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Review

Recent developments in low molecular weight complement inhibitors

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ABSTRACT

As a key part of the innate immune system, complement plays an important role not only in defending against invading pathogens but also in many other biological processes. Inappropriate or excessive activation of complement has been linked to many autoimmune, inflammatory, and neurodegenerative diseases, as well as ischemia–reperfusion injury and cancer. A wide array of low molecular weight complement inhibitors has been developed to target various components of the complement cascade. Their efficacy has been demonstrated in numerous *in vitro* and *in vivo* experiments. Though none of these inhibitors has reached the market so far, some of them have entered clinical trials and displayed promising results. This review provides a brief overview of the currently developed low molecular weight complement inhibitors, including short peptides and synthetic small molecules, with an emphasis on those targeting components C1 and C3, and the anaphylatoxin receptors.

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1. Introduction

As a central component of the innate immune system, complement is a key player in the body's defense against invading microorganisms. In addition, it is also involved in the clearance of self-antigens and apoptotic cells (Flierman and Daha, 2007), forms a bridge to adaptive immunity, and plays an important role in inflammation (Barrington et al., 2001), tissue regeneration (DeAngelis et al., 2006), and tumor growth (Markiewski et al., 2008). For exercising these various functions, the complement cascade relies on a complex interplay of soluble and cell–surface-bound proteins. Traditionally, complement activation is described to be initiated through three major pathways (classical, lectin, and alternative pathways), which are all mainly based on the detection of surface structures by pattern-recognition proteins (Fig. 1). The classical pathway (CP) is mainly activated when complement component C1q interacts with patches of surface-bound antibodies (IgM and IgG), but also with C-reactive protein, serum amyloid P, pentraxin 3, and other ligands on the surface of apoptotic or microbial cells (Gewurz et al., 1995; Marschang et al., 1997; Nauta et al., 2004; Paidassi et al., 2008; Steel and Whitehead, 1994; Warren et al., 2002). These interactions lead to sequential activation of the serine proteases C1r and C1s, which then cleave C4 and C2 to form the CP C3 convertase (C4bC2a). In the case of the lectin pathway (LP), the same C3 convertase is generated by mannose-binding lectin (MBL)-associated serine proteases (MASPs) in response to the bind-

ing of MBL or ficolins to a wide array of carbohydrate structures on microbial, apoptotic, or necrotic cell surfaces (Jensen et al., 2007; Kuraya et al., 2005). The MASPs that cleave C4 and C2 in the LP are structurally analogous to C1r and C1s in the CP (Gal et al., 2007).

The alternative pathway (AP) not only represents an individual recognition pathway, but also serves as an amplification loop of the classical and lectin pathways. *In vivo* studies have shown that the AP alone can contribute to >80% of the total activation induced by either pathways (Harboe and Mollnes, 2008). There is a constant low-level activation of the alternative pathway as a result of the spontaneous hydrolysis of C3 to C3(H₂O), which is called “tick-over”. This hydrolysis allows the formation of an initial AP C3 convertase (C3(H₂O)Bb) in the presence of the two serine proteases factor B (FB) and factor D (FD). The C3 convertases from all pathways cleave native C3 into its two active fragments, the anaphylatoxin C3a and the opsonin C3b, the latter of which gets covalently attached to nearby surfaces. In the absence of complement regulators, surface-deposited C3b can be gradually amplified through the formation of the final AP C3 convertase (C3bBb) and cleavage of more C3. The AP can also be triggered directly by properdin, various proteins, lipids, and carbohydrate structures on foreign surfaces (Harboe et al., 2006; Kimura et al., 2008; Spitzer et al., 2007; Xu et al., 2008). C3b can be either degraded into fragments iC3b and C3d that cannot participate in convertase formation any longer but harbor important signaling functions, or it can initiate the formation of C5 convertases in both the CP/LP (C3bC4bC2a) or the AP (C3bC3bBb). These C5 convertases cleave C5 into the potent anaphylatoxin C5a and into C5b, thereby forming a membrane attack complex (MAC; C5b-9), which can lyse the target cells or foreign microorganisms (Fig. 1).

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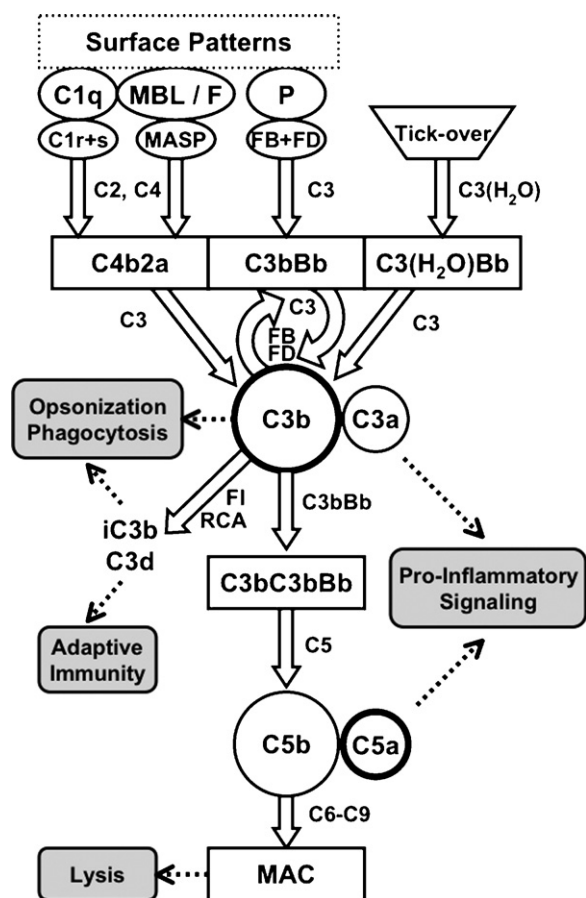


Fig. 1. Simplified representation of the complement cascade. Pattern-recognition proteins such as C1q, MBL, ficolins (F), or properdin (P) bind to surface patches on microbial or apoptotic cells and initiate the formation of C3 convertase complexes, which all cleave C3 into C3a and C3b. In addition, low levels of C3b are constantly produced via spontaneous hydrolysis of C3 (tick-over). Opsonization with C3b leads to the local generation of additional C3 convertases (self-amplification) and the C5 convertase. In the terminal pathway, this C5 convertase cleaves C5 into the potent anaphylatoxin C5a and into C5b, which forms the lytic membrane attack complex (MAC) pores. Regulators of complement activation (RCA) modulate convertase activity and enable factor I (FI) to degrade C3b into fragments iC3b and C3d, which participate in signaling and link to adaptive immune responses.

In addition to these three major activation mechanisms, there are several bypass routes that have been shown to directly trigger complement response at various stages. For example, extrinsic proteases such as thrombin or kallikrein are able to cleave and activate C3, which may indicate cross-talks with other systems such as the coagulation cascade (Markiewski et al., 2007; Ricklin and Lambris, 2007). IgM has been reported to be able to bind to MBL and directly activate the lectin pathway (McMullen et al., 2006; Zhang et al., 2006). Furthermore, C2/C4 can be bypassed in the case of complement activation induced by antibodies or oligosaccharides (Selander et al., 2006; Wagner et al., 1999). Similarly, C5 can be cleaved by thrombin, bypassing C3 (Ganter et al., 2007; Huber-Lang et al., 2002, 2006). Silica and asbestos fibers have been reported to be able to cleave C5 via mechanisms involving free radical generation and kallikrein activation (Governia et al., 2000, 2002, 2005). In addition, C3a and C5a can be generated directly from C3 and C5 by proteases found in the allergenic feces produced by dust mites (Maruo et al., 1997).

Complement is non-specific in that it can attack both foreign invaders and host cells. Under normal conditions, host cells are protected from potential complement-mediated damage by various fluid-phase and membrane-bound complement regulatory pro-

teins, including C1 inhibitor (C1-Inh), C4b-binding protein (C4BP), factor H (FH), complement receptor 1 (CR1; CD35), complement receptor Ig (CRIg), decay accelerating factor (DAF; CD55), membrane cofactor protein (MCP; CD46), and CD59 (Holers, 2008; Mollnes and Kirschfink, 2006). However, deficiencies of these protective components or excessive activation of complement in response to certain pathological conditions can overwhelm this protective mechanism. Such unbalanced activation has been associated with a growing number of diseases and pathological disorders (Mollnes and Kirschfink, 2006; Sjöholm et al., 2006). In addition, the anaphylatoxin C5a has been shown to contribute to tumor growth in mice (Markiewski et al., 2008). In these situations in which complement has deleterious effects, it is desirable to modulate its activation by using appropriate complement inhibitors (Ricklin and Lambris, 2007). The effectiveness of such therapeutic interventions has been demonstrated in numerous pre-clinical and clinical studies using both bio-pharmaceuticals (e.g. recombinant proteins and antibodies) and small complement inhibitors (Table 1). Furthermore, the U.S. Food and Drug Administration (FDA) recently approved human C1-Inh (Cinryze, ViroPharma) for hereditary angioedema (Cocchio and Marzella, 2009) and eculizumab (Soliris, Alexion Pharmaceuticals), a humanized anti-C5 monoclonal antibody for the treatment of paroxysmal nocturnal hemoglobinuria caused by an absence of the glycosphosphatidylinositol-anchored complement regulatory proteins DAF/CD55 and CD59 (Inoue et al., 2003; Rother et al., 2007).

Thus, complement inhibitors are not only needed for the treatment of complement-related disorders but also as invaluable tools for understanding the roles played by key complement components in disease models. Whereas all the complement-inhibiting drugs in clinical use and the majority of those in trials represent large bio-therapeutics (Ricklin and Lambris, 2007), there is an urgent need for low molecular weight complement inhibitors that are therapeutically effective. Despite their large efficacy and many advantages, protein drugs generally have several drawbacks: They are often expensive to produce, difficult to formulate, potentially immunogenic, and their oral bioavailability and tissue penetration are often poor. Thus, to date, these drawbacks have limited the full potential of complement inhibitors. For example, the failure of the anti-C5 mAb pexelizumab (Alexion Pharmaceuticals) in clinical studies for the treatment of acute myocardial infarction may have been partly caused by its poor tissue penetration (APEX AMI Investigators et al., 2007).

In contrast to protein inhibitors, low molecular weight drugs do not suffer from these disadvantages, and therefore they hold promise as candidates for the treatment of acute as well as chronic diseases associated with inappropriate or excessive complement activation. A large number of low molecular weight compounds have been reported to be capable of inhibiting complement; these early inhibitor candidates have been extensively reviewed in the past (Asghar, 1984; Lambris et al., 1993; Makrides, 1998). However, most of these inhibitors have proved to be plagued by a variety of problems, including poor selectivity, high toxicity, low potency, and short half-life, and will not be discussed here. Instead, this review will focus on the development of more recent low molecular weight (under 2 kDa) complement inhibitors, including small molecules, peptides, and peptidomimetics that target key complement proteins, proteases, and anaphylatoxin receptors.

2. Inhibitors targeting complement protein-protein interactions

Compared with many other pathways, the proper function of the complement cascade seems to rely on an exceptionally large number of protein-protein interactions. Despite some promising

Table 1
Examples of low molecular weight complement inhibitors used in disease models.

Compound	Target	Conditions studied	Model
Compstatin	C3	Transplantation	Human islet, in vitro (Tjernberg et al., 2008) Pig kidney, ex vivo (Fiane et al., 1999)
		Bioincompatibility	Artificial surface-induced, in vitro (Lappegard et al., 2008; Lappegard et al., 2005; Nilsson et al., 1998; Schmidt et al., 2003)
		Inflammation	<i>Escherichia coli</i> -induced, in vitro (Mollnes et al., 2002) Heparin/protamine complex-induced, baboon (Souluka et al., 2000)
		Age-related macular degeneration	Rabbit, monkey (Francois et al., 2009)
SB290157	C3aR	Lung inflammation	Guinea pig, LPS-induced (Ames et al., 2001)
		Arthritis	Rat, adjuvant-induced (Ames et al., 2001)
		Acute respiratory distress syndrome (ARDS)	Rat, cobra venom factor-induced (Proctor et al., 2006)
		Allergic asthma	Mouse (Baelder et al., 2005)
		Lupus nephritis I/R injury	Mouse (Bao et al., 2005a) Mouse, focal cerebral (Ducruet et al., 2008) Rat, intestinal (Proctor et al., 2004)
PMX53	C5aR	Inflammatory bowel disease (IBD) ARDS	Rat, TNBS-induced (Woodruff et al., 2005)
			Rat, cobra venom factor-induced (Proctor et al., 2006)
		Sepsis	Mouse, cecal ligation/puncture (Huber-Lang et al., 2002)
		Multiple organ injury	Rat, ruptured abdominal aortic aneurysm (Harkin et al., 2004)
		Inflammatory pain	Rat, mouse (Ting et al., 2008)
		Lupus nephritis Huntington's disease	Mouse (Bao et al., 2005b) Rat, 3-nitropropionic acid-induced (Woodruff et al., 2006)
		Tumor growth	Mouse (Markiewski et al., 2008)
		I/R injury	Rat, hepatic (Arumugam et al., 2004) Rat, renal (Arumugam et al., 2003) Rat, intestinal (Proctor et al., 2004)
		PMX205	C5aR
Huntington's disease	Rat, 3-nitropropionic acid-induced (Woodruff et al., 2006)		
Alzheimer's disease	Mouse (Fonseca et al., 2009)		
C089	C5aR	Allergic asthma	Rat (Abe et al., 2001)
		Thrombotic glomerulonephritis	Rat (Kondo et al., 2001)
JPE1375	C5aR	Renal allograft transplantation Tubulointerstitial fibrosis	Mouse (Gueler et al., 2008) Mouse (Boor et al., 2007)
C1s-INH-248	C1s	I/R injury	Rabbit, myocardial (Buerke et al., 2001)

efforts, the inhibition of such protein–protein interactions using low molecular weight drugs is still a challenging endeavor (Wells and McClendon, 2007). The interaction interfaces are usually much larger compared to, e.g. the pocket of enzymes, and amino acid residues involved in such interactions are often not contiguous. In addition, the contact surfaces are usually shallow and lack any grooves that would enable tight binding of small compounds. It is telling, therefore, that all the physiological complement regulators, including the protease inhibitor C1-Inh, are relatively large proteins. Despite this challenge, use of low molecular weight compounds is a valid and promising approach to regulate complement activation, as shown by the discovery of short peptides that can selectively inhibit the normal functions of C1q and C3.

2.1. C1q-selective inhibitors

The classical pathway has been identified as the major complement activation mechanism in pathological conditions such as hyperacute xenograft rejection (Platt, 1996). Inhibiting the hexameric pattern-recognition molecule C1q can effectively control classical pathway activation at its earliest stages, while leaving the lectin and alternative pathways intact to fight invading pathogens. Both small molecules and short peptides have been identified that can inhibit C1q–antibody interactions without activating the classical pathway. However, many of the small molecule inhibitors that have been reported thus far, such as derivatives of bisphenol disulfates (Bureeva et al., 2005), steroids and triterpenoids (Bureeva et al., 2007), have generally had a low potency against complement. In addition, there are concerns about their safety, pharmacokinetics, and selectivity (Assefa et al., 1999, 2001; Dinkova-Kostova et al., 2005; Roos et al., 2002). Despite these obstacles, a novel class of peptide-based inhibitors has emerged in recent years and brought new momentum to this substance group.

The cyclic peptide 2J ([CEGPFGRHDLTFC]W), selected from a phage-displayed library, was reported to bind to the globular head domains of C1q and inhibit its interaction with IgG in a dose-dependent fashion. The disulfide bond in peptide 2J has been shown to be necessary for this activity. Binding of peptide 2J did not lead to activation of C1q, probably because of the monomeric nature of this interaction, as opposed to the multimeric binding that is exhibited by surface-deposited antibody complexes. In the study, peptide 2J showed activity across a wide range of species, including humans, chimpanzees, monkeys, rats, and mice, with an IC₅₀ of 2–6 μM (Roos et al., 2001). Its efficacy has been demonstrated by the fact that it can significantly reduce C3 and C4 deposition onto pig cells exposed to human serum. Peptide 2J was recently found to share homology (GXFGXXDXXXC) with human beta-defensin 2 (LPGVFGGIGDPVTCL), which has also been reported to interact with C1q and inhibit complement activation via the classical pathway, but not the alternative pathway (Bhat et al., 2007). A molecular modeling study has shown that the proposed consensus binding site appears on the same side of the peptide 2J and human beta-defensin 2 helix displays. The binding of this consensus sequence probably involves a hydrophilic interaction between the aspartic acid residue in the peptides and an arginine residue in the C1q binding site (Comis and Easterbrook-Smith, 1985). Peptide 2J could be a promising inhibitor candidate considering its ability to bind to both murine and human C1q. However, follow-up studies with the inhibitor have not been reported so far.

2.2. C3-specific inhibitors

In many pathological situations in which complement plays a critical role, several complement pathways are activated at the same time (Harboe and Mollnes, 2008). In these cases, damage to the host tissue can be caused directly or indirectly by the gener-

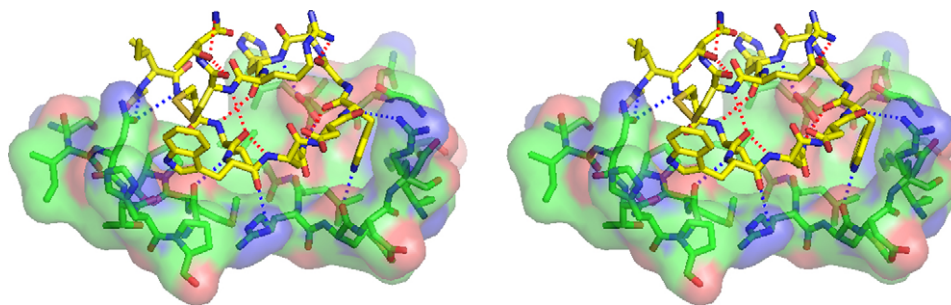


Fig. 2. Stereo representation of the polar interactions within the complex between C3c and a compstatin derivative. Red dotted lines indicate intramolecular contacts within the compstatin derivative and blue dotted lines represent intermolecular contacts between the compstatin derivative and C3c. Only C3c residues within 5 Å of the compstatin derivative are shown. The figure was prepared from PDB file 2QKI (Janssen et al., 2007) by using PyMOL (www.pymol.org). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

ation of opsonins, the MAC, and anaphylatoxins, regardless of the initiator (Fig. 1) (Lucchesi and Tanhehco, 2000). Therefore, an intervention at the central level of C3 is an attractive strategy because this approach can effectively modulate the production of all the critical complement mediators. In fact, more than half of the naturally occurring complement regulatory proteins that have been identified (FH, CR1, CRiG, DAF, and MCP) inhibit at the C3 level. As a consequence, C3 inhibitors can offer great therapeutic benefits with regard to many diseases, including AMD, in which a large variety of complement components are involved in the disease pathogenesis (Gehrs et al., 2006; Ricklin and Lambris, 2008).

Compstatin, a 13-residue cyclic peptide (H-[CVVQDWG-HHRC]T-NH₂) discovered by screening a phage peptide library, is able to selectively bind to primate C3 (and its C3b and C3c fragments) and inhibit the cleavage of C3 by both the AP and CP C3 convertases (Ricklin and Lambris, 2008; Sahu et al., 1996). The recently determined co-crystal structure of fragment C3c in complex with a compstatin derivative (Ac-[CVVQDWGAHRC]T-NH₂) has revealed that the binding site is located on a shallow groove between domains MG4 and MG5 of the β -chain of C3c and is far distant from the C3 convertase cleavage site (Janssen et al., 2007). This co-crystal structure is considered to closely resemble binding of the compstatin analogue to intact C3 because of a lack of conformational differences in the binding domains between C3 and C3c. As the MG4/5 domains are likely to be involved in the initial binding of C3 to the C3 convertase, it has been hypothesized that binding of compstatin may sterically hinder this essential interaction and thereby prevent the cleavage of C3 (Ricklin and Lambris, 2008; Rooijackers et al., 2009). The fact that compstatin is more efficacious in inhibiting the alternative pathway than the classical pathway is in agreement with this proposed mechanism, since it binds to both C3 and C3b in the AP convertase C3bBb but cannot bind to the CP C3 convertase that involves C4b. Other inhibitors of the alternative pathway that bind to the same side on C3b, such as CRiG (Wiesmann et al., 2006) or the anti-C3b mAb S77 (Katschke et al., 2009) are likely to act in a similar way as compstatin.

The structure–activity relationships (SAR) relating to compstatin have been discussed in great details elsewhere (Holland et al., 2004; Katragadda et al., 2006; Magotti et al., 2009; Mastellos et al., 2004; Ricklin and Lambris, 2008), and the present review will therefore focus on the most recent developments in this area. The bound conformation of the compstatin derivative that was revealed by co-crystallization proved to be clearly distinct from that seen for the average free conformation of compstatin in solution, as determined by NMR. The observed difference suggests a major conformational change upon peptide binding to C3c that involves a shift in the essential beta-turn (Janssen et al., 2007; Morikis et al., 1998). The bound conformation is stabilized by factors including a Cys²–to–Cys¹² disulfide bond, a beta-turn encompassing Gly⁸ and Arg¹¹, several intramolecular polar interactions (i.e. Arg¹¹–Gly⁸,

Val³–His¹⁰, Val³–Gln⁵/Cys¹², Gln⁵–Try⁷), and a sulfur–aromatic interaction between Cys¹² and Trp⁴. This conformation is also optimized for forming a tight intermolecular contact interface to the shallow pocket on the C3c surface via hydrophobic (side chains of residues 3, 4, 7, and 10) and polar (Acetyl, and residues 1, 2, 4, 5, 6, 7, and 10) interactions with C3c (Fig. 2).

The potency of compstatin was improved by 264-fold by using a combination of rational ligand-based design, molecular modeling, and biophysical studies even before the co-crystal structure had been obtained (Katragadda et al., 2006). The biggest improvement came from substitution of Val⁴ for Trp(Me)⁴. Val⁴ was identified earlier to be exchangeable in an alanine scan (Morikis et al., 1998). The Trp⁴ substitution established stronger hydrophobic interactions between the peptide and C3c, according to the crystal structure. Methylation of the Trp⁴ indole nitrogen further strengthened this hydrophobic interaction, as evidenced by the smaller entropy penalty ($-T\Delta S = 6.94$ kcal/mol) and slower dissociation rate ($k_{\text{off}} = 0.011$ s⁻¹) in the [Trp(Me)⁴]-Ac-compstatin analogue than in the [Trp⁴]-Ac-compstatin analogue ($-T\Delta S = 8.79$ kcal/mol, $k_{\text{off}} = 0.134$ s⁻¹) (Katragadda et al., 2006; Magotti et al., 2009). More recently, we have developed a novel compstatin analogue with a more than 2-fold increase in potency over the [Trp(Me)⁴]-Ac-compstatin (manuscript in preparation). Thus, potent compstatin analogues are now available for research and clinical applications.

Compstatin derivatives have been shown to be safe and effective in a series of ex vivo and in vivo experiments (Table 1). Recently, a compstatin derivative successfully completed a phase I clinical trial under the name POT-4 (Potentia Pharmaceuticals, Inc.) for the treatment of AMD. Interestingly, compstatin analogues form a gel-like deposit in the eye after intravitreal injection, from which the active peptide is slowly released over time (Francois et al., 2009). This distinct pharmacokinetic behavior may prove highly advantageous for the treatment of chronic eye diseases like AMD and is expected to reduce the frequency of intravitreal injections. Based on other experimental data, compstatin derivatives also hold great promise for the treatment of acute phase disorders from biomaterial incompatibility to autoimmune diseases such as rheumatoid arthritis (Table 1) (Ricklin and Lambris, 2007; Ricklin and Lambris, 2008).

3. Inhibitors targeting serine proteases

The complement cascade contains a series of serine proteases, which are involved in pathway initiation (C1r, C1s, MASPs and C2), amplification (FB and FD) and regulation (FI) (Sim and Tsiftoglou, 2004). From a pharmaceutical standpoint, proteases represent suitable targets for low molecular weight inhibitors because of the small size and distinct shape of their catalytic sites. Due to this high druggability and their central involvement in the com-

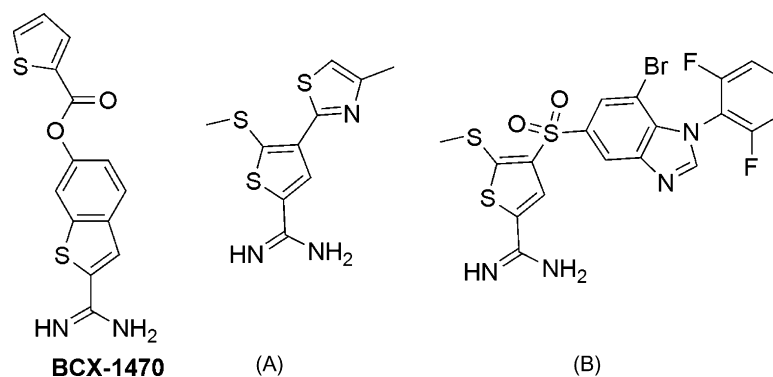


Fig. 3. Examples of small molecule C1s inhibitors. Whereas the potent inhibitor of C1s (and FD) BCX-1470 (left) has been tested in clinical trials, a novel class of compounds based on the lead structure **A** (middle) is being developed and produced an inhibitor (**B**; right) with improved specificity and affinity for C1s.

plement cascade, serine proteases have been among the earliest targets for developing complement-specific therapeutics. However, the large number of structurally similar catalytic domains of serine proteases in complement and other systems makes it often difficult to discover inhibitors that show a sufficiently high selectivity for a particular complement protease. Futhan (FUT-175, Nafamostat) is a well-known example of such broad-spectrum, small molecule serine protease inhibitors, which inhibits many systems such as pancreatic and coagulation enzymes but also the classical and alternative pathways of complement activation (Schwartz et al., 2008). Although a number of low molecular weight complement protease inhibitors have been reported to be selective, none have been widely used for in vivo studies so far.

3.1. C1s inhibitors

C1s is an 80-kDa serine protease that mediates the proteolytic activity of the C1 complex by cleaving both C2 and C4. BCX-1470, initially discovered in 1997 as a FD inhibitor with an IC_{50} of 96 nM, turned out to be a very potent C1s inhibitor, with an IC_{50} of 1.6 nM (Fig. 3) (Makrides, 1998; Szalai et al., 2000). BCX-1470 has also been tested for safety in clinical trials with healthy volunteers but the outcome of these trials have not officially been published (Morikis and Lambris, 2002; Pugsley et al., 2003). Whereas no further clinical trials have been reported, a new patent may indicate a future use of the compound in the treatment of AMD (Romano, 2008). More recently, a novel type of small molecule C1s inhibitor has been reported (Subasinghe et al., 2004, 2006; Travins et al., 2008). A lead compound (Fig. 3; molecule **A**, $K_i = 3.5 \mu\text{M}$) featuring a thiopheneamidine motif has been discovered by screening small molecule libraries (Subasinghe et al., 2004). The docking of this lead compound into a crystal structure of the catalytic domain of C1s indicated that the amidine moiety forms a salt bridge with Asp⁶¹¹ of C1s, and the thiophene moiety fully occupies the S1 binding pocket. The 5-methylthio substituent on the thiophene ring was found to be important for effective binding. Furthermore, a 4-arylsulfonyl group was found to confer better selectivity as a result of a hydrogen bond formed between the sulfonyl group and Lys⁶¹⁴, which sits at the entrance to the C1s binding pocket. Over the past few years, the affinity of the lead compound has been significantly improved, from the micromolar range to the nanomolar range, by optimizing the 4-arylsulfonyl group. Compound **B** (Fig. 3, $K_i = 10 \text{ nM}$) is among the most potent C1s-selective inhibitors reported thus far, with good selectivity over other proteases such as urokinase-type plasminogen activator, tissue plasminogen activator, Factor Xa, thrombin, and plasmin. However, in vivo data are still lacking, and there is concern about the possible in vivo toxicity of the thiophene moiety (Treiber et al., 1997).

Development of a peptidomimetic C1s inhibitor, C1s-INH-248 (M.W. 520.5), was based on the thrombin inhibitor D-Phe-Pro-Arg. This inhibitor was reported to be very potent against human and rabbit C1s (IC_{50} of 2 and 0.7 nM, respectively) (Buerke et al., 2001). It showed over 1000-fold higher selectivity for C1s compared to C1r, MASP-1, thrombin, and other related serine proteases such as plasma kallikrein or factors XIa and XIIa, and it did not inhibit the lectin pathway. Its cardioprotective effects have been demonstrated in a rabbit model of ischemia–reperfusion injury (Buerke et al., 2001, 2006). Despite these positive indicators, however, C1s-INH-248 may have a short half-life in vivo, and its structure has not been disclosed (Ricklin and Lambris, 2007).

3.2. Inhibitors of FB and C2

Despite its central involvement in both the AP C3 and C5 convertases, only little progress has been reported on designing small inhibitors of FB. Truncation of the complement C2 receptor inhibitor trispanning (CRIT), a regulator that is, e.g. found in human hemopoietic cells and some parasites, derived a 11-residue peptide (CRIT-H17; HEVKIKHFSPY), which was reported to bind FB, inhibit its FD-mediated cleavage, and reduce complement activation in a hemolysis assay (Inal et al., 2005). Furthermore, a short peptide (Ac-SHLGLAR-H) was recently described to be able to bind to the catalytic domain of human FB and inhibit FB-mediated C3 cleavage reversibly in a dose-dependent manner, with an IC_{50} of 19 μM (Le et al., 2007). It also inhibited the enzymatic activity of the C3 convertase C3bBb when the general serine protease inhibitors leupeptin and phenylmethylsulfonyl fluoride did not. Its IC_{50} value for the alternative pathway was determined to be 15 μM . This peptide was designed on the basis of C3 sequence at the cleavage site of FB. The aldehyde group was designed to react with the catalytic serine side-chain hydroxyl group to form a tetrahedral intermediate, thus achieving competitive inhibition. A serine is preferred in position 1 over charged or hydrophobic residues. In addition, peptides shorter than seven residues were suggested to be poor substrates of FB and therefore less potent. Unfortunately, this peptide also inhibited trypsin ($IC_{50} = 0.7 \mu\text{M}$) and thrombin ($IC_{50} = 24 \mu\text{M}$). It may also have C3a-like activity because it shares the same sequence with the C-terminus of C3a (SHLGLAR).

Very recently, the same authors have published that the same peptide (Ac-SHLGLAR-H) also inhibits the enzymatic activity of complement component C2, which is structurally similar to FB, with an IC_{50} of 4.2 μM . They also reported a hexapeptide inhibitor (Ac-RLLLAR-H) with increased potency of C2 inhibition ($IC_{50} = 0.33 \mu\text{M}$) (Halili et al., 2009). Given the similarities with the FB-inhibiting peptide described above, this new C2 inhibitor may share similar potential drawbacks and nothing is reported on the specificity of the new hexapeptide so far.

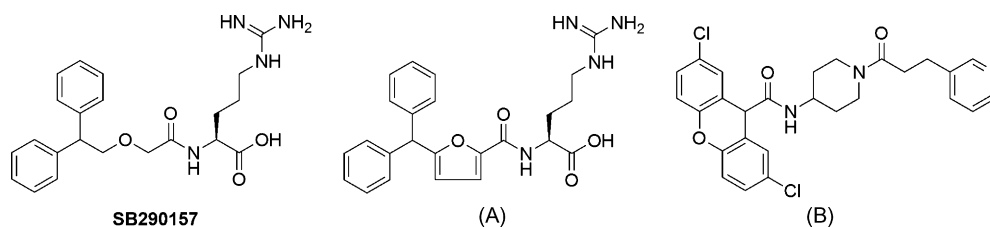


Fig. 4. Structures of C3aR antagonists. SB290157 (left) and its optimized derivative (A; middle) represent antagonists that contain an arginine moiety, whereas molecule B (right) follows a different structural concept that includes a pyridine moiety and an amino-piperidine linker.

4. Inhibitors targeting anaphylatoxin receptors

The complement system comprises three known anaphylatoxin receptors, i.e. C3aR, C5aR and C5L2, which all belong to the family of G protein-coupled receptors (GPCR) and represent potential targets for pharmaceutical intervention. Whereas the functions of the C3aR and C5aR have been extensively and conclusively described, it is still a matter of debate whether C5L2 is a decoy receptor or functional receptor (Lee et al., 2008). While C3a and C5a bind to C3aR and C5aR, respectively, with high affinity, they are quickly degraded to C3a-desArg and C5a-desArg that have greatly reduced potency. However, both C5a and C5a-desArg bind C5L2 with affinity in the nanomolar range (Cain and Monk, 2002). C3a and C5a are potent inflammatory mediators with distinct hemodynamic effects (Proctor et al., 2009). Targeting anaphylatoxin receptors can effectively regulate complement-associated downstream signaling stimulated by C3a and C5a while preserving other important early-stage complement functions, such as opsonization and MAC formation. While a selective low molecular weight inhibitor of C5L2 is yet to be discovered for functional studies, a variety of small and promising antagonists for C3aR and C5aR have been developed and used in many disease models (Table 1).

4.1. Antagonists of the C3a receptor (C3aR)

Release of C3a can lead to a wide range of cellular responses, including contraction of smooth muscle, chemotaxis, increases in vascular permeability, and the activation of leukocytes, such as macrophages, mast cells, and eosinophils (Gerard and Gerard, 2002; Hawlisch et al., 2004). Data from *in vivo* experiments have indicated that C3aR plays an important role in inflammatory pulmonary diseases and ischemia/reperfusion injury of the brain (Gerard and Gerard, 2002; Hawlisch et al., 2004; Mocco et al., 2006). It should be noted, though, that novel functions have been reported for C3a in recent years, some of which are not necessarily mediated by C3aR (Honczarenko et al., 2005; Jinsmaa et al., 2000; Nordahl et al., 2004; Ohinata and Yoshikawa, 2008; Ratajczak et al., 2004). Furthermore, C3a, along with other cationic amphiphilic neuropeptides, has been shown to be able to activate G protein independent of the C3aR, via sialic acid residues displayed on the surface of the cell membrane (Emadi-Khiav et al., 1995).

Currently available C3aR antagonists are built on two different types of scaffold: The first type has an arginine moiety (Fig. 4, SB290157, molecule (A)), while the second one features an amino-piperidine linker and a pyridine moiety (Fig. 4, molecule (B)). SB290157 (IC_{50} = 200 nM) was developed in 2001 by using library screening and chemical optimization (Ames et al., 2001). This compound was able to inhibit C3a-induced calcium mobilization in C3aR-expressing rat basophilic leukemia cells and human neutrophils (IC_{50} of 27.7 and 28 nM, respectively), C3a-induced receptor internalization in human neutrophils, C3a-mediated ATP release from guinea pig platelets, and C3a-induced potentiation of the contractile response to field stimulation of perfused rat caudal artery. Its *in vivo* therapeutic efficacy has been demonstrated in

several disease models (Table 1). However, Mathieu and coworkers demonstrated in 2005 that SB290157 had potent agonist activities in a variety of assays involving different cell types that overexpress C3aR (Mathieu et al., 2005). The authors concluded that SB290157 was a partial agonist that displays antagonist activity only in systems with very low receptor densities. Compound A (Fig. 4, pIC_{50} = 7.2) is a modification of SB290157 (Denonne et al., 2007b); it has a higher affinity because of its more rigid linker. SAR studies have shown that the arginine residue and the ether oxygen are essential for strong binding. The aryl substitution has dramatic effects on affinity as well as functional selectivity. However, both antagonists have low bioavailability and a short *in vivo* half-life because of the presence of the arginine moiety. Therefore, further optimization is clearly warranted.

Compound B (Fig. 4, pIC_{50} = 5.8) was discovered by small molecule library screening (Denonne et al., 2007a). It was the first C3aR antagonist without an arginine moiety. However, attempts to obtain more potent analogues with antagonist activity have not been successful so far. Although modifications at the xanthene or pyridine groups have provided analogues with higher affinity, these compounds act as agonists of the C3aR. In addition, *in vivo* data have shown that compound B and its analogues have a poor drug metabolism and pharmacokinetic profile, probably because of the amino-piperidine core.

4.2. Antagonists of the C5a receptor (C5aR)

C5aR, along with its native agonist C5a, modulates a wide variety of important cell-dependent activities, including phagocytosis, degranulation, chemotaxis, peroxide production, granule enzyme release, vasodilation, and cellular apoptosis (Lee et al., 2008). Numerous experiments have established the involvement of the C5aR in various inflammatory, autoimmune, and neurodegenerative disorders, in liver regeneration (DeAngelis et al., 2006), and in tumor growth (Markiewski et al., 2008; Monk et al., 2007). The development of low molecular weight C5aR antagonists was a hot topic even before the cloning of the C5aR in 1991 (Lee et al., 2008). Over the past decade, many small potent C5aR antagonists have been reported and used in various animal models as well as in humans (Table 1).

The most useful C5aR antagonists are peptidomimetics (Fig. 5), including cyclic PMX53 (Finch et al., 1999), PMX205 (March et al., 2004), and linear JPE1357 (Schnatbaum et al., 2006). All three molecules originated from C089 (Fig. 5), which was derived from the C-terminus of C5a and represented the first full C5aR antagonist (Kontekatis et al., 1994). These antagonists all bind to the second binding site (effector site) of C5a at the transmembrane region of C5aR near the extracellular interface (Monk et al., 2007). Residues in positions 1, 4, and 6 are proposed to be important for binding, while position 5 is responsible for the antagonist activity. The sequence Pro-DXaa is critical for the preference for an important reverse turn structure, which is further stabilized by a lactam ring in the cyclic analogues PMX53 and PMX205 (March et al., 2004). It is interesting to note that the binding pocket can accommo-

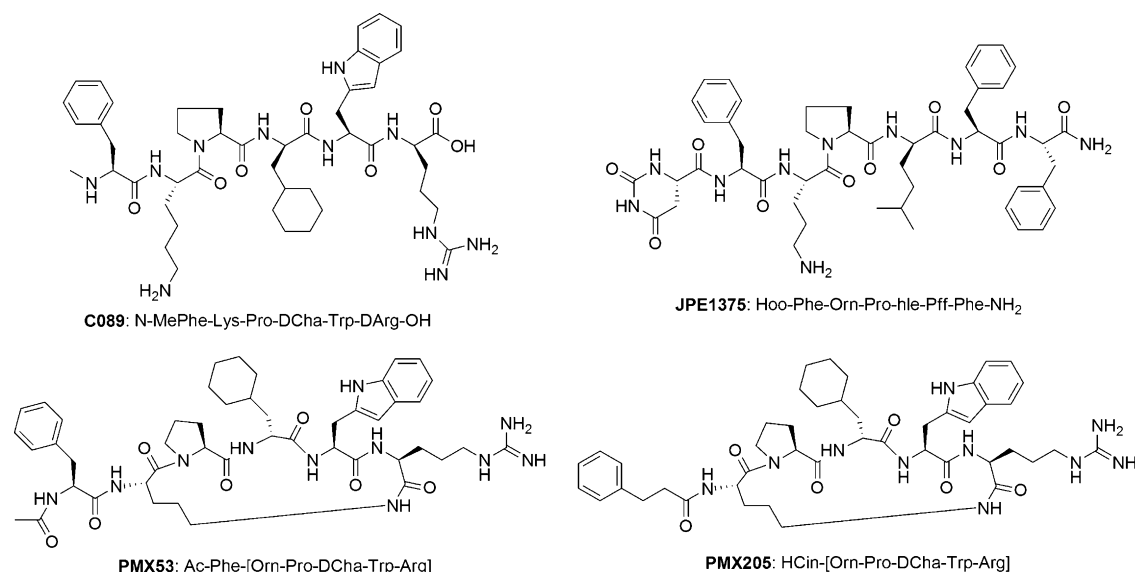


Fig. 5. Structures of the peptidomimetic C5aR antagonists PMX53, PMX205, JPE1375, and C089. Though all these antagonists were originally derived from a C-terminal peptide fragment of C5a, they have been optimized in different ways and are either linear (C089, JPE1375) or cyclic (PMX53, PMX205).

date either a charged guanidinyll group or a hydrophobic phenyl group at the C-terminus of these peptide antagonists (Schnatbaum et al., 2006). N-terminal modification affects the antagonist activity to varying degrees, depending on the species (Woodruff et al., 2001). Both PMX53 and JPE1375 effectively inhibit the release of glucosaminidase from human polymorphonuclear neutrophils in vitro with similar IC₅₀ values (31 and 41 nM, respectively). Both inhibitors are orally available and have good in vivo stability as a result of their peptidomimetic nature. Despite their functional similarities, the microsomal stability and receptor selectivity of JPE1375 are superior to those of PMX53. In addition, JPE1375 was 18 times more effective than PMX53 in blocking the chemotaxis of mouse J774A.1 cells (Schnatbaum et al., 2006).

PMX53 has been shown to be safe and well tolerated in Phase I clinical trials for rheumatoid arthritis and psoriasis (Kohl, 2006). However, a Phase Ib trial of PMX53 in patients with active rheumatoid arthritis failed to show a reduction in synovial inflammation despite that a comparable serum level of PMX53 was shown to be able to block C5aR-mediated cell activation in vitro (Vergunst et al., 2007). Similarly, PMX53 administered intra-articularly or intravenously did not show measurable effects on rat acute synovitis induced by intra-articular antibody injection (Mizuno et al., 2000). Given the prevalence of AMD and the large involvement of complement in its etiology, both PMX53 and JPE1375 have been considered for the treatment of the disease. In the case of JPE1375, the recent closure of Jerini Ophthalmic (a subsidiary of Jerini targeting eye diseases) will likely affect the current development of the compound for the treatment of advanced stages of dry AMD. Whereas clinical trials of PMX53 for AMD have also been discontinued because of insufficient activity, ongoing pre-clinical studies of the compound for other indications will be continued according to Arana Therapeutics. PMX205 (Fig. 5), an analogue of PMX53 with a hydrocinamate moiety in the place of Ac-Phe¹, demonstrated a much-improved pharmacokinetic profile and in vivo efficacy in rat inflammatory bowel disease (10- to 30-fold lower oral dose requirement) and neurodegeneration models (two-times better in crossing blood brain barrier), an improvement that was attributed to its resistance to intestinal metabolism and increased lipophilicity (Woodruff et al., 2005, 2006). Even though no clinical development of PMX205 has been announced so far, the compound showed beneficial effects concerning reduction of memory loss in a mouse model of Alzheimer's disease very recently (Fonseca et al., 2009).

Many small molecule C5aR antagonists (Fig. 6) that are not based on peptides have been reported in the literature, including W-54011 (Sumichika et al., 2002), NDT9520492 (Waters et al., 2005), NGD 2000-1 (Lee et al., 2008), CP-447,697 (Blagg et al., 2008), and NDT 9513727 (Brodbeck et al., 2008). W-54011, a racemic compound discovered as a result of a high-capacity screening and chemical optimization, is a potent competitive small molecule C5aR antagonist (IC₅₀ = 2.2 nM) with good oral availability (Waters et al., 2005). It was shown to be effective in inhibiting C5a-induced calcium mobilization, chemotaxis, and generation of reactive oxygen species in human neutrophils. Pre-treatment of gerbils with W-54011 inhibited C5a-induced neutropenia. The presence of W-54011 almost completely abrogated the enhancement of platelet-neutrophil microaggregate formation and the redistribution of neutrophil P-selectin glycoprotein ligand-1 induced by

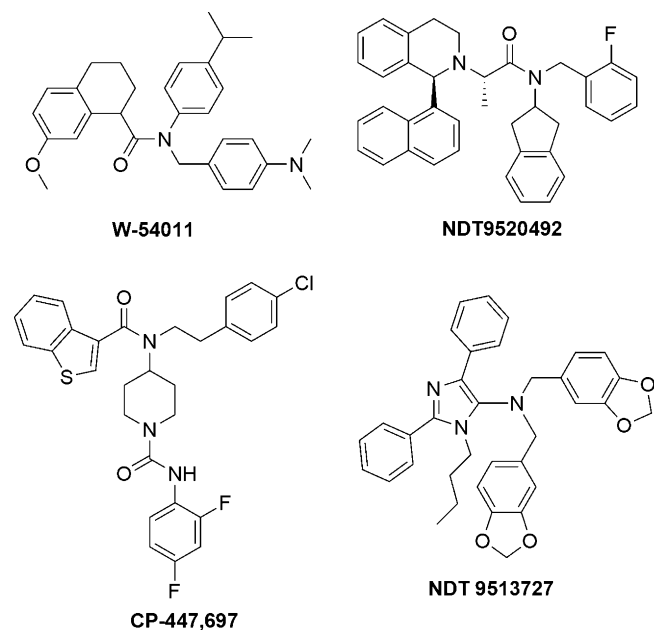


Fig. 6. Structures of small molecule C5aR antagonists. In contrast to peptidomimetic antagonists, small molecules are often selected from compound libraries and further optimized, and therefore show a much larger variability concerning scaffolds.

haemodialysis membrane-treated plasma (Itoh et al., 2008). W-54011 also inhibited the production of cysteinyl-leukotrienes, which are important mediators involved in the pathophysiology of allergic asthma, in human lung tissue that was stimulated by C5a and human serum (Hama et al., 2007). However, pre-clinical studies have been complicated by the hydrophobicity of W-54011 and its narrow species specificity for primates and gerbils.

Like W-54011, NDT9520492 is also species-specific for primates and gerbils (Waters et al., 2005). It was able to inhibit [³⁵S]GTPγS binding to wild-type human C5aR with a K_i of 15.2 nM. NGD 2000-1 is structurally similar to NDT9520492 and did not show significant therapeutic effects in a Phase II asthma study (Monk et al., 2007). Although it showed slight improvement at a maximum dose of 100 mg in a Phase II trial for rheumatoid arthritis, it was also found to inhibit cytochrome P450 3A4. Therefore, further clinical trials were halted (Lee et al., 2008). Potent analogues based on CP-447,697 (IC_{50} = 31 nM) have recently been reported, but they have shown poor bioavailability and a short half-life (Blagg et al., 2008). NDT 9513727 is a novel competitive reverse agonist that was recently disclosed by Neurogen. In cell-based assays, it inhibits a wide range of C5a-induced functions, including [³⁵S]GTPγS binding, calcium mobilization, chemotaxis, degranulation, oxidative burst, and CD11b cell-surface expression, with an IC_{50} of 1.1–9.2 nM. Studies in gerbils and cynomolgus macaques have demonstrated its ability to inhibit C5a-induced neutropenia, and it has proved to be highly selective for C5aR but not for C5L2 and more than 50 neurotransmitter and hormone receptors. In vivo experiments in rats and monkeys have also demonstrated that it is orally available, with a desirable pharmacokinetic profile. However, like other small molecule C5aR antagonists, it also shares the problem of narrow species selectivity and displayed only a rather moderate in vivo IC_{50} of 0.6 μM due to a very high plasma protein binding (>99%), likely as a result of its hydrophobicity (Brodbeck et al., 2008).

The species selectivity of peptide and small molecule C5aR antagonists is likely caused by the existence of differences in the binding sites and poor C5aR sequence homology among the various species (Waters et al., 2005). Results of receptor mutation studies have suggested that as compared to their peptide counterparts, small molecule antagonists bind more deeply within transmembrane domain V in gerbils, humans, and non-human primates, in which a Trp residue is conserved and critically involved in the binding. The lack of binding of small molecule C5aR antagonists in the case of mouse, rat, and dog is a result of the mutation of the Trp to Leu, Val, or Gly.

5. Perspectives

Complement has long been recognized as a valid and promising target for therapeutic intervention in a number of disease states. The confirmed involvement of complement in the etiology of prevalent diseases such as AMD, and the recent FDA approval of complement-targeting drugs (i.e. Soliris and Cinryze) have certainly fueled the efforts of developing complement drugs. Differential targeting of complement components is essential for the success of this endeavor, a challenge that is complicated by our limited understanding of the complement system and its complex functions and of the many disease mechanisms in which it is involved. In some situations, it is best to block anaphylatoxin receptors at a late stage of the complement cascade. In other situations, inhibition of a specific pathway at early stages, or at the C3 level, is required. Our increasing knowledge about the complement interaction network and the steady release of (co-)crystal structures of key complement components, such as FB (Janssen et al., 2009) or the C3 convertase (Rooijackers et al., 2009), are

likely to provide better tools for designing novel complement inhibitors.

Even though the quest for complement therapeutics began more than 25 years ago (Asghar, 1984), there is still a shortage of small complement inhibitors targeting different stages of the complement cascade. Peptides or peptidomimetics are among the most successful complement inhibitors because of their improved safety, selectivity, and accessibility. Their drawbacks, such as a short half-life, can be minimized through recent technological advancements in peptide optimization. Compstatin and C5aR antagonists are good examples of such efforts. Despite these achievements, however, more effort is clearly needed in terms of developing low molecular weight complement inhibitors that can selectively block the classical pathway, the alternative pathway, or C5L2 in vivo. The availability of a complete range of such inhibitors will greatly benefit our efforts to decipher the roles of complement in various complement-associated pathological conditions and the development of effective drugs for clinical applications.

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