(s): M.-K.Y., R.C.R., and D.P.F. are named inventors on a patent (US14356,821), involving complement C3a receptor agonists and antagonists, that is owned by the University of Queensland. The remaining authors declare no competing interests.

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■ ABBREVIATIONS USED

ANOVA, analysis of variance; Boc, *tert*-butyloxycarbonyl; BOP, benzotriazol-1-yloxytris(dimethylamino)-phosphonium hexafluorophosphate; C3a, Complement protein component 3; C3aR, Complement C3a receptor; Ccl2, Cd244, cluster of differentiation 244; CCL2, chemokine (C−C motif) ligand 2; dCha, D-cyclohexylalanine; CNS, central nervous system; DCM, dichloromethane; DTPA, diethylenetriaminepentaacetic acid; DIPEA, *N*,*N*-diisopropylethylamine; DMF, *N*,*N*-dimethylformamide; DMSO, dimethylsulfoxide; DPX, dibutylphthalate polystyrene xylene; ED1, monoclonal antibody clone directed against the rat CD68 protein; Et3SiH, triethylsilane; GPCR, G-protein-coupled receptor; H-bond, hydrogen bond; hC3a, human Complement protein C3a; HBSS, Hank's balanced salt solution; HEK-293, human embryonic kidney 293 cells; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid); HMDMs, human monocyte-derived macrophages; Hprt, hypoxanthine-guanine phosphoribosyltransferase; HRMS, high-resolution mass spectrometry; iCa^{2+}, intracellular calcium; Ig, immunoglobulin; IL, interleukin; LAD2, laboratory of allergic diseases 2; LDA, lithium diisopropylamide; LPS, lipopolysaccharide; MAC, membrane attack complex; NBO, natural bond orbital; OCT, optimal cutting temperature; PCR, polymerase chain reaction; PBS, phosphate-buffered saline; rp-HPLC, reversed-phase high-performance liquid chromatography; RT, room temperature; SCF, stem cell factor; SEM, standard error of mean; TFA, trifluoroacetic acid; THF, tetrahydrofuran; TMS, trimethilsilane; TNF, tumor necrosis factor α; TOF, time of flight

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