Discovery and Design of First Benzylamine-Based Ligands Binding to an Unlocked Conformation of the Complement Factor D

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Supporting Information

ABSTRACT: Complement Factor D, a serine protease of the S1 family and key component of the alternative pathway amplification loop, represents a promising target for the treatment of several prevalent and rare diseases linked to the innate immune system. Previously reported FD inhibitors have been shown to bind to the FD active site in its self-inhibited conformation characterized by the presence of a salt bridge at the bottom of the S1 pocket between Asp189 and Arg218. We report herein a new set of small-molecule FD ligands that harbor a basic S1 binding moiety directly binding to the carboxylate of Asp189, thereby displacing the Asp189-Arg218 ionic interaction and significantly changing the conformation of the self-inhibitory loop.

KEYWORDS: Alternative complement pathway, factor D inhibitors, fragment-based drug discovery, structure-based design

Complement factor D (FD), the rate-limiting enzyme of the alternative pathway (AP), plays a key role in pathway activation and amplification by cleaving Factor B (FB) in its complex with C3b to generate active convertases on target surfaces, leading to membrane-attack complex (MAC) formation and cell lysis.^{1,2} Dysregulation of AP activity predisposes individuals to diverse disorders such as age-related macular degeneration (AMD) and paroxysmal nocturnal hemoglobinuria (PNH).^{3,4} FD is a trypsin-like serine protease that, in its latent conformation, is autoinhibited by the self-inhibitory loop (residues 214 to 218) inducing a nonproductive arrangement of the catalytic triad (His57, Ser195, Asp102) and blocking Asp189 at the bottom of the S1 pocket by forming a salt bridge with Arg218 of the self-inhibitory loop. Neutral nonpeptidic inhibitors binding to the “closed” S1 pocket, previously described by us (Chart 1), were derived by structure-based drug design and fragment-based screening.^{5–7} The X-ray crystal structures of compounds 1–3 bound to human FD revealed that this class of inhibitors extends from the S1 to S2' specificity pockets with the Asp189-Arg218 salt bridge in place. A structurally related center

proline-based analog has entered recently Phase II clinical trials for the treatment of PNH.⁸

The discovery of 2-indole-carboxamide 4 (Scheme 1) by WaterLOGSY NMR screening of a FD structure-based fragment

Scheme 1. Fragment Growing Based on the Indole-Carboxamide S1-Binding Motif of Ligand 4

library as a low-affinity ligand of latent FD (K_d value of 1600 μ M, determined by ^1H , ^{15}N -HSQC NMR) has been a key accomplishment of our previously reported efforts.^{5,6} The crystal structure of 4 in complex with FD (Figure 1) revealed the indole-carboxamide to be positioned in the unique S1 pocket of latent FD, which is characterized by a “closed” self-inhibitory loop conformation and a salt-bridge between Asp189 and Arg218. The amide of 4 forms H-bonds with Thr214 and Arg218, and the indole NH is within H-bond distance to the Arg218 backbone. The benzyl ether portion of 4, lacking electron density in the crystal complex, is exposed to solvent space. Importantly, the S1', S1 β , and S2'

Chart 1. First Reported Noncovalent, Reversible FD Inhibitors

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Figure 1. (A) Crystal structure of **4** (PDB 5FBI; green color) in complex with human FD. H-bonds of the CONH₂ to the carbonyl of Thr214 and NH₂ of Arg218 (self-inhibitory loop) and of the indole-NH with the Arg218 carbonyl are highlighted (dashed lines), as well as the Asp189-Arg218 salt bridge. The benzoate portion is solvent exposed and not visible in the electron density map. (B) Superposition of **4** (green color) and **5** (cyan) in complex with FD, closely overlapping in S1 (closed conformation). (C) X-ray structure of **6** (green) bound to FD. The basic NH₂ is at close distance to Asp189 and Ser190 and to two bound waters (dashed lines). Arg218 adopts an outward conformation with the guanidine pointing toward solvent. The Arg218 carbonyl interacts with the amide NH of **6**. A conserved water at the oxyanion hole, observed also in the FD/**4** complex (A), is highlighted.

recognition sites of FD, identified as hot spots for binding,⁶ remain unoccupied. This prompted us to embark in a parallel drug discovery effort on a *de novo* growing approach from the P1 indole-2-carboxamide fragment to target the adjacent S1' and S1 β regions, ultimately aiming at new chemotype FD inhibitors offering the potential for a differentiating overall ADMET profile.

A closer inspection of the binding pose of **4** in the X-ray complex suggested to us that the indole C4-position could provide a suitable vector for growing toward the S1' region. Furthermore, a water molecule is forming two H-bonds with the side chain hydroxyl of Ser195 and the NH of Gly193 constituting part of the oxyanion hole (Figure 1A). We therefore considered to explore the NHCO (anilide) and CONH (amide) linker at the indole-C4, which could engage in either a direct contact with the Gly193 NH or in a water-mediated interaction with Gly193 and Ser195 (Figure S1).

In order to probe the design concept, we synthesized compound **5** (Scheme 1) bearing a symmetrical adamantyl residue at the C4 anilide spacer. The latter was selected for its hydrophobic bulk and spherical shape, proposed by modeling to favor hydrophobic contacts to the surface of the S1' pocket. In addition, a distal basic NH₂ was incorporated to improve aqueous solubility of the ligand to allow *K_d* determination by NMR. Compound **5** showed only very weak chemical shift perturbations in the presence of FD in the protein-observed ¹H,¹⁵N-HSQC NMR spectrum at the highest tested concentration (2000 μ M). Most gratifyingly, the X-ray structure of **5** bound to FD was resolved after crystal soaking (1.67 Å resolution) confirming the binding pose as modeled (Figure 1B). The 2-indolyl-carboxamide common to **4** and **5** closely overlaps in S1 adopting a conserved locked conformation with the Arg189-Asp218 salt bridge in place. The adamantyl of **5** occupies S1' similarly as was reported for *N*-(1-adamantyl)-*N'*-(4-guanidinobenzyl)urea (WX-293T) bound to urokinase.⁹ The basic NH₂ of **5** points toward the solvent and is involved in interactions with the backbone carbonyl of His57 and Ser215 mediated by two water molecules. The carbonyl oxygen of the anilide forms a 3.0 Å H-bond contact to the Gly193 NH at the oxyanion hole, with a different orientation to that observed for (S)-proline-based inhibitor **1**.

The design concept for **6** (Scheme 1) is illustrated in Figure S1. In brief, the linker carbonyl was modeled to form a H-bond with an enzyme-bound water and the amide NH with the side chain oxygen of solvent exposed Ser217, respectively. While the hydrophobic phenyl moiety would not be in direct van der

Waals contact to the enzyme, the *meta*-position could be in close proximity to the flexible acidic 60-loop on top of the S1' pocket. The *meta*-aminobenzyl moiety was selected as we reasoned that due to the lack of contact to S1' such an ionic interaction could be beneficial for solubility and potency, despite being solvent exposed.

Compound **6** showed weak binding affinity to FD, determined by ¹H,¹⁵N-HSQC NMR (*K_d* of 1000 μ M), in a similar range compared to **4**. Again, we succeeded to resolve the X-ray crystal complex at high 1.67 Å resolution (Figure 1C). Strikingly, the binding pose of **6** is flipped virtually by 180° with the benzylic NH₂ forming a direct salt-bridge with the COOH of Asp189 at a 2.9 Å distance. In addition, H-bonding of the NH₂ to the Ser190 hydroxyl and two waters bound deep in S1 were observed. Intriguingly, the self-inhibitory loop is displaced due to major conformational movements, thereby disrupting the Asp189-Arg218 salt bridge of FD. As a consequence, the side chain of Arg218 is redirected with its guanidine residue exposed to solvent space. The indole scaffold of **6** is shielded from solvent on either side by the side chains of Lys192 and Arg218, respectively, both adopting an extended conformation. The terminal CONH₂ of **6** is engaged in a water-mediated H-bond to the carbonyl of His57 adopting a noncatalytic "outward" conformation. Furthermore, the NH of the reversed amide linker of **6** is in H-bond contact with the carbonyl of Arg218, while the spacer carbonyl interacts with the oxyanion hole mediated by a conserved enzyme-bound water. The latter perfectly overlays with that observed in the crystal structure of FD/**4** (Figure 1A), forming H-bonds with Gly193 (NH) and Ser195 (OH). The S1 pocket in its "unlocked" conformation remains quite narrow, similar to the closed S1 conformation of FD in complex with proline-based inhibitors (Chart 1).^{5,6}

Benzylamines and related benzamides are well-known privileged structural elements that are readily accommodated by the S1 binding hot spot of many trypsin-like S1 proteases and that have found successful applications in the design of highly potent, selective inhibitors of multiple members of this enzyme family.^{10,11} Remarkably, we had not identified any potent hits bearing such functional motifs in our previous high-throughput screens based on either FD-mediated thioesterolysis or membrane-attack complex (MAC) formation.⁵ To the best of our knowledge, **6** is the first basic benzylamine-derived ligand reported to date, which binds in a canonical manner to the S1 pocket of FD by forming a salt-bridge to Asp189 upon adopting an

“unlocked” S1 pocket conformation. The existence of such an open conformation of FD was confirmed by the seminal X-ray crystallography work reported by Forneris et al. (PDB 2XWB; not available at the time of our work).¹² These authors observed a major rearrangement of the self-inhibitory loop in the ternary protein complex formed between S195A mutant FD and C3bFB. This repositioning provided access to the nonprime site for substrate binding, albeit no electron density was observed for the scissile loop of FB bearing the P1 Arg234. Furthermore, the His57 side chain conformation resulted in an active configuration, in contrast to the complex structure with **6** (*vide supra*). In the self-inhibited state, Arg218 of FD forms a salt bridge with Asp189 at the bottom of S1, whereas the Arg218 side chain points outward in C3bFB-FD. The R218A mutation disrupts a crucial interaction responsible for maintaining the self-inhibited state of FD. Interestingly, the crystal structure of the highly flexible state of the R218A FD variant (PDB 4CBN) displays a 6:4 distribution of the inactive vs active conformation of the catalytic triad,¹³ while only the inactive conformation is observed in wild-type FD (PDB 1DSU¹⁴). Consistently, the R218A FD variant shows increased catalytic activity toward artificial peptides. The major differences between the crystal structure of FD-bound **6** and the C3bFB-FD complex, including the respective His57 conformation and the arrangement of the self-inhibitory loop, are depicted in Figure S2.

Apparently, electrostatic interactions with the S1 Asp189 residue are not sufficient to provide high-affinity fragments for FD, in contrast to other serine proteases^{10,11} in which Asp189 is more accessible to ligand contacts in the apo structures. We speculate that the locked conformation of FD (observed in its apo crystal structure PDB 1DSU) may be lower in energy, as FD circulates in blood in an inactive latent state that is switched to its active conformation by an allosteric mechanism upon binding to the large C3bFB protein complex.

Intrigued by the unexpected binding of **6**, we next performed a substructure search in the Novartis compound archive using the 3-(aminomethyl)aniline pharmacophore as the query. An NMR ¹⁹F reporter assay (FAXS¹⁵) was set up to enable K_d determination of weak-affinity ligands at higher throughput and under reduced protein consumption compared to K_d determination by ¹H, ¹⁵N-HSQC titration. Inhibitor **1** was used as the ¹⁹F reporter to screen for active site binders, to rank-order hits based on their relative competition against **1**, and to determine K_d values reliably up to 1000 μ M. Among several ligands identified, **7** (Scheme 2), an in-house pan-kinase inhibitor, attracted most of our interest due to its good binding affinity (NMR K_d of 50 μ M) and potency in the thioesterolysis assay (IC₅₀ of 20 μ M).

The predicted binding pose of **7** suggested the S1 β pocket (composed of the Cys191-Cys220 disulfide bridge and the side chains of Val219, Leu17, His146, and Ile143) to accommodate the phenol moiety. We had previously identified by *in silico* FD active site mapping analysis⁶ the S1 β subpocket to be a potential hot spot for binding that could provide an attractive opportunity for ligand design. The exploitation of S1 β for the generation of potent and selective S1 protease inhibitors had been reported previously, e.g., for thrombin, coagulation Factor Xa, and urokinase.¹⁶

The X-ray crystal complex of **7** was obtained by soaking FD crystals of the space group P1, which we identified as the first crystal form of FD without crystal contacts in the proximity of the S1 β region (Figure 2). The 1H-pyrazolo[3,4-*d*]pyrimidine of **7** adopts a slightly twisted out-of plane conformation relative to the benzylic residue. The pyrimidine nicely overlays in a coplanar fashion with the phenyl portion of indole **6**. However, in the X-ray

Scheme 2. Evolution of the S1-Binding Benzylamine Fragment by Substructure Searches, Merging, and Structure-Based Morphing

Figure 2. Crystal structure of **7** bound to FD with close-up of the S1 and S1 β pockets. Portions of the self-inhibitory loop are not shown as these were not clearly visible in the electron density.

crystal complex of **7**, part of the self-inhibitory loop is not clearly visible. The anilinic N3 and N4 atoms linking the *meta*-benzylamino and the *meta*-phenol residue to the center scaffold are pointing toward the Arg218 carbonyl at 3.1 and 3.4 Å distance, respectively. The S1'/S2' subpockets are entirely unoccupied by **7** (Figure S3). Instead, the phenol is nicely accommodated by the S1 β pocket with the phenyl ring making favorable sulfur–aryl interactions¹⁷ with the Cys191-Cys220 disulfide bridge and the OH forming an H-bond with the imidazole N δ of His146.

The unprecedented FD-binding features of ligands **6** and **7** paved the way for several subsequent avenues of structure-guided design (Scheme 2). The biaryl amide topology of **6** in its binding pose to FD projects the indole-2-carboxamide portion toward the solvent, thereby limiting the options to expand the ligand further into the prime pockets. Likewise, the pyrazolo[3,4-*d*]pyrimidine of **7**, and particularly the N-1 suitable for chemical substitution, appeared less attractive for growing due to an unfavorable distance to the protein surface. We therefore envisaged the *de novo* design of S1 benzylamine-based fragments bearing suitable exit vectors that provide alternative attachment points for growing and targeting the oxyanion hole, S1', and/or the S1 β subpocket. The design concepts for two new series based on the biaryl amine scaffold derived from **7** and on the biphenyl (reversed) amide template from **6**, respectively, are discussed in the subsequent sections.

First, we envisaged to morph the pyrazolo[3,4-*d*]pyrimidine of **7** to the related 1-amino-quinazoline scaffold offering a promising growing vector into S1' by tethering aliphatic substituents to the NH₂ group (Scheme 2). In addition, we considered to introduce a

properly positioned COOH residue to the putative S1' binding motif, which could potentially interact with the oxyanion hole. Carboxylic and phosphonic acids interacting at the oxyanion hole are found in various crystal structures of S1 proteases such as factor VIIA,¹⁸ urokinase,¹⁹ and chymase.²⁰ The first prototype analog we prepared, 2,4-diamino-quinazoline **8** (Scheme 3),

Scheme 3. Structure-Based Guided Evolution of the Benzylamine Hit **7 to Biaryl Amine Derivatives **8–10** Extended toward the Oxyanion Hole and S1'**

showed an NMR K_d of 170 μ M and an IC_{50} of 75 μ M (thioesterolysis). The cocrystal structure of **8** with FD matched the predicted binding mode (Figure 3). Specifically, the N-

Figure 3. X-ray structures of (S)-**8** (green) and (1R,2S)-**9** (yellow) bound to FD. H-bonds of the NH at the C-4 position of the center quinazoline to the backbone of Arg218 (self-inhibitory loop), and the COOH moiety to Gly193 (NH) and Ser195 (NH, OH) are highlighted (dashed lines).

isopropyl residue is accommodated by S1' and the COOH forms a H-bond network to Gly193 and Ser195. In order to better fill the hydrophobic S1' pocket, racemic *cis*-2-amino-cyclohexanecarboxylic acid derivative **9** (Scheme 3) was prepared but was found to be just equipotent to **8**. Only the (1R,2S)-configured enantiomer was observed in the cocrystal complex with FD (Figure 3).

The corresponding phenyl portions of **8**, **9** (quinazolines), and **6** (indole), respectively, are closely overlapping in a coplanar manner (Figure S4). The upper part of the fused phenyl ring, however, is exposed to solvent space and not in binding contact to the protein. The closely related and less lipophilic 2,4-diaminopyrimidine **10** (Scheme 3) showed indeed a similar binding affinity, suggesting that the annulated phenyl of **8** is not contributing significantly to binding affinity.

Remarkably, compounds **8–10** constitute the first weakly active FD ligands representing a betaine-based chemotype characterized by a terminal basic amine binding to S1 in an unlocked conformation and a spatially distant COOH interacting to the oxyanion hole. The two moieties are predicted to be able to make an ionic interaction in solution. Betaine-type inhibitors of other S1 proteases were reported previously.^{18,19}

In parallel, we explored the biphenyl amide scaffold (Scheme 2) as a complementary design concept emerging from the superposition of benzylamine **6** and (S)-proline-based **1**^{5,6} in their FD-bound crystal complexes (Figure 4). The carboxamide

Figure 4. Superposition of **1** (PDB SFBE, orange) and **6** (green) bound to FD adopting two different topologies of the S1 pocket: (a) a closed conformation in complex with **1** in which Arg218 of the self-inhibitory loop forms a salt-bridge with Asp189; (b) a more open "unlocked" conformation in complex with **6** in which Arg218 is in a flipped-out position.

spacer of **6** closely overlaid with the P1 phenyl residue of **1** in an almost coplanar fashion, while the phenyl portion of **6** (stacking onto the Cys191-Lys192 peptide bond lining the deeper portion of S1) overlaid with the carboxylic ester moiety of **1**. This prompted us to merge the two structural elements placed within S1, thereby forming a hydrophobic planar biphenyl template. Fragment **11** (Scheme 2), available in the Novartis archive, was indeed identified by FD-observed ¹H,¹⁵N-HSQC NMR to be a very weak binder (K_d > 2000 μ M). Its docking pose in the unlocked S1 conformation of FD of the X-ray complexes of **6** suggested the *meta*-position at the "upper" phenyl portion of **11** to be viable for ligand growing either toward the prime site or the S1 β region. The docking model suggested a potential H-bond interaction of a 3-carboxamide spacer (3-CONHR, Scheme 2), when oriented toward S1 β , with the Arg218 carbonyl similar to the NH at the 3-position of pyrazolo[3,4-*d*]pyrimidine **7** (Figure S5).

A small library of biphenyl-methylamines decorated with aliphatic or arylalkyl substituents attached either to a reversed carboxamide (**12a–e**) or an amide spacer (**13a–c**) was synthesized, and binding affinities to FD were determined by the ¹⁹F NMR reporter assay (Table 1). NMR K_d values correlated nicely with the corresponding IC_{50} values measured in the thioesterolysis assay. All compounds showed a significant increase in FD binding affinities compared to fragment **11**.

The X-ray cocrystal structure of **12b** showed six FD molecules in the asymmetric unit (C222 space group; 2.25 Å resolution), three of which form a complex with **12b** characterized by an open self-inhibitory loop conformation, similar to that observed for **6** and **7** (Figure 5A) but with the Arg218 side chain displaced from S1 forming crystal contacts within the unit cell. The benzylic NH₂ of **12b** is again found in a highly conserved position similar to that of **6** and **7** and in binding contact to Asp189 (COOH) and Ser190 (OH). The biphenyl portion extends between the hydrophobic chains of Arg218 and Lys192, while the *sec*-butyl group makes only minimal contacts to S1'. This observation and the flat SAR seen for **12a–e** suggested that the van der Waals contacts to the hydrophobic S1' site are suboptimal for these ligands and that further optimization or extension into S2' is required to increase binding. We recently disclosed a preliminary account on the

Table 1. FAXS NMR Binding Affinity (K_d) and *in Vitro* Potency (IC_{50}) Data for Biphenyl Amides 12–14

Entry	Cpd	R	NMR K_d (μM) ^a	FD thio IC_{50} (μM) ^{b,c}
1	12a	CH ₃	680	690
2	12b		120	120
3	12c		220	257
4	12d		100	43
5	12e		160	140
6	13a		80	310
7	13b		20	54
8	13c		80	98
9	14		<10	3.4 ^d

^aDissociation constant K_d determined by ^{19}F NMR using **1** as reporter.⁸ ^bHalf-maximal inhibition of FD, determined in a thioesterolysis assay with Z-Lys-thiobenzylester as substrate. ^cData represent mean values of duplicate measurements. ^dFD ELISA IC_{50} = 11 μM .²

Figure 5. (A) X-ray overlay of **12b** (cyan) and **13b** (orange), both binding to the “unlocked S1” pocket. The His57 adopted either a flipped-outward (cyan) or a catalytic triad-like (orange) conformation. (B) X-ray complex of **13b** spanning S1–S1 β . Two conformations of the benzylamine portion of **13b** (orange and violet) and succinic acid (green and cyan), compatible with the electron density map, are shown.

design of potent cyclopentyl-(1*R*,2*R*)-dicarboxamide inhibitors of FD spanning S1–S2' by merging **11** with **2**.²¹

The predicted docking pose of **13a** (Table 1) toward S1 β indicated a preference for benzylic substituents (Figure S6). The conformationally rigid analog (S)-**13b** showed a 4-fold improvement in NMR binding affinity vs **13a**. The cocrystal structure of FD/**13b** confirmed the 1,2,3,4-tetrahydronaphthalene to be positioned in S1 β and the amide NH in close distance to the Arg218 carbonyl (Figure S6).

Most noteworthy, we observed the presence of a single succinic acid molecule originating from the crystallization buffer to be positioned close to the oxyanion hole (Figure 5B). One of the two COOH moieties forms a H-bond with the N^e-imidazole nitrogen of His57, as well as with Gly193 (NH) and Ser195 (OH), while the second carboxylate is not involved in any polar contacts. Further, the His57 side chain was for the first time observed to adopt the catalytically active triad conformation very similar to that observed in the ternary C3bFB-FD complex (PDB 2XWB)¹² (Figure S7). The highly conserved binding pose of the common biphenyl-methylamine pharmacophore of **12b** and **13b** (Figure 5A) prompted us to prepare the merged analog **14** (Table 1) targeting S1' and S1 β simultaneously.

Strong chemical shift perturbations were observed for **14** in the 1H , ^{15}N -HSQC spectrum (Figure S8), and binding to the protein was in slow exchange on the NMR time-scale in line with an IC_{50} value of 3.4 μM . Hence, **14** showed a remarkable 35- and 15-fold increase in potency vs **12b** (filling only S1') and **13b** (occupying only S1 β), respectively. Compound **14** was also shown to block proteolytic FD activity and cleavage of factor B (IC_{50} of 11 μM) in an ELISA assay format.²

In summary, we have discovered a new class of basic P1 benzylamine-based FD inhibitors targeting an unlocked conformation of the S1 pocket and binding directly to the COOH residue of Asp189. X-ray crystal structure determination was critical to unveil the flipped binding pose of a weak-affinity ligand to an unprecedented FD active site conformation in which the Asp189-Arg218 interaction in S1 is displaced due to conformational movements of the self-inhibitory loop. The principal design concepts that further emerged by substructure searches and structure-guided approaches of this work were subsequently exploited to develop orally potent and selective FD inhibitors and will be reported in due course.²²

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmedchemlett.8b00104.

Full experimental procedures for compounds **5**, **6**, **8**–**10**, **12a**, **12c**–**e**, **13b**, and **14**; crystallographic data collection and refinement statistics for crystal structures (PDF)

Accession Codes

PDB codes for the X-ray crystal structures described in this study have been deposited in the Protein Data Bank under the accession codes 6FTY, 6FTZ, 6FUG, 6FUH, 6FUI, 6FUJ, and 6FUT.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

ADMET, absorption, distribution, metabolism, excretion, and toxicity; AP, alternative pathway; FB, factor B; FD, factor D; FBS, fragment-based screening; SBDD, structure-based drug design; AMD, age-related macular degeneration; PNH, paroxysmal nocturnal hemoglobinuria; MAC, membrane attack complex

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