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## Structural aspects and design of low-molecular-mass complement inhibitors

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### Abstract

We present a mini-review on the structure-based design of three promising complement inhibitors. Firstly, we review compstatin, a 13-residue cyclic peptide that binds to C3 and inhibits the cleavage of C3 to C3a and C3b. Secondly, we review a six-residue cyclic peptide that binds to C5aR and antagonizes the binding of C5a to its receptor C5aR. Finally, we review three small molecules that bind to Factor D and inhibit the enzymic action of Factor D, during which Factor D proteolytically cleaves Factor B in complex with C3 or C3b.

### Introduction

The complement system is ubiquitous and is constantly active within living species. The complement system can be activated by, and act against, both self and non-self. Under normal conditions, efficient regulation prevents activation against self; however, under pathological con-

ditions, complement regulation breaks down and activation against self is inevitable. Therefore, it is crucial to develop an array of externally administered therapeutic agents, which could control the various steps of complement activation under conditions of cellular stress or disease. There have been several efforts towards this direction, but there is currently no efficient anti-complement drug available clinically.

There are some excellent reviews on complement inhibitors, including an exhaustive database and classification of complement inhibitors of any molecular mass [1], a review of the most promising complement inhibitors [2] and various aspects of therapeutic complement inhibition [3]. Here, we will present a mini-review on the structural aspects of complement inhibitor design. Studies on structure-based complement inhibitor design are rather scarce, so this mini-review focuses on the structure-based development of the three currently most promising complement inhibitors.

A summary of the steps in complement activation follows, with selective emphasis on the complement components that are targeted by the inhibitors reviewed here. The essentials of complement activation are reviewed in [4]. The role of the complement system is to recognize invading

Key words: complement system, drug design, NMR.

Abbreviations used: Ac, acetyl group; C5aR, C5a receptor; dCha, D-cyclohexylalanine; dX, D-isomer of amino acid X; Nme, N-methylamide.

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foreign pathogens, damaged host cells and immune complexes, and to participate in their elimination. The complement system causes direct lysis of target cells, opsonizes (tags) target cell surfaces for recognition and disposal by phagocytes, and activates chemotactic movement of leucocytes. There are three pathways of complement activation, the classical, the alternative and the lectin. The pathways of complement activation involve cascades of reactions with participation of several proteins and protein complexes. Collectively, the three pathways involve four stages: recognition, protease activation, opsonization, and lysis [4].

In the classical pathway, complement component C1q recognizes target cells, through electrostatic and hydrophobic interactions, and also antigen–antibody complexes. In the lectin pathway, complement component mannose-binding lectin recognizes neutral sugars on target cell surfaces. In the alternative pathway, there is no single recognition molecule and activation occurs spontaneously, followed by rapid inactivation, unless there is an encounter of a target cell surface with the generated C3b fragment of C3. Complement activation in the alternative pathway depends on the presence of both neutral and charged sugars on the target cell surfaces [4].

Factor D is one of nine serine proteases that catalyses the first step of the alternative pathway of complement activation [4]. Unlike other pancreatic proteases that have broad specificities for catalysis of peptide bond cleavage, Factor D catalyses the cleavage of a single peptide bond of Factor B bound to C3 with hydrolysed thioester bond, C3(H<sub>2</sub>O)B. The products of this reaction are the small fragment Ba and the alternative pathway fluid-phase C3 convertase C3(H<sub>2</sub>O)Bb. This C3 convertase cleaves C3 into C3a and C3b. Factor D also cleaves Factor B in complex with surface-bound C3b (C3bB complex) to form the C3 convertase C3bBb. Additional complexes resulting from the alternative pathway C3 convertase are the surface-bound properdin-stabilized convertase C3bBbP, and the C5 convertases (C3b)<sub>2</sub>Bb and (C3b)<sub>2</sub>BbP. Complement component C3b also participates in the production of the classical pathway C5 convertase, C4b3b2a. All three pathways converge to complement component C3, which is subsequently cleaved to anaphylatoxic peptide C3a and C3b. The end result is the production of C3b, the opsonin that binds covalently to target cell surfaces and acts as a signal for phagocyte recognition.

A subsequent step in complement activation involves C5 and its cleavage to anaphylatoxic peptide C5a and C5b by the C5 convertase. This is a step towards the formation of the membrane attack complex or C5b678(9)<sub>n</sub>, which binds to lipid surfaces of micro-organisms and causes direct lysis. The anaphylatoxic peptide C5a is a chemoattractant of leucocytes, to which they can induce damage.

The critical role that C3, C5a, and Factor D play in complement activation makes them good targets for the development of complement inhibitors. This mini-review focuses on the low-molecular-mass inhibitors of C3, C5a, and Factor D currently available. Owing to both the complexity and multiplicity of complement component interactions, we expect that further promising small molecules targeting the inhibition of other complement components will be available in the future. Firstly, we present a C3 inhibitor called compstatin, which is a 13-residue cyclic peptide. Secondly, we present an un-named C5a inhibitor, which is a six-residue cyclic peptide. Finally, we present Factor D inhibitors that have led to a commercial application.

## The C3 inhibitor compstatin

### Identification

Compstatin was identified by screening a phage-displayed random peptide library against C3b [5]. The rationale behind this approach was to identify a peptide that binds to C3b that could possibly influence the interactions of C3b with other complement components. The result of this study was the identification of a 27-mer peptide that bound to C3, C3b and C3c, but not C3d [5]. A synthetic peptide with the sequence of the phage-displayed peptide, reversibly inhibited complement activation in both the classical and the alternative pathways [5]. The 27-mer peptide was truncated to a 13-mer peptide, without losing inhibitory activity, with the sequence Ile-[Cys-Val-Val-Gln-Asp-Trp-Gly-His-His-Arg-Cys]-Thr-NH<sub>2</sub> (brackets denote sites of cyclization; [5]), later named compstatin [6].

### Structure

Compstatin contains an 11-residue ring, cyclized through a disulphide bridge between Cys2 and Cys12, with two additional residues outside the ring. Elimination of the two outer residues resulted in an active peptide but with lower inhibitory activity than its parent peptide [5,6]. Also, linear analogues with reduced and alkylated cys-

teine or with cysteines replaced by alanines resulted in inactive peptides [5–9]. Efforts to reduce further the size of the cyclic ring of compstatin by preparing analogues with an increasing number of step-wise deletions starting from each terminus, resulted to inactive analogues, suggesting that the 11 member ring is the minimal sequence capable of inhibitory activity [7]. An alanine scan of substitutions within the 11 member ring demonstrated that Val<sup>3</sup>, Gln<sup>5</sup>, Asp<sup>6</sup>, Trp<sup>7</sup>, and Gly<sup>8</sup> resulted in either loss (Val<sup>3</sup>, Gln<sup>5</sup>, Gly<sup>8</sup>) or significant decrease (Asp<sup>6</sup>, Trp<sup>7</sup>) of inhibitory activity [6,8].

The three-dimensional structure of compstatin was determined using NMR and two restrained computational methods with NMR-derived restraints [6,10]. Compstatin forms a Type I  $\beta$ -turn at the opposite site of the disulphide bridge, spanning residues 5–8 (Figure 1). Interestingly, these are four out of the five residues that were deemed necessary for inhibitory activity by the alanine scan, the fifth residue being Val<sup>3</sup> outside the  $\beta$ -turn [6]. Examination of the molecular surface of compstatin revealed the presence of both a polar cluster and a hydrophobic cluster, located at opposite sites to each other. The polar cluster is formed by the segment of residues 5–11, and contains the  $\beta$ -turn. The hydrophobic part consists of the structurally contiguous segment of residues 1–4 and 12–13 at the linked termini, and contains the disulphide bridge and Val<sup>3</sup>, essential for activity (Figure 1) [9]. It should be noted that

the side chain of Trp<sup>7</sup> bends over and caps the  $\beta$ -turn with orientation away from the polar cluster. This is due to the dual hydrophobic and polar character of tryptophan, because of the presence of the aromatic ring and the indole amide, respectively. Similarly Thr<sup>13</sup> has dual hydrophobic and polar character because of the presence of a methyl and a hydroxyl group, respectively. Removal of the positive N-terminal charge when Ile<sup>1</sup> was blocked by acetylation resulted in a 3-fold more active analogue ([7–9]; A. M. Soulika, D. Morikis, M. R. Sarrias, A. Sahu, M. Roy, L. Spruce and J. D. Laubris, unpublished work). This change effectively enhanced the hydrophobic cluster at the linked termini. It should also be noted that the end residue of the  $\beta$ -turn is Gly<sup>8</sup>, which is the most abundant residue at this position for Type I  $\beta$ -turns [11]. Interestingly, a Gly<sup>8</sup>Ala substitution resulted in the most drastic loss of inhibitory activity [6]. This is attributed to the lack of a side chain that allows conformational freedom necessary for the formation of the Type I  $\beta$ -turn [6]. It was clear from all of the above studies that there is a correlation between structure and inhibitory activity of compstatin.

To gain insight into the role of key residues in the formation of structure and/or inhibitory activity, seven analogues were designed to introduce structural perturbations that would either enhance or disrupt structure. These analogue designs were studied by NMR to examine the consistency of their structural features with the structure of their

**Figure 1**

**Three-dimensional structure of compstatin**

(A) The lowest energy structure of compstatin, with only selected residues and the disulphide bridge indicated for clarity. (B) A tube representation of the backbone of the structural ensemble of compstatin structures, showing the disulphide bridge and the Type I  $\beta$ -turn. (C) A different view of the structural ensemble of compstatin in a tube representation of the backbone alone. The molecular graphics of the Figure have been generated using the structure with Code 1AIP [6] from the Rutgers/SDSC Protein Data Bank (PDB) [54], and the software MOLMOL [55].

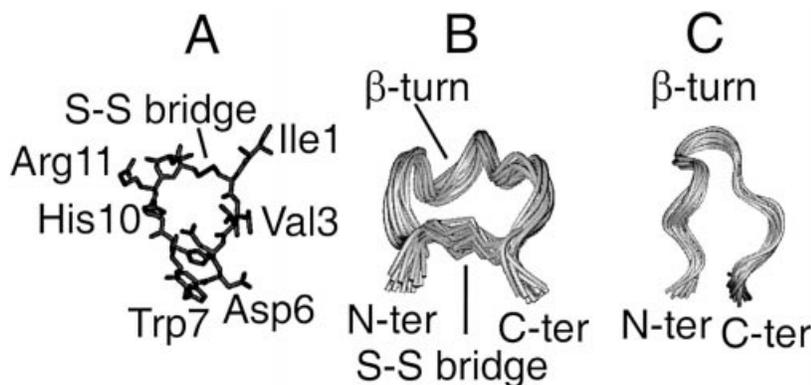


Table 1

## Benchmarks in the design of C3 inhibitor compstatin

<sup>a</sup>Original design. <sup>b</sup>Variation of original design. <sup>c</sup>This analogue was initially thought to resist proteolytic cleavage of the N-terminal residue by serum proteases, but its function was understood in terms of elimination of N-terminal charge and strengthening of the formation of the hydrophobic cluster. <sup>d</sup>Structure-based rational design. <sup>e</sup>Second generation of structure-based phage-displayed design. \*A. M. Soulika, D. Morikis, M. R. Sarrias, A. Sahu, M. Roy, L. Spruce and J. D. Lambris, unpublished work.

Peptide	Sequence	IC <sub>50</sub> ( $\mu$ M)	Reference
27 residue C3-binding peptide <sup>a</sup>	I[CVVQDWGHHRC] TAGHMANLTSHASI	19	[5]
Compstatin ring only <sup>b</sup>	[CVVQDWGHHRC]-NH <sub>2</sub>	33	[5,6]
Compstatin <sup>b</sup>	I[CVVQDWGHHRC]T-NH <sub>2</sub>	12	[5]
Ac-compstatin <sup>c</sup>	Ac-I[CVVQDWGHHRC]T-NH <sub>2</sub>	4	[7,9]*
Ac-V4A/H9A/T13I <sup>d</sup>	Ac-I[CVAQDWGAHRC]I-NH <sub>2</sub>	4	[9]
Ac-T13I <sup>d</sup>	Ac-I[CVVQDWGHHRC]I-NH <sub>2</sub>	3.2	[9]
Ac-H9A <sup>d</sup>	Ac-I[CVVQDWGAHRC]T-NH <sub>2</sub>	2.9	[9]
Ac-I1L/H9W/TG3I <sup>e</sup>	Ac-L[CVVQDWGWHRC]G-NH <sub>2</sub>	2.9	*

parent peptide, Ac-compstatin [9]. Parallel inhibitory activity studies were performed that allowed for structure–activity correlation [9]. These studies suggested that the sequence of compstatin has a propensity for turn formation, but the disulphide bridge, together with the  $\beta$ -turn, are critical for structural stability: the  $\beta$ -turn sufficiently separates the two legs of the peptide, while the disulphide bridge prevents them from drifting apart. In combination, the disulphide bridge and the  $\beta$ -turn facilitate the formation of the hydrophobic cluster. Increasing the hydrophobicity of the cluster results in increased activity [9]. Also, structural stability, hydrophobic clustering and residues of the  $\beta$ -turn segment are necessary for inhibitory activity, but none of them alone is sufficient for inhibitory activity [9]. Flexibility of the  $\beta$ -turn may be important for activity [9]. The side chain of Trp<sup>7</sup> may participate in contact with C3, possibly through hydrogen bond formation [9].

Additional inhibitory activity studies were performed on analogues that were designed to test the characteristics of compstatin that were deemed necessary for structure and function. Conservative replacement analogues showed that valine is preferable to leucine at position 3, arginine is preferable to lysine at position 11, and isoleucine is preferable to threonine at position 13. Also, some combinations of conservative replacements have compensatory effects in gaining or losing activity [9]. The structure-based design of compstatin analogues has yielded two analogues that are more

active than the parent peptide (Ac-H9A and Ac-T13I, Table 1), and one analogues that is about equally active as the parent peptide (Ac-V4A/H9A/T13I, Table 1). Other substitutions that promote turn formation of specific type (some involving D-amino acids), and a retro-inverso analogue that is resistant to proteolytic cleavage did not show inhibitory activity [7,9].

A second round of structure-based design of random phage-displayed studies has been performed, where key residues of the hydrophobic cluster and  $\beta$ -turn, Cys<sup>2</sup>, Val<sup>3</sup>, Gln<sup>5</sup>, Asp<sup>6</sup>, Trp<sup>7</sup>, Gly<sup>8</sup>, and Cys<sup>12</sup>, were kept invariable and the remaining residues were allowed to combinatorially vary, using the template: Xaa-Cys-Val-Xaa-Gln-Asp-Trp-Gly-Xaa-Xaa-Xaa-Cys-Xaa (A. M. Soulika, D. Morikis, M. R. Sarrias, A. Sahu, M. Roy, L. Spruce and J. D. Lambris, unpublished work). These studies resulted in four C3-binding clones of which one was more active than compstatin (Ac-I1L/H9W/T13G). Table 1 summarizes the benchmarks in the design of compstatin analogues.

Kinetic studies that examine the binding of compstatin and analogues to C3, using surface plasmon resonance, are also available [7,12]. These studies implicate differences in the binding mechanism between compstatin and one of the most active analogues to date, the more flexible Ac-H9A. It is possible that conformational changes in the active site of C3 occur upon binding of compstatin, but not upon binding of Ac-H9A [7,9]. This may be because of the higher flexi-

bility of the Ac-H9A analogue. Also, functional data have shown that compstatin binds to C3 and inhibits the cleavage of C3 to C3a and C3b by C3 convertase [5]. This inhibition is not because of sterically disallowed access to C3 cleavage, and does not affect the interaction of C3b with Factor B, or the formation of C3 convertases C3b(H<sub>2</sub>O)B and C3b(H<sub>2</sub>O)BP [5]. A working model for binding of compstatin to C3 is currently available, based on the combination of the kinetic, functional and structural data. This model implicates hydrophobic interactions (hydrophobic cluster), hydrogen bonding (Trp<sup>7</sup>), and shape complementarity ( $\beta$ -turn) between compstatin and the binding site of C3b [9]. Work in progress to determine the three-dimensional structure of C3-bound compstatin is expected to provide valuable insights into the mechanism of binding and into possible conformational changes in the part of compstatin upon association.

### Activity

Compstatin has been tested for complement inhibitory activity in several studies and has been shown to be an excellent candidate for becoming a therapeutic agent. Benchmark experiments that demonstrated the potential of compstatin for complement inhibition are summarized chronologically below. Firstly, it was shown that compstatin inhibits complement activation *in vitro* in normal human serum. Indeed, compstatin inhibits the alternative pathway complement activation at twice the molar concentration of C3 [5]. Secondly, compstatin was shown to inhibit complement activation in whole blood induced by biomaterials, in models of extracorporeal circuits that resemble the *in vivo/ex vivo* interface used in cardiopulmonary bypass, dialysis and plasmapheresis. These studies also showed that the presence of compstatin did not affect blood cell counts [13]. Thirdly, it was shown that compstatin prolonged the lifetime of a model of kidney xenograft from pig to human perfused *ex vivo* with human blood [14–16]. This is a model for a potential pig to human xenotransplantation situation. Fourthly, compstatin was shown to totally inhibit *in vivo* complement activation in primates, induced by heparin–protamine, without side effects. Complement activation due to administration of heparin–protamine activation is typical of cardiac surgery [17]. Fifthly, compstatin and active analogues showed little or no inhibition of clotting or of key enzymes in the clotting cascade, and did not appear to have significant cytotoxicity [8]. Finally,

compstatin shows interesting species specificity in studies using a real-time surface plasmon resonance-based assay [12]. These studies showed that compstatin binds primate C3 but not lower mammalian species C3. In addition, compstatin does not bind human C4 and C5 that are structural homologues of C3 [12].

### The C5aR cyclic antagonist acetyl-Phe-[Orn-Pro-D-cyclohexylalanine-Trp-Arg] (Ac-Phe-[Orn-Pro-dCha-Trp-Arg])

#### Identification

Currently the most promising C5aR antagonist is the cyclic peptide Ac-Phe-[Orn-Pro-dCha-Trp-Arg] [18]. Cyclization of the bracketed part of the sequence is achieved through the side chain amine of arginine and the backbone carbonyl of ornithine (macrocycle in brackets). Since there is no specific name associated with this peptide, we will call it generically cyclic C5aR peptide antagonist. An excellent recent review on complement anaphylatoxins, their receptors, and their inhibitors can be found in [19].

This antagonist is the product of several studies by different groups, spanning a period of about 15 years of research, and has evolved from studies of the interaction of C5a with its receptor C5aR. Early site-directed mutagenesis studies identified that C-terminal basic Lys<sup>68</sup> and Arg<sup>74</sup> of C5a interact with C5aR [20]. Several fragments of C5a were tested for binding to C5aR [21,22] that identified the C-terminal octapeptide (Ac-His-Lys-Asp-Met-Gln-Leu-Gly-Arg) segment of C5a as the minimal sequence for binding to C5aR [21]. Studies of octapeptide analogues showed that hydrophobic enhancement of the middle part of the sequence favourably affected the binding to C5aR [23]. Acetylation was present in the N-terminus and cyclohexylalanine (Cha) was introduced in the middle sequence. However, these studies were deemed disappointing as these peptides showed only agonist, but not antagonist, activity [23]. Things changed as subsequent studies showed that a hexapeptide analogue with the sequence NmePhe-Lys-Pro-dCha-Trp-dArg showed antagonist, but not agonist, activity against C5aR [24]. Also, these studies showed that tryptophan is preferred over phenylalanine or any other hydrophobic residue at position 5.

#### Structure

The structure of the hexapeptide analogue NmePhe-Lys-Pro-dCha-Trp-dArg was deter-

mined by NMR in  $d_6$ -DMSO or  $Me_2SO$  solvent and was found to be unusually well-defined for a linear peptide [25,26]. In particular, the peptide structure encompasses an inverse  $\gamma$ -turn spanning the Lys-Pro-dCha residues within a distorted Type II  $\beta$ -turn spanning residues Lys-Pro-dCha-Trp. Important features of this structure are the presence of an unusually strong hydrogen bond between dCha-NH and Lys-CO that stabilized the  $\gamma$ -turn, and a very weak, possibly transient, hydrogen bond between Trp-NH and Lys-CO [25]. The authors of this study focused their efforts on understanding the role of charge, hydrophobicity, isomerization of the C-terminal residue and variable length of this analogue [18,25]. They also designed cyclic analogues to reduce the conformational freedom of their peptides and studied three of them by NMR in  $d_6$ -DMSO [18,25]. The structure of the analogue Nme[Phe-Trp-Pro-dCha-Trp-dArg], where cyclization was achieved by covalently linking N- and C-termini, converged to two sets of major conformers, with added complication caused by the presence of *cis-trans* isomerization at the linked termini. The dominant characteristic of these structures was the presence of an inverse  $\gamma$ -turn as in the linear peptide [18]. They also undertook the NMR studies of the following two cyclic analogues with different isomers at the N-terminus: Ac-Phe-[Orn-Pro-dCha-Trp-dArg] and Ac-Phe-[Orn-Pro-dCha-Trp-Arg], where cyclization was achieved with a peptide bond between the side chain amine of ornithine and the main chain carboxylate of D-arginine or arginine. Note that ornithine was preferred over lysine for cyclization (ornithine resembles lysine but has one less methylene group). The structure of Ac-Phe-[Orn-Pro-dCha-Trp-dArg] showed that the  $\gamma$ -turn remained intact in the segment Orn-Pro-dCha stabilized by a strengthened hydrogen bond between dCha-NH and Orn-CO, but the Type II  $\beta$ -turn was shifted to span residues dCha-Trp-dArg-Orn (side chain of the ornithine residue) and was stabilized by a hydrogen bond between Orn-NH (side chain) and dCha-CO [25]. In this analogue, the backbone of the middle hydrophobic residue dCha is, in essence, stabilized by two hydrogen bonds, the backbone C-terminal negative charge is eliminated and the N-terminus is neutralized by acetylation. Further optimization at the C-terminus resulted in Ac-Phe-[Orn-Pro-dCha-Trp-Arg], which is currently the most potent antagonist of C5aR [18]. The structure of Ac-Phe-[Orn-Pro-dCha-Trp-Arg] showed a shifted inverse  $\gamma$ -turn, spanning

residues dCha-Trp-Arg, stabilized by strong hydrogen bonding between Arg-NH and dCha-CO [18]. This new  $\gamma$ -turn enforces a backbone conformation similar to Ac-Phe-[Orn-Pro-dCha-Trp-dArg], but with different side chain orientation [18]. Table 2 summarizes the benchmarks in the design of the C5aR peptide antagonist.

Overall, these NMR studies demonstrate that conformational restriction of the backbone in a five-residue macrocycle is essential for binding and, together with the particular side chain type composition, for antagonism without agonist properties, although the specific location of the observed turns and hydrogen bonds may not be as important. Final answers on the importance of the specific structure of the cyclic C5aR peptide antagonist can be given if its structure can be determined when bound to C5aR. It is possible that the structure of the cyclic C5aR peptide antagonist will change upon binding; however, its small length does not leave much space for significant structural changes. Also, the structures of the bound complex should be analysed in aqueous solution instead of in  $d_6$ -DMSO. The use of the organic solvent was justified because of the low solubility of their peptides. The authors of this study also collected NMR spectra of Ac-Phe-[Orn-Pro-dCha-Trp-dArg] in  $d_6$ -DMSO/water mixtures and found no significant changes in the measured 3-bond backbone coupling constants, suggesting that similar structures in  $d_6$ -DMSO and water were expected [18,25].

The three-dimensional structure of C5a determined by NMR in solution forms a four-helix bundle [27–29] with a tail of a small six-residue fifth helix at the C-terminus comprising residues 69–74 [29]. Figure 2 shows the three-dimensional structure of the eight-residue and six-residue C-terminal segment of C5a, which includes the fifth helix [29]. This segment must be rather flexible since early NMR studies had suggested an unstructured conformation [27]. The C-terminal region of C5a was used to design the initial peptides that, after iterative modelling, have yielded the design of the C5aR antagonist peptide Ac-Phe-[Orn-Pro-dCha-Trp-Arg]. Currently the modelled structure of C5aR, based on the structure of rhodopsin, suggests seven transmembrane helices (I–VII) and an extracellular extended and predominantly charged N-terminal region [26,30,31]. It has been shown that C5a interacts with C5aR at two distinct sites [32,33]; the first site involves the core of C5a and the N-terminus of C5aR and the second involves the C-terminal

region of C5a and an interhelical region of C5aR [32–34]. It is not clear what the functional relationship is between sites 1 and 2 or if there is sequential binding [34]. It has been proposed that Ac-Phe-[Orn-Pro-dCha-Trp-Arg] antagonizes C5a by disrupting the interaction on site 2, with C-terminal Arg<sup>6</sup> of the peptide and Arg<sup>206</sup> of C5aR being involved [35].

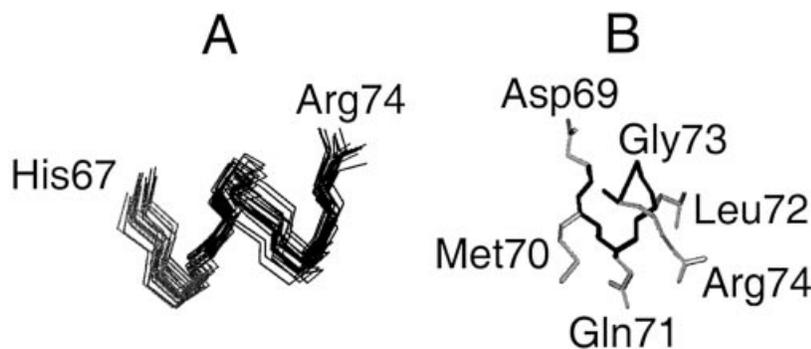
Most recent docking studies of the linear antagonist NmePhe-Lys-Pro-dCha-Trp-dArg on a model of C5aR suggest that (a) the C-terminal arginine residue of the peptide inserts into the interhelical region of helices III, V, and VI (site 2, as mentioned above), with the guanidinium group of Arg<sup>6</sup> interacting with aromatic residues between helices V and VI, (b) the hydrophobic group of

dCha<sup>3</sup> inserts favourably within the hydrophobic interface of helices III and VII, and (c) the accommodation of the peptide in the active site of C5aR may be accompanied by major structural rearrangement of C5aR helices [26]. As the authors of this study point out, it is difficult to understand the role of Trp<sup>5</sup> in activating C5aR, as inversion of the position of dCha<sup>4</sup> and Trp<sup>5</sup> (dCha<sup>4</sup>-Trp<sup>5</sup> into Trp<sup>4</sup>-dCha<sup>5</sup>) results in a conversion from antagonist into agonist [26]. Conversion of NmePhe-Lys-Pro-dCha-Trp-dArg into an agonist can also be accomplished by a mutation on C5aR [26]. It is obvious that more structural, experimental and theoretical data are needed for the receptor, and the complex of the receptor with the cyclic C5aR peptide antagonist, for a more

**Figure 2**

**Three-dimensional structure of the C-terminal segment of C5a**

(A) A backbone trace of the structural ensemble of the six-residue (in black) and eight-residue (in grey and black) C-terminal segment of C5a, His<sup>67</sup>-Lys<sup>68</sup>-Asp<sup>69</sup>-Met<sup>70</sup>-Gln<sup>71</sup>-Leu<sup>72</sup>-Gly<sup>73</sup>-Arg<sup>74</sup>, that participates in binding to C5aR. (B) A stick model of a representative structure of the C-terminal six-residue segment Asp<sup>69</sup>-Met<sup>70</sup>-Gln<sup>71</sup>-Leu<sup>72</sup>-Gly<sup>73</sup>-Arg<sup>74</sup> (backbone in black and side chains in grey). Modelling based on the sequence of the last six-residue segment has resulted in the identification of the C5aR peptide antagonists. The molecular graphics of the Figure were generated using the structure with PDB Code 1KJS [29] from the Rutgers/SDSC Protein Data Bank [54], and the software MOLMOL [55].



**Table 2**

**Benchmarks in the design of the C5aR antagonist peptides**

<sup>a</sup>A, agonist property; Anti-A, anti-agonist property; +, positive property, —, negative property.  
<sup>b</sup>Original eight residue design. <sup>c</sup>Introduction of Cha. <sup>d</sup>Introduction of tryptophan at position 5 and chirality change at position 4. <sup>e</sup>Introduction of cyclization and re-introduction of N-acetylation. <sup>f</sup>Currently most potent peptide with IC<sub>50</sub> for receptor affinity 0.3 μM and IC<sub>50</sub> for antagonism 0.02 μM [18].

Peptide	Peptide sequence	Anti-A <sup>a</sup>	A <sup>a</sup>	Reference
Peptide-1 <sup>b</sup>	Ac-HK D M Q L G R	—	+	[23]
Peptide-2 <sup>c</sup>	Ac-HK D Cha Cha Cha G R	—	+	[23]
Peptide-3 <sup>d</sup>	NmeF K P dCha WdR	+	—	[24]
Peptide-4 <sup>e</sup>	Ac-F[K P dCha WdR]	+	—	[25]
Peptide-5 <sup>e</sup>	Ac-F[O P dCha WdR]	+	—	[25]
Peptide-6 <sup>f</sup>	Ac-F[O P dCha W R]	+	—	[18]

detailed model of the mechanism of binding and antagonism.

### Activity

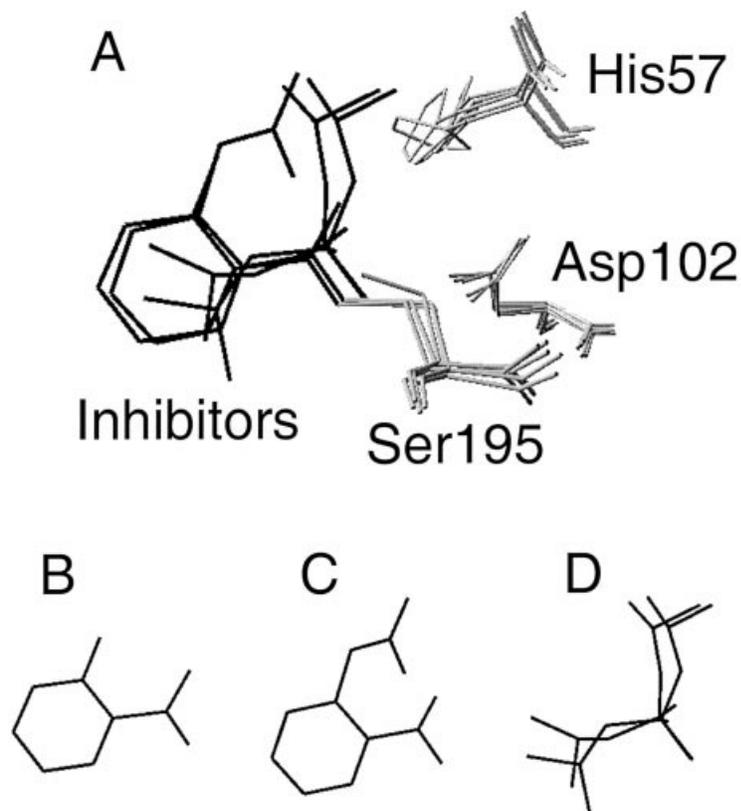
Currently the most potent C5aR antagonist is the peptide Ac-Phe-[Orn-Pro-dCha-Trp-Arg] with an  $IC_{50}$  in the nanomolar range, and it is an excellent candidate to become a therapeutic agent. Studies of the first linear peptide that exhibited antagonist and not agonist properties, showed that it inhibited C5a-induced degranulation and C5a-stimulated G-protein activation [24]. Various studies have shown that Ac-Phe-[Orn-Pro-dCha-

Trp-Arg]: (a) antagonized the binding of C5a to its receptor C5aR on human polymorphonuclear leucocytes *in vitro*, (b) inhibited C5a-induced neutrophil chemotaxis and macrophage cytokine production *in vitro*, (c) inhibited neutropenia associated with septic shock induced by lipopolysaccharide in rats *in vivo*, (d) inhibited reverse-passive Arthus reaction and endotoxic shock in rats *in vivo*, and (e) protected the small intestine of rats *in vivo*, in situations of ischaemia/reperfusion injury [18,25,36–41]. Finally, Ac-Phe-[Orn-Pro-dCha-Trp-Arg] abrogated the ability of hepatocytes to proliferate in a novel role of

**Figure 3**

**Three-dimensional structural of portion of the catalytic site of Factor D with bound inhibitors**

(A) The catalytic triad of Factor D, His<sup>57</sup>, Asp<sup>102</sup>, and Ser<sup>195</sup> (in grey), with Ser<sup>195</sup>-bound inhibitors (in black). Superposition of five structures by fitting the co-ordinates of the backbone heavy atoms. The following structures from the Rutgers/SDSC Protein Data Bank [54] have been used: (i) free Factor D (PDB Code 1HFD, [49]), (ii) Factor D with bound isatoic anhydride inhibitor (PDB Code 1BIO, [49]), (iii) Factor D with bound 3,4-dichloroisocoumarin inhibitor (PDB Code 1DIC, [48]), (iv) Factor D bound di-isopropyl fluorophosphates inhibitor form in conformation A (PDB Code 1DFP, [47]), and (v) Factor D bound di-isopropyl fluorophosphates inhibitor form in conformation B (PDB Code 1DFP, [47]). (B) Serine-bound isatoic anhydride (serine not shown; PDB Code 1BIO). (C) Serine-bound 3,4-dichloroisocoumarin (serine not shown, PDB Code 1DIC). (D) Serine-bound di-isopropyl fluorophosphates inhibitor form in conformations A and B (serine not shown; PDB Code 1DFP). The molecular graphics of the Figure were generated using the software Swiss PDB Viewer [56].



complement in liver regeneration in an *in vivo* model with mice [42].

## Inhibitors of Factor D

### Identification

We believe that the identification of Factor D inhibitors reviewed here is based on earlier work that has shown substituted isocoumarines as weak inhibitors of complement serine proteases [43]. It has also been shown that di-isopropyl fluorophosphate blocks catalytic activity of serine proteases [44]. A general review on Factor D inhibitors can be found in [45]. Here we will focus on the use of structural information in the design of small Factor D inhibitors. The process of a successful identification of Factor D inhibitors involved the determination of the three-dimensional structure of both free Factor D and Factor D with bound inhibitors on the catalytic serine that block the initiation of catalysis [46–49].

### Structure

The structural basis of the catalytic mechanism with which serine proteases cleave peptide bonds is well understood [50] and an excellent review of the structural aspects of Factor D can be found in [51]. Briefly, a catalytic triad comprises serine, histidine and aspartate residues in close proximity. The hydroxyl group of serine acts as a nucleophile and attacks the peptide carbonyl group of the prospective cleavage site of the substrate protein, forming a tetrahedral transition-state intermediate. The nearby histidine participates in the formation of the tetrahedral transition state by receiving a proton. The protonated form of histidine is favoured by Coulombic interaction between histidine and aspartate, which raises the  $pK_a$  of histidine. Subsequently, the tetrahedral intermediate breaks down to an acyl intermediate and a cleaved substrate bond. Finally, the acyl intermediate hydrolyses to two (cleaved) fragments of the substrate protein and the intact serine protease. In addition to the catalytic triad, this process is accomplished with the aid of the oxyanion hole that both accommodates and stabilizes the negatively charged tetrahedral transition state, the specificity pocket that provides favourable interaction with the particular side chain in the substrate that occupies the position before the cleavage of the peptide bond (typically an arginine residue), and the non-specific substrate binding site. The catalytic serine of Factor D is an excellent target for binding of an inhibitor that will not allow catalysis to take place.

A number of three-dimensional crystallographic structures of free and inhibitor-bound Factor D have been determined [46–49,52]. Figure 1(A) shows a superposition of the catalytic triad of Factor D, His<sup>57</sup>, Asp<sup>102</sup>, and Ser<sup>195</sup> and Ser<sup>195</sup>-bound inhibitors, from five structures: free Factor D [49], Factor D with bound isatoic anhydride inhibitor [49], Factor D with bound 3,4-dichloroisocoumarin inhibitor [48], and Factor D with bound di-isopropyl fluorophosphates inhibitor form in two conformations [47]. Figure 3(B–D) shows the Ser<sup>195</sup>-bound inhibitors where Ser<sup>195</sup> is deleted for clarity. It is believed that the tightly bound inhibitors do not permit the dynamic rearrangement of the catalytic site that is necessary for the enzymic activation to take place. Also, the conformational space of the catalytic site, between Ser<sup>195</sup> and His<sup>57</sup>, is occupied by the bulky or branched inhibitors, disallowing access and cleavage of the proper peptide bond of C3b(H<sub>2</sub>O)B [45]. Modified inhibitors have been designed with hydrophobic components and functional groups that enhance binding by favourably interacting with the surrounding hydrophobic environment [45]. Out of these studies, a number of compounds that are potent complement inhibitors were identified, among them the commercial compound named BCX-1470 [45].

### Activity

The end compound BCX-1470 has the highest inhibitory activity of Factor D, with an IC<sub>50</sub> of approx. 100 nM [45]. This is two orders of magnitude higher than the inhibitory activity of another commercially available general protease inhibitor, called FUT-175, when it inhibits Factor D [45]. It should be noted that the Factor D inhibitors also inhibit C1s and other serine proteases *in vitro*, although many of the common serine protease inhibitors cannot inhibit Factor D. This may be because of the high specificity of Factor D, which is known to cleave proteolytically only a single enzyme, the C3 convertase C3b(H<sub>2</sub>O)B [45].

BCX-1470 has been shown to be safe in animal toxicity studies [45] and has blocked the development of reverse-passive Arthus reaction-induced oedema in rats [45,53]. BCX-1470 has also been tested in clinical trials with healthy human volunteers [45]. It is believed that BCX-1470 may be a candidate therapeutic for complement activation during cardiopulmonary bypass surgery [45].

## Summary

Each of the inhibitors we have presented here has unique abilities to inhibit complement activation. Compstatin is an inhibitor that can shut off all three pathways of complement activation, because of its action on C3, the complement component of convergence of all three pathways. The C5aR antagonist can shut off the C5a-mediated pro-inflammatory effect. The inhibitors of Factor D can shut off the alternative pathway because they block the formation of C3 convertase, which in turn is responsible for the amplification of complement activation. Complement activation through more than one pathway is typical in clinical conditions. Also typical is the cross-activation of complement pathways.

Although there is progress in the structure-based design of the inhibitors we presented here, the lack of three-dimensional structures of target–inhibitor complexes for compstatin and cyclic C5aR peptide antagonist does not allow for a detailed understanding of the inhibition mechanism. Such understanding can lead to better designs with improved function. Structural information of complexes is crucial in the cases where the inhibitor changes or adjusts its structure upon binding. This is typical for the larger inhibitor peptides (e.g. > 5–6 residues) that may have more conformational freedom for structural changes upon binding. In favourable cases of larger peptide inhibitors or for small peptide inhibitors, the three-dimensional structure of the inhibitor alone may be a good template that is complementary to the structure of the active site. Complementarity here is understood as favourable hydrophobic interactions, electrostatic interactions, hydrogen bonding interactions and steric interactions (shape complementarity).

In certain instances, the static time-averaged three-dimensional structures may not be sufficient to understand binding, either because the binding site does not have the right size and shape or because there is no entrance channel that leads from the solvent to the active site. When dynamic rearrangements may be necessary for binding, molecular simulation using computational methods may also be necessary; however, availability of a three-dimensional structure is a requirement to initiate computational studies, such as stochastic dynamics, molecular dynamics, Monte Carlo calculations or electrostatic calculations.

In the case of Factor D inhibitors, three-dimensional structures of the complexes are available. Typically, an inhibitor for a catalytic process imitates the transition-state tetrahedral intermediate. Since the transition-state intermediates are short-lived transient states, direct study using crystallography or NMR is not possible. Alternatively, a catalytic process can be understood using a pseudo-substrate and/or a pseudo-cofactor. However, additional computational modelling and molecular simulation is usually required. Typically, one introduces theoretical structural perturbations and calculates dynamic properties of the enzyme to understand the catalytic mechanism.

The work reviewed here shows the use of NMR to obtain structural information in solution for numerous peptide analogues, and the use of crystallography to obtain structures of large proteins or complexes, in the course of inhibitor design. Some of the C5aR work is based on modelling of the structure of the receptor. We propose that, in addition to continuing efforts in obtaining three-dimensional structures of complexes, computational studies that will simulate the internal dynamics of proteins, the dynamic interactions of the components of the complexes, as well as their interactions with the solvent, are necessary.

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