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**Journal**

ACS Medicinal Chemistry Letters, 11(5)

**ISSN**

1948-5875



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**Publication Date**

2020-05-14

**DOI**

10.1021/acsmedchemlett.9b00663

Peer reviewed



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ACS Medicinal Chemistry Letters

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# Factor H-Inspired Design of Peptide Biomarkers of the Complement C3d Protein

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Cite This: *ACS Med. Chem. Lett.* 2020, 11, 1054−1059 Read Online

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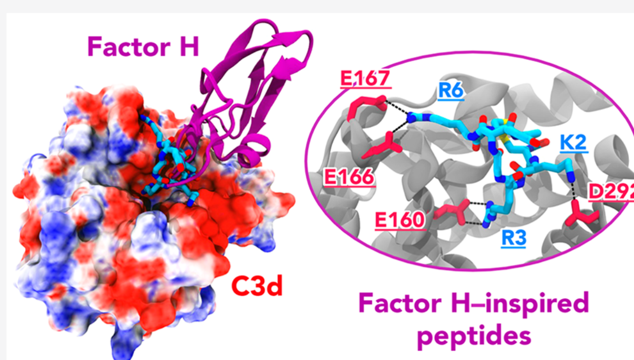
**ABSTRACT:** C3d is a hallmark protein of the complement system, whose presence is critical to measure the progression of several immune diseases. Here, we propose to directly target C3d through small peptides mimicking the binding of its natural ligand, the complement regulator Factor H (FH). Through iterative computational analysis and binding affinity experiments, we establish a rationale for the structure-based design of FH-inspired peptides, leading to low-micromolar affinity for C3d and stable binding over microsecond-length simulations. Our FH-inspired peptides call now for further optimization toward high-affinity binding and suggest that small peptides are promising as novel C3d biomarkers and therapeutic tools.




**KEYWORDS:** *Structure-based drug design, protein−ligand binding, molecular dynamics, hit discovery*

The complement system is an effector arm of the immune system, which eliminates pathogens, promotes homeostasis, and forms a bridge between innate and adaptive immunity.[1,2] Complement proteins are key actors in severe pathologies, such as age-related macular degeneration (AMD), a widespread eye disease leading to permanent vision loss over age 60.[3] Under-regulation of the complement system exacerbates this disease, contributing to the formation and growth of drusen under the retinal pigment epithelium.[4] To inhibit disease progression, monitoring complement activity is a key strategy.[5] In this respect, the complement protein C3d is considered a hallmark of complement activation.[1] Indeed, under-regulation of complement results in the accumulation of C3d in tissues, making C3d a natural biomarker of complement activation. When the complement system is dysregulated, C3b (the precursor of C3d) is rapidly amplified on cell surfaces. This triggers the function of the complement regulator Factor H (FH), which promotes the degradation of C3b into C3d, and the accumulation of the latter in tissues.[6] Hence, C3d is a promising clinical biomarker for AMD. Given the physiology of the eye, a fluorescent molecule targeting C3d can be used as a noninvasive diagnostic biomarker for AMD. Previous studies have shown that fluorescently labeled monoclonal antibodies conjugated to C3d can be candidates as diagnostic tools.[7] Virtual screening studies have also been performed to search for small molecules with intrinsic fluorescence and affinity for C3b, the precursor of the C3d.[5] However, these small molecules exhibited low solubility and low binding affinity toward C3d with low dissociation constants ($K_d$) between 1 and 500 $\mu$M.

Here, we propose to directly target the C3d through small peptides mimicking the binding of FH. Peptides are increasingly entering in the drug pipeline and obtaining FDA approvals, as they can have excellent stability and can be administered by injection and also orally, while also having low production cost compared to monoclonal antibodies. Hence, peptides can be promising to target C3d and can be useful as diagnostic and therapeutic tools. In the future, these peptides may be further conjugated to FDA-approved fluorophores for direct imaging of drusen in the eye.

Crystallographic structures and biochemical studies[8,9] have made available critical information characterizing the binding interaction between C3d and FH. Leveraging this existing knowledge, we hypothesize that small peptides binding C3d can be designed as inspired from FH, preserving the key interactions that contribute to a thermostable binding interface. Hence, we initiate the design of small peptides (e.g., 10−15 residues of about 1500−2000 Da molecular weight), mimicking the binding of the complement regulator

**Special Issue:** In Memory of Maurizio Botta: His Vision of Medicinal Chemistry

**Received:** December 30, 2019
**Accepted:** February 28, 2020
**Published:** February 28, 2020

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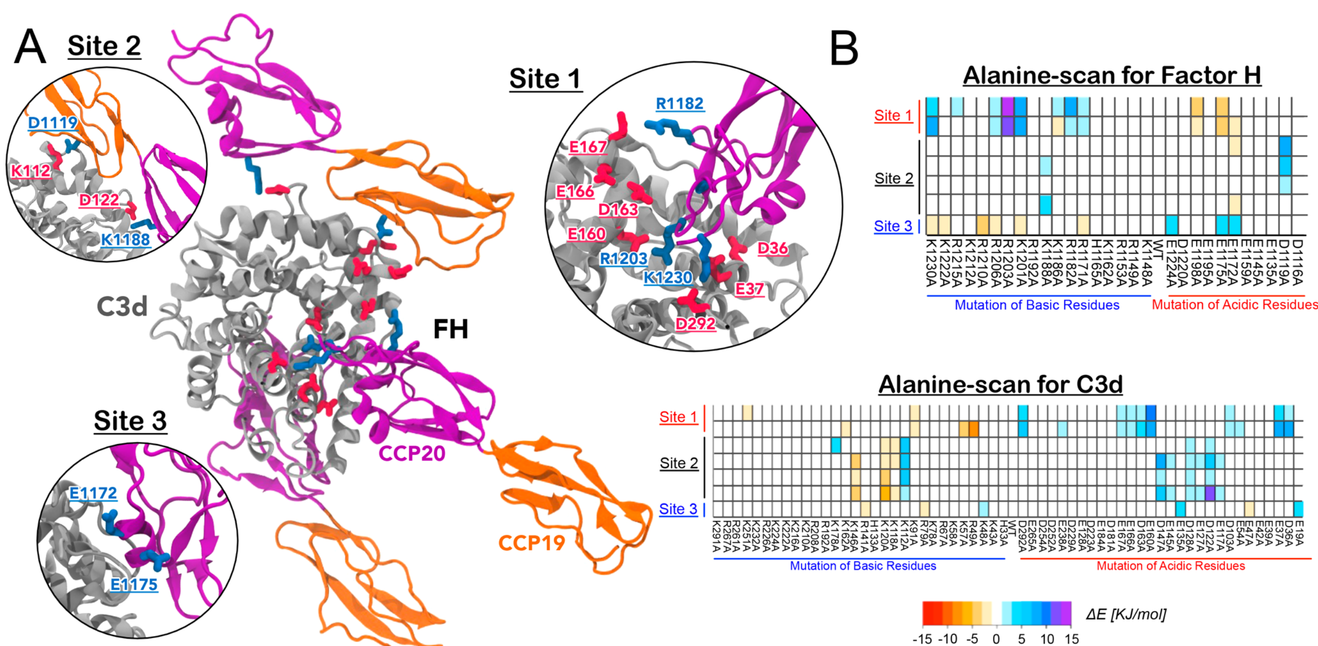


**Figure 1.** (A) Binding modes of Factor H (FH) to the complement protein C3d, as from crystallographic structures. FH is composed of the Complement Control Proteins (CCP) 19 and 20, which bind at three distinct sites of C3d: Site 1, referred as the "acidic patch", Site 2, and Site 3. C3d is shown as gray ribbons, while FH is shown highlighting the CCP19 (orange) and CCP20 (violet) in different colors. A close-up view of the three sites is reported, showing critical interactions. (B) Change in free energy of binding for the C3d−FH complex (compared to the wild-type) upon alanine-scan of FH (top) and C3d (bottom panel). Two X-ray structures are used for analysis: 3OXU[8] and 2XQW,[9] considering several chains. Negative values (red) correspond to gain of binding upon alanine mutations, while positive values (blue) correspond to loss of binding. Adapted with permission from ref 10. Copyright 2015 Wiley-VCH Verlag GmbH & Co. KGaA.

FH to C3d. Toward this aim, we performed computational analysis of the C3d−FH interaction, assessing the preferred site for the binding of FH and identifying the key residues participating in the interaction. Based on this information, molecular dynamics (MD) has been employed to guide the design of FH-inspired peptides, flanked by microscale thermophoresis (MST) experiments, used to test their ability to bind C3d. Our iterative approach shows that FH-inspired peptides can bind C3d in the low-$\mu$M range, thereby supporting the concept that small peptides mimicking FH can serve as C3d biomarkers. These outcomes pose the basis for future optimization of our identified "hit", aimed at reaching nM binding. This is a promising strategy to design novel biomarkers and therapeutic tools for age-related macular degeneration.

## RATIONALE FOR THE DESIGN OF FH-INSPIRED PEPTIDES

FH is a 155 kDa protein composed of modular protein elements, called complement control proteins (CCP), which are covalently linked forming "beads-on-a-string" structures.[8,9] Each CCP is stabilized by two disulfide bridges, contains up to three beta strands, and typically contains a conserved tryptophan. Structures of the C3d−FH complex have suggested that FH CCP19 (6321 Da) and CCP20 (7633 Da) can bind at three possible sites (Figure 1A) of C3d (31 772 Da). Site 1 involves a negatively charged surface of C3d, referred to as the "acidic patch", binding to positively charged FH CCP20, while Site 2 predominately involves interactions between CCP19 at a ridge of C3d not far from the "acidic patch". Located on the opposite face of C3d, Site 3 involves binding of the slightly positive, basic surface of C3d to the positively charged FH CCP20 module.

A previous computational study[10] suggested that Sites 1 and 2 are most likely physiologically relevant due to stable salt bridges, hydrogen bonds, and aliphatic interactions. On the other hand, Site 3 is likely less relevant given the low interfacial surface area, unfavorable electrostatic interactions, and proximity to a thioester moiety that may be inaccessible when covalently bound to a cell surface. In terms of binding affinity, CCP20 binds C3d with a $K_d$ of 410 nM, while CCP19 binds C3d with a dissociation constant ($K_d$) of 1.14 $\mu$M.[9] These two binding domains contribute to high avidity of FH for C3d. Indeed, FH CCP19−20 bind C3d with a $K_d$ of 180 nM, a higher affinity interaction than CCP19 or CCP20 in isolation. Taken together, these studies indicate that the binding of small peptides at one (and preferred) site of C3d is unlikely to fully disrupt the interaction between FH and C3d. As also previously shown,[5] this is highly beneficial for the design of diagnostic biomarkers, since it does not result in the dysregultation of the complement function. To achieve the design of FH-inspired peptides binding C3d, it is desirable to target the preferred binding site, which displays stronger binding.[5] Hence, the investigation of the binding properties of the FH natural ligand is a fundamental step. Considering the electrostatic nature of the C3d−FH interaction, we performed an electrostatic analysis, to identify the preferred binding site and key residues of FH responsible for binding.[10] Specifically, we performed a computational alanine-scan—i.e., the systematic mutation in alanine of each ionizable amino acid one at a time—computing the change in free energy of binding upon mutation. We used the AESOP (Analysis of Electrostatic Similarities of Proteins) method, developed by Morikis (details


https://dx.doi.org/10.1021/acsmedchemlett.9b00663
ACS Med. Chem. Lett. 2020, 11, 1054−1059

are in the Supporting Information).[11] The AESOP protocol enables predicting changes in the free energy of binding upon residue perturbation and has been shown to be efficient in computational alanine-scan studies.[12−15] The method identifies residues that cause "loss of binding" upon alanine mutation (i.e., displaying positive values in the change in free energy of binding with respect to the wild-type) and that are therefore mainly responsible for tight interactions.

As a result, FH seems to electrostatically prefer the binding at Site 1, which displays the highest number of residues affected by alanine mutation (Figure 1B, top panel). Sites 2 and 3 are less susceptible to alanine mutation, indicating that the electrostatic character of the interaction at this level is weaker. At Site 1, residues R1282, R1203, and K1220 of CCP20 show the highest "loss of binding" upon alanine-scan. Notably, by performing alanine-scan of the C3d counterpart, negatively charged residues of the "acidic patch" (i.e., D36, E37, E160, D163, E166, E167, D292) also show "loss of binding" indicating the key role of ionic interactions at Site 1 (Figure 1B, bottom panel). This indicates that the formation of the C3d−FH complex at Site 1 profits from strong ionic interactions between positively charged residues of FH and the "acidic patch". Interestingly, CCP20 binds C3d with a $K_d$ of 410 nM, which is lower (i.e., higher in affinity) than the $K_d$ of CCP19 for C3d (1.14 $\mu$M).[9] Considering that alanine mutation of the CCP20 residues R1282, R1203, and K1220 at Site 1 displays a remarkable "loss of binding", while the CCP19 residues at Sites 2 are less susceptible to alanine mutation, the increased binding affinity of CCP20 can be related to strong electrostatic interactions at Site 1. Accordingly, by inspection of the crystallographic complexes (Figure 1A), Site 1 displays proper accommodation of the FH charged residues. Site 2 shows two pairs of ionic interactions between D1119−K112 and K1188−D122. At Site 3, positively charged residues of FH do not find a negatively charged counterpart in C3d.[10] Overall, visual inspection of the crystallographic interactions suggests targeting Site 1 for the design of small binding peptides. However, free energy analysis through the AESOP computational protocol allows a systematic evaluation of the critical residues responsible for tight binding, facilitating our peptide design. We note that electrostatic analysis has been performed over two crystal structures of the C3d−FH complex, and considering different crystallized chains, which capture the three binding sites and also display local differences. This consensus constitutes a solid basis for structure-based drug design. Building on the systematic evaluation of the electrostatic interactions between C3d and FH, we selected Site 1 as a target for the design of FH-derived peptides.

## ■ ITERATIVE RATIONAL DESIGN

Here, we performed iterative rational design of FH-inspired peptides binding at Site 1 of C3d. During our initial design, we preserved the R1282, R1203, and K1220 of FH, as these residues show the highest "loss of binding" upon alanine-scan (Figure 1B) and favorably accommodate into the C3d "acidic patch". For naming our peptides, we adopted a scheme that identifies the C3d target site (i.e., Site 1) and the peptide numbers. Accordingly, S1P1 is the first peptide that targets Site 1. Our first designed peptide is constituted by the sequence [CKRGYRC]gpgAK (brackets denote cyclization, lower-case denotes residues not present in the sequence of FH). This initial peptide has acetylated N-termini and amidated C-termini and includes a cyclized portion, which has been used to constrain the conformational space in an effort to promote binding to C3d. This cyclization reduces the conformational entropy of the peptide and is expected to promote binding. Subsequently, miscroscale thermophoresis (MST)[16,17] experiments have been used to estimate the dissociation constant ($K_d$) for the C3d−S1P1 complex. As a result, we found weak binding with a $K_d$ of 2.1 ± 1.6 mM (Figure 2). The dose−response observed was not fully formed, contributing to the higher uncertainty of the $K_d$.

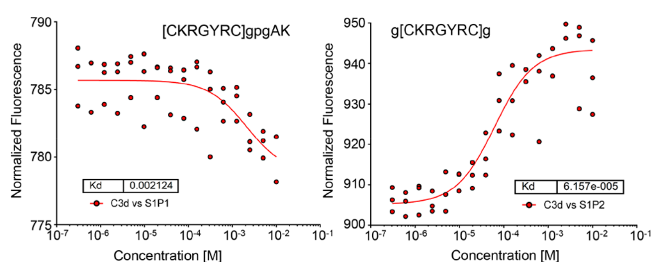


**Figure 2.** Thermophoretic response of fluorescently labeled C3d is observed at varying concentrations of the S1P1 (left) and S1P2 (right) peptides (sequence on top of the graphs). For S1P1, the dose−response curve suggests binding, but is not fully formed, due to low affinity of S1P1 for C3d. The dissociation constant ($K_d$) is estimated at 2.1 ± 1.6 mM. For S1P2, the dose−response curve is fully formed and $K_d$ is estimated as 61.6 ± 20.0 $\mu$M. Units of the inset for $K_d$ are M.

To understand the origin of the low binding affinity detected through MST, and to gain insights on how to improve the binding of S1P1 through rational design, we employed MD simulations, which are useful for drug discovery.[18,19] All-atom MD has been performed on the S1P1 peptide in solution and on the full C3d−S1P1 complex, reaching ∼1 $\mu$s for each system. This enabled characterizing the structural stability of S1P1 in solution and within C3d and easily identifying regions of the peptide affected by high flexibility, which do not positively contribute to the binding. Analysis of the per-residue Root Mean Square Fluctuations (RMSF) indicates large fluctuations at the level of the uncyclized region (i.e., residues A11, K12) of S1P1, both in solution and bound to C3d (Figure 3A). As expected, when bound to C3d, residues 1−10 of S1P1 display lower fluctuations. However, residues A11 and K12 display high fluctuations as in the solvent, suggesting that the uncyclized tail of S1P1 might not favor C3d binding. Visual inspection of the ∼1 $\mu$s trajectory of the C3d−S1P1 complex reveals that S1P1 quickly changes configuration during the first ∼30 ns and assumes a different binding mode (Figure 3B). In this configuration, R3 binds E167/E166/D163, while R6 binds E37. Cluster analysis reveals that this new complex is relatively stable (except for the uncyclized flexible tail, which assumes different configurations), as it belongs to the first three most populated clusters, accounting together for the ∼80% of sampled configurations. The relative stability of this configuration can be ascribed to stable interactions of R3/R6 with the negatively charged residues of C3d, which are preserved along ∼1 $\mu$s of MD (Figure 3C). We note that R3/R6 correspond to the FH R1203/R1282, which have been shown through electrostatic analysis to be critical for binding (Figure 1B). This indicates that arginine residues at positions 3 and 6 of our peptide can act as "ionic anchors", suggesting that they should be preserved during optimization (see below). Finally,

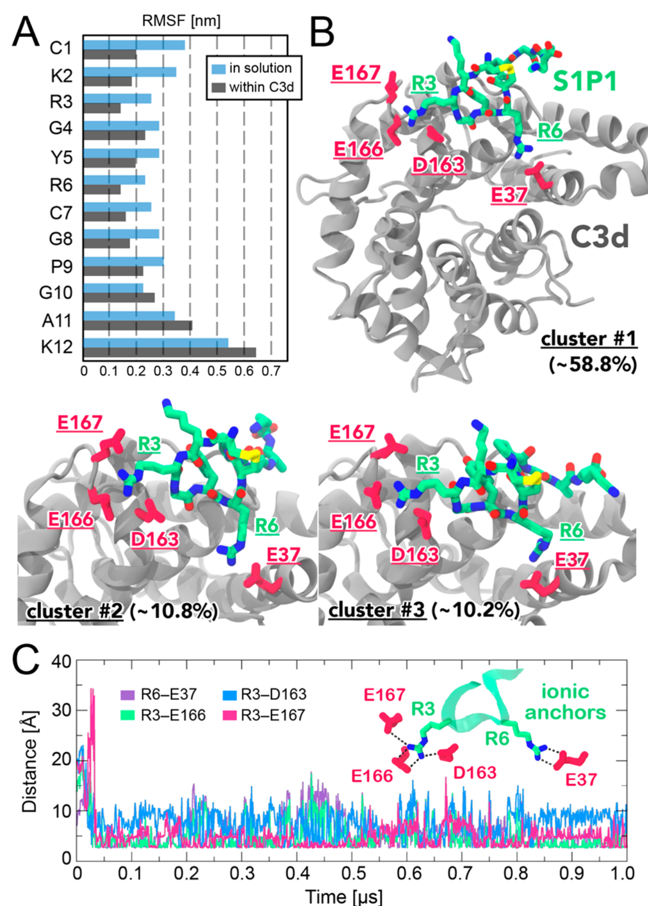


Figure 3. (A) Root Mean Square Fluctuations (RMSF) for the S1P1 peptide residues, computed over ∼1 $\mu$s of MD of the peptide in solution and within C3d. (B) Conformations of S1P1 bound to C3d, as from MD simulations. Representative snapshots from the three most populated clusters are shown (cluster populations are in brackets). (C) Time evolution along MD of critical ionic interactions, which act as "ionic anchors" and are established between the R3–R6 residues of S1P1 and negatively charged residues of C3d.

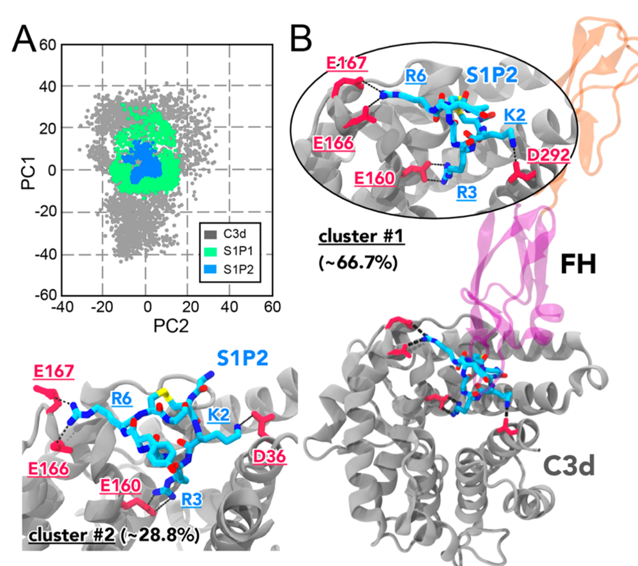


Figure 4. (A) Conformational space adopted by C3d (gray), S1P1 (green), and S1P2 (blue) during MD simulations, as detected by plotting the two first Principal Components (PC1 vs PC2). (B) Conformations of S1P2 bound to C3d, as from MD simulations. Representative snapshots from the two most populated clusters are shown (cluster populations are in brackets). Cluster 1 is superposed to FH, highlighting the overlap of S1P2 with FH.

it is notable that critical ionic interactions are formed over the first ∼50 ns of MD (Figure 3C), indicating that our results can also be achieved over shorter MD runs. This would help reproduce our results for drug discovery purposes by running shorter MD simulations.

Building on this information, we hypothesized that the uncyclized region (positions 8–12) may not contribute favorably to C3d binding. Hence, we designed a novel peptide removing the flexible tail, S1P2, with sequence g[CKRGYRC]-g. This peptide preserves the critical core region including R1282/R1203, and includes glycine residues to each end. By using MST, we assessed the dissociation properties of the C3d–S1P2 complex, observing a dose–response curve and measuring a $K_d$ of 61.6 ± 20.0 $\mu$M (Figure 2). MD simulations of the C3d–S1P2 complex, performed over ∼1 $\mu$s in analogy with C3d–S1P1, reveal an increased stability of the S1P2 peptide. To comparatively assess the mobility of the FH-derived peptides in complex with C3d, we performed Principal Component Analysis (PCA), which characterizes the conformational space adopted by the protein–ligand complex.[20] This analysis enabled monitoring differences in the mobility of the S1P1 and S1P2 peptides, informing on their stability at the level of the binding site, and thereby providing a further indication on their ability to stongly bind C3d.[21]

As a result, S1P1 explores a relatively broad conformational space along the first two Principal Components (PC1 vs PC2, Figure 4A). Instead, S1P2 adopts a restricted conformational space, as arising from a more stable anchoring at the level of C3d. For the sake of completeness, we also report the PC plot the C3d protein (gray, Figure 4A), noting that C3d more broadly explores the conformational space, with respect to the peptides, due to its bigger size, flexible loops and terminal chains. Cluster analysis reveals that the first two clusters account for ∼95% of the total population and display conserved interactions between the R3/R6 "ionic anchors" and negatively charged residues of C3d (Figure 4B). S1P2 also engages in ionic interactions with C3d through K2, which binds D922/D36. Notably, these interactions remarkably overlap with the interactions established by the FH R1182, R1203, and K1230 residues with C3d, as observed in crystal structures (Figure 1A). The conservation of critical ionic interactions and the high population of the first two clusters, indicate that our engineering of S1P1 toward S1P2 leads to a more stable binding. In turn, this observation rationalizes the ameliorated binding affinity of S1P2 for C3d, measured through MST (Figure 2). Hence, the S1P2 peptide constitutes now a stepping-stone for further optimization, toward the design of high-affinity peptides binding C3d.

Overall, we show that small peptides mimicking the binding of the complement regulator FH can be employed to directly target C3d, posing the foundation for novel biomarkers and diagnostic strategies. Through iterative computational analysis and binding affinity experiments, we established a rationale for the design of FH-inspired peptides. Building on this information, iterative design and optimization led to a novel peptide—namely S1P2—with low-micromolar affinity for C3d and stable binding over $\mu$s-length MD. This peptide constitutes now the starting "hit" compound for further optimization to

reach nM binding affinity against C3d and for conjugation to fluorophores in order to image complement activity. This is ongoing in our laboratories by integrating computational methods, binding assays, and fluorescence spectroscopy.[5] As a final remark, we note that although the "acidic patch" at Site 1 is particularly receptive for the design of targeting ligands,[22,23] however, while we cannot exclude binding at the other accessory sites, this grants future investigations to exploit alternative binding and/or multitarget strategies.[24,25] This strategy is promising to design biomarkers and novel diagnostic strategies to detect immune diseases related to the dysregulation of the hallmark protein C3d.

## ■ ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsmedchemlett.9b00663.

> Supporting Information and methods. Strucutral models, electrostatic analysis and molecular dynamics simulations. Binding assays and data analysis. (PDF)

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### Author Contributions

R.E.S.H. performed the computational and experimental studies. N.T.Z., R.H., and Y.B.N analyzed the data. D.M., V.I.V., and G.P. conceived and directed this research. R.E.S.H. and G.P. wrote the manuscript. All authors provided critical comments and edited the manuscript.

### Funding

This work has been supported by the National Institutes of Health, through grant R01 EY027440. This work was partially supported by the National Science Foundation under Grant No. CHE-1905374. The Extreme Science and Engineering Discovery Environment (XSEDE) provided computer time through grant TG-MCB160059.

### Notes

The authors declare no competing financial interest.

The AESOP code has been originally developed by the Morikis research group and is currently maintained and improed by the Palermo laboratory. The AESOP code is freely availabe at: https://aesop.readthedocs.io/en/latest/index.html and on Github at: https://github.com/biomodel/aesop/blob/master/docs/index.rst.

## ■ ABBREVIATIONS

C3d, complement control protein 3; FH, Factor H

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*ACS Med. Chem. Lett.* 2020, 11, 1054−1059