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Small-molecule factor D inhibitors selectively block the alternative pathway of complement in paroxysmal nocturnal hemoglobinuria and atypical hemolytic uremic syndrome

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ABSTRACT

Paroxysmal nocturnal hemoglobinuria and atypical hemolytic uremic syndrome are diseases of excess activation of the alternative pathway of complement that are treated with eculizumab, a humanized monoclonal antibody against the terminal complement component C5. Eculizumab must be administered intravenously, and moreover some patients with paroxysmal nocturnal hemoglobinuria on eculizumab have symptomatic extravascular hemolysis, indicating an unmet need for additional therapeutic approaches. We report the activity of two novel small-molecule inhibitors of the alternative pathway component Factor D using *in vitro* correlates of both paroxysmal nocturnal hemoglobinuria and atypical hemolytic uremic syndrome. Both compounds bind human Factor D with high affinity and effectively inhibit its proteolytic activity against purified Factor B in complex with C3b. When tested using the traditional Ham test with cells from paroxysmal nocturnal hemoglobinuria patients, the Factor D inhibitors significantly reduced complement-mediated hemolysis at concentrations as low as 0.01 μM. Additionally the compound ACH-4471 significantly decreased C3 fragment deposition on paroxysmal nocturnal hemoglobinuria erythrocytes, indicating a reduced potential relative to eculizumab for extravascular hemolysis. Using the recently described modified Ham test with serum from patients with atypical hemolytic uremic syndrome, the compounds reduced the alternative pathway-mediated killing of *PIGA*-null reagent cells, thus establishing their potential utility for this disease of alternative pathway of complement dysregulation and validating the modified Ham test as a system for pre-clinical drug development for atypical hemolytic uremic syndrome. Finally, ACH-4471 blocked alternative pathway activity when administered orally to cynomolgus monkeys. In conclusion, the small-molecule Factor D inhibitors show potential as oral therapeutics for human diseases driven by the alternative pathway of complement, including paroxysmal nocturnal hemoglobinuria and atypical hemolytic uremic syndrome.

Introduction

The complement system provides an important defense against bacteria, fungi, and viruses.[1,2] Mutations (e.g. *PIGA, factor H, factor I*, etc.) and autoantibodies (e.g. Factor H autoantibodies) that lead to unregulated complement activation are implicated in the pathogenesis of many disorders including paroxysmal nocturnal hemo-

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globinuria (PNH) and atypical hemolytic uremic syndrome (aHUS).[3-5]

Complement activation is initiated by: 1) the lectin pathway, 2) the classical pathway, and 3) the alternative pathway of complement (APC) which converge on the common central component C3. The APC also serves as an amplification loop for the lectin and classical pathways. It has been estimated that the APC may account for 80% of complement activation products even when the complement cascade is initiated by the classical and lectin pathways.[6]

The APC is constitutively activated at low levels *via* slow spontaneous hydrolysis of an internal thioester within C3 that generates C3(H_2O). This activated C3(H_2O) in solution phase binds Factor B to generate the proconvertase C3(H_2O)B, which is processed by the serine protease Factor D to the APC C3 convertase, C3(H_2O)Bb. This C3 convertase then cleaves additional C3 molecules to generate C3a and C3b, the latter of which can covalently attach to available surfaces.[7] Deposited C3b can then elicit a rapid localized amplification, especially when complement regulation is impaired, described as follows in a process designated the amplification loop. Deposited C3b can pair with Factor B, which is cleaved by Factor D to generate a second form of the APC C3 convertase, C3bBb. Membrane-bound C3bBb then cleaves additional C3 to generate further C3b deposits, which pair with additional Factor B molecules to repeat the cycle. The end result of this amplification is C3b opsonization, release of the anaphylatoxins C3a and C5a, and assembly of the terminal complement complex (also known as the membrane attack complex, MAC) on the target surface. The entire process is depicted in Figure 1A.

Paroxysmal nocturnal hemoglobinuria is caused by a somatic *PIGA* mutation that prevents expression of glycosylphosphatidylinositol (GPI) anchored proteins on the surfaces of affected cells, among them the complement regulators CD55 and CD59 which normally protect host cells from complement-mediated lysis. CD55 interferes with C3 convertase formation following C3b attachment, preventing spurious amplification of initial activation events,[8] whereas CD59 prevents localized MAC assembly following amplification.[9] Due to the absence of both regulators from erythrocyte membranes, PNH manifests with chronic hemolytic anemia primarily due to APC activation on mutant erythrocyte membranes. PNH is effectively treated with the humanized monoclonal antibody, eculizumab.[10,11] Eculizumab binds C5 and blocks its cleavage to C5a and C5b and thus protects mutant erythrocytes from MAC formation and lysis. However, due to the absence of CD55, the mutant erythrocytes in PNH patients on eculizumab treatment continue to be opsonized by C3b and thus are susceptible to extravascular hemolysis.[12] In most PNH patients on eculizumab, this leads to asymptomatic chronic extravascular hemolysis; however, a subset of PNH patients on eculizumab have symptomatic hemolysis and require red blood cell transfusions.[10,13] Moreover, rare patients (approx. 3.5% of the Japanese population) carry a genetic C5 variant (2654G>A, Arg885His) that prevents eculizumab binding and causes eculizumab resistance.[14] Another limitation of eculizumab is that it must be administered intravenously indefinitely every two weeks to block intravascular hemolysis and prevent thrombosis. Thus, novel complement inhibitors are needed to address these limitations.

Atypical hemolytic uremic syndrome is also caused by APC dysregulation. aHUS presents with signs and symptoms of thrombotic microangiopathy (TMA) including thrombocytopenia, non-immune hemolytic anemia, peripheral blood schistocytes, and often end-organ damage to the kidneys and central nervous system. Most cases are caused by mutations in APC genes or autoantibodies directed against APC regulatory proteins.[15-17] These mutations either disable the regulatory proteins that help degrade cell surface C3b, including haploinsufficient mutations of Factor H (most commonly), Factor I, MCP, and thrombomodulin, or enhance the function of proteins that drive the APC, such as gain-of-function mutations in C3 or factor B.[15,17-19] The result is activation of terminal complement on various target tissues, including renal and vascular endothelium, and cell death mediated by the MAC. Distinguishing aHUS from other TMAs such as thrombotic thrombocytopenic purpura (TTP) is challenging. Recently, we described a serum-based assay that distinguishes most cases of aHUS from TTP.[20] This assay (modified Ham test) measures the ability of human serum to kill *PIGA*-null reagent cells. Due to APC dysregulation in the serum of most aHUS patients, the *PIGA*-null cells that fail to express CD55 and CD59 are more readily killed by aHUS serum than by serum from healthy individuals or TTP patients.

Complement Factor D is a promising therapeutic target to treat PNH and aHUS. Factor B is its only natural substrate, and of all complement proteins, Factor D has the lowest abundance in serum and is the rate-limiting component of the APC.[21] Its crystal structure has been elucidated in a series of studies.[22-24] Here, we describe two novel, small-molecule inhibitors that bind Factor D and inhibit its cleavage of Factor B. We demonstrate *in vitro* that these inhibitors block PNH cell hemolysis, mitigate C3 fragment

Table 1. Clinical data on paroxymal nocturnal hemoglobinuria patients studied.

Patient ID	Age, sex	Erythrocyte clone size Type II/ III (%)	Granulocyte clone size (%)	LDH (U/L)	Hemoglobin (g/dL)	Direct Coombs C3	Direct Coombs IgG	Absolute reticulocyte count (K/cu mm)	Months on eculizumab at q14 days dosing at start of study
1	51, F	3/64	96	266	9.5	Pos	Neg	251.1	57
2	58, M	1/98	99	281	10.3	Pos	Neg	321.6	107
3*	72, F	1/13	81	842	9.7	Neg	Neg	138.3	0

LDH: lactate dehydrogenase; q14 days: every 14 days; M: male; F: female;; Pos: positive; Neg: negative. *Transfused within 60 days.

Table 2. Clinical data on atypical hemolytic uremic syndrome patients studied.

Patient ID	Age, sex	Disease phase (weeks)	ADAMTS13 activity (%)	ADAMTS13 inhibitor (%)	PLT count (x10^9/L)	LDH (mg/dL)	Cr (mg/dL)	Genetic mutation	Previous treatments	Current treatment	Response to eculizumab	Serum tested
1	47, F	Acute	75	NA	9	2505	2.3	CFHR3-CFHR1 homozygous deletion; CFH heterozygous c.2850G>T, p.Gln950His	–	–	–	No
									PEx (x5) Eculizumab (x8)	–	Yes	Yes
		Remission (20 w)	NA	NA	314	228	1.9	None				
2a	59, M	Acute	15	67	39	676	2.3	None	PEx (x4)	–	–	Yes
2b		Remission (12 w)	NA	NA	330	170	1.4			Eculizumab (x12)	Yes	Yes
3	35, F	Acute	18	76	10	1270	2.4	None	–	–	–	No
		Remission (82 w)	NA	NA	108	75	1.1		–	Eculizumab (x77)	Yes	Yes
4	23, F	Acute	102	NA	62	2820	6.3	None	–	–	–	No
		Remission (60 w)		NA	NA	205	178	3.5	Eculizumab (x37)	Dialysis	Yes	Yes

F: female; M: male; w: weeks; CFHR: complement Factor H-related proteins; CFH: complement Factor H; ADAMTS13: a disintegrin and metalloprotease with thrombospondin type 1 motif, 13; NA: not available; PEx: plasma exchange.

accumulation on PNH cell surfaces, and block dysregulated APC activity in aHUS serum. We also show that the modified Ham test can be used to evaluate novel complement inhibitors in aHUS, and, finally, we demonstrate APC suppression in serum obtained from non-human primates following oral administration of one inhibitor.

Methods

Human samples

Three PNH patients and 4 aHUS patients were enrolled in this study (Tables 1 and 2). Patients were diagnosed with aHUS as previously defined.[25,26] All patients gave written informed consent. This study was approved by the Institutional Review Board and conducted in accordance with the Declaration of Helsinki. Blood was collected in EDTA and serum separation tubes (*Online Supplementary Appendix*).

Factor D inhibitors

Compounds ACH-3856 and ACH-4471 were synthesized by Achillion Pharmaceuticals and fully characterized by proton nuclear magnetic resonance (^{1}H-NMR), high performance liquid chromatography (HPLC), and mass spectrometry.

Assays for Factor D binding, Factor D enzymatic activity, and hemolysis mediated by APC and the classical pathway

Binding kinetics and affinities of compounds to human Factor D were determined by surface plasmon resonance. Inhibition of Factor D enzymatic activity was evaluated with 80 nM purified human Factor D and the small synthetic thieoster substrate Z-Lys-SBzl, or with 0.8 nM purified human Factor D and its natural substrate C3bB. Inhibition of APC-mediated hemolytic activity was assessed with 8% normal human serum and rabbit erythrocytes; inhibition of hemolysis by the classical pathway was assessed with 0.5% normal human serum and antibody-sensitized sheep erythrocytes. Methods for these assays are described in the *Online Supplementary Appendix*.

Inhibition of APC-mediated cell killing using PNH and aHUS patient samples: Ham test and modified Ham test

Inhibition of hemolytic assay by Factor D inhibitors was assessed using PNH erythrocytes at 1% hematocrit in GVB0/MgEGTA (pH 6.4) and 20% acidified human serum (Ham test), as previously described.[27] Based on the same principle, the modified Ham test was performed to assess the efficacy of Factor D inhibitors in APC-mediated killing caused by aHUS patient serum.[20] Both assays are described in the *Online Supplementary Appendix*.

Inhibition of C3 fragment deposition

C3 fragment deposition on PNH erythrocytes from PNH Patient 2 (blood group O) by 60% acidified C5-depleted human serum was assessed by flow cytometry. Erythrocytes were washed and prepared as in the hemolytic assay. Sample preparation and C3 fragment deposition measurement by flow cytometry are described in detail in the *Online Supplementary Appendix*.

APC activity in cynomolgus monkeys

The in-life portion of these studies was performed at Ricerca Laboratories (Concord, OH, USA) in accordance with Care and Use of Laboratory Animals guidelines and the United States Department of Agriculture Animal Welfare Act. Experimental procedures were reviewed and approved by Ricerca's Institutional Animal Care and Use Committee (IACUC) prior to initiation. ACH-4471 was formulated in solution at 80 mg/mL in PEG400:water (70:30) (w:w). Three male cynomolgus monkeys were dosed by oral gavage with ACH-4471 at 200 mg/kg twice, 12

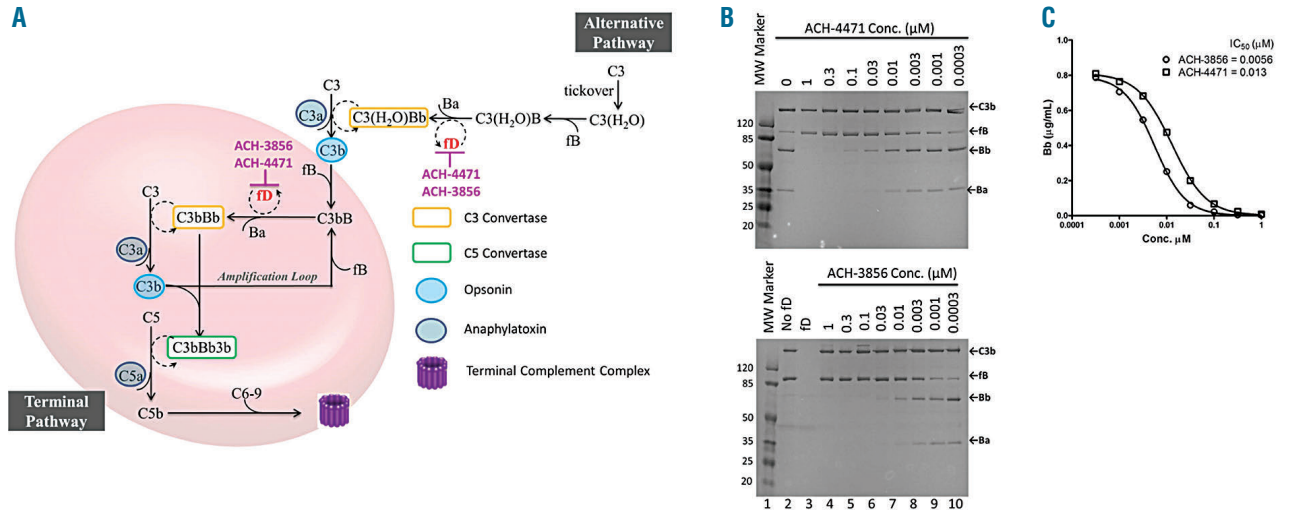


Figure 1. Inhibition of Factor D proteolytic activity. (A) Depiction of Factor D inhibitors in the alternative complement pathway (APC). Factor D (fD) participates in C3 convertase generation by cleavage of Factor B (fB) at two steps in the APC cascade: generation of the initial C3 convertase [C3(H_2O)Bb] following spontaneous APC activation (tickover); and the production of surface-bound C3 convertase (C3bBb) which mediates dramatic amplification of the initial activation (amplification loop) and the consequent opsonization of target surfaces by C3b, formation of the terminal complement complex (aka, membrane attack complex, MAC), and release of the anaphylatoxins C3a and C5a. An erythrocyte is shown to depict the membrane-bound events. Additional regulatory proteins not shown here can promote (properdin) or attenuate (Factor H, Factor I, multiple membrane-bound proteins) APC activity. (B) Factor D proteolytic activity against Factor B in complex with C3b. Two stained gels from a single representative experiment show dose-dependent inhibition by compounds of Factor B (fB) cleavage to its products Ba and Bb. Control reactions included one omitting C3b and fB (labeled "fD") and one omitting fD (labeled "No fD"). (C) Dose-response curves and IC_{50} values from the representative experiment of (B). Average IC_{50} values ± standard deviation for ACH-3856 and ACH-4471 were 0.0058 ± 0.0005 μM and 0.015 ± 0.003 μM in 4 independent experiments.

Table 3. Compound binding to and inhibition of Factor D enzyme.

Compound	fD binding				fD activity			
	On ka (M^{-1} s^{-1})	Off kd (s^{-1})	affinity KD (μM)	N	C3bB IC50 (μM)	N	Thioester IC50 (μM)	N
ACH-3856	1.7e7 ± 7e6	0.0086 ± 0.0061	0.00048 ± 0.00016	2	0.0058 ± 0.005	4	0.036 ± 0.003	83
ACH-4471	2.6e6 ± 4e5	0.0015 ± 0.0001	0.00057 ± 0.00004	2	0.015 ± 0.003	4	0.035 ± 0.001	3

fD: Factor D; N: number. Mean ± standard deviation from N independent experiments. fD binding parameters were assessed by surface plasmon resonance; representative sensorgrams are shown in *Online Supplementary Figure S2*. fD activity was measured both against its natural substrate C3bB and against a small synthetic thioester substrate.

hours apart. Serial blood samples were collected at specified time points through 30 hours. Plasma ACH-4471 concentration was determined by LC-MS/MS with a lower limit of quantitation of 2.44 ng/mL. Pharmacokinetic parameters were calculated with non-compartmental analysis using Phoenix 6.2 WinNonlin (Pharsight, Princeton, NJ, USA) from individual plasma concentration *versus* time using the linear-trapezoidal method. Serum APC activity was measured in duplicate using the APC-specific Wieslab assay (Euro Diagnostica, Malmö, Sweden). Activity at each time point was normalized to pre-dosing activity in the same animal. Serum Factor D concentrations were determined using the Human Complement Factor D Quantikine ELISA Kit (R&D Systems).

Results

Inhibition of Factor D proteolytic activity

The potency of the small-molecule Factor D inhibitors ACH-3856 and ACH-4471 was investigated by biophysical and biochemical methods. Both compounds showed high binding affinity to human Factor D, shown by surface plasmon resonance studies with recombinant enzyme (Table 3 and *Online Supplementary Figure S1*), with respective KD values of 0.00036 μM and 0.00054 μM. Additionally, the compounds inhibited the proteolytic activity of purified Factor D against its natural substrate Factor B in complex with C3b, blocking production of Bb fragment in a dose-dependent manner with respective IC50 values of 0.0058±0.0005 μM and 0.015±0.003 μM (Table 3 and Figure 1B and C). The compounds act as catalytic inhibitors, established by inhibition of Factor D protease activity against a small synthetic substrate (N-α-Cbz-L-lysine thiobenzyl ester) (Table 3 and *Online Supplementary Figure S2*), distinguishing them from the anti-Factor D monoclonal antibody inhibitor lampalizumab which binds an exosite and sterically blocks Factor B access to the active site.[28] Additionally, the compounds showed selectivity as they inhibited in a standard assay for rabbit erythrocyte hemolysis upon APC activation but not in a counterpart assay for the complement classical pathway (Table 4), and moreover did not inhibit a panel of 12 human serine proteases (*Online Supplementary Table S1*), thus establishing their limited potential for off-target effects.

PNH and aHUS patients' characteristics

Paroxymal nocturnal hemoglobinuria and aHUS are diseases of complement dysregulation that could be amenable to Factor D inhibitors. As proof of concept, we assessed the Factor D inhibitors *in vitro* using erythrocytes and serum obtained from PNH patients and serum from aHUS patients. Patients' characteristics are shown in Table 1 (PNH) and Table 2 (aHUS). Erythrocytes were recovered

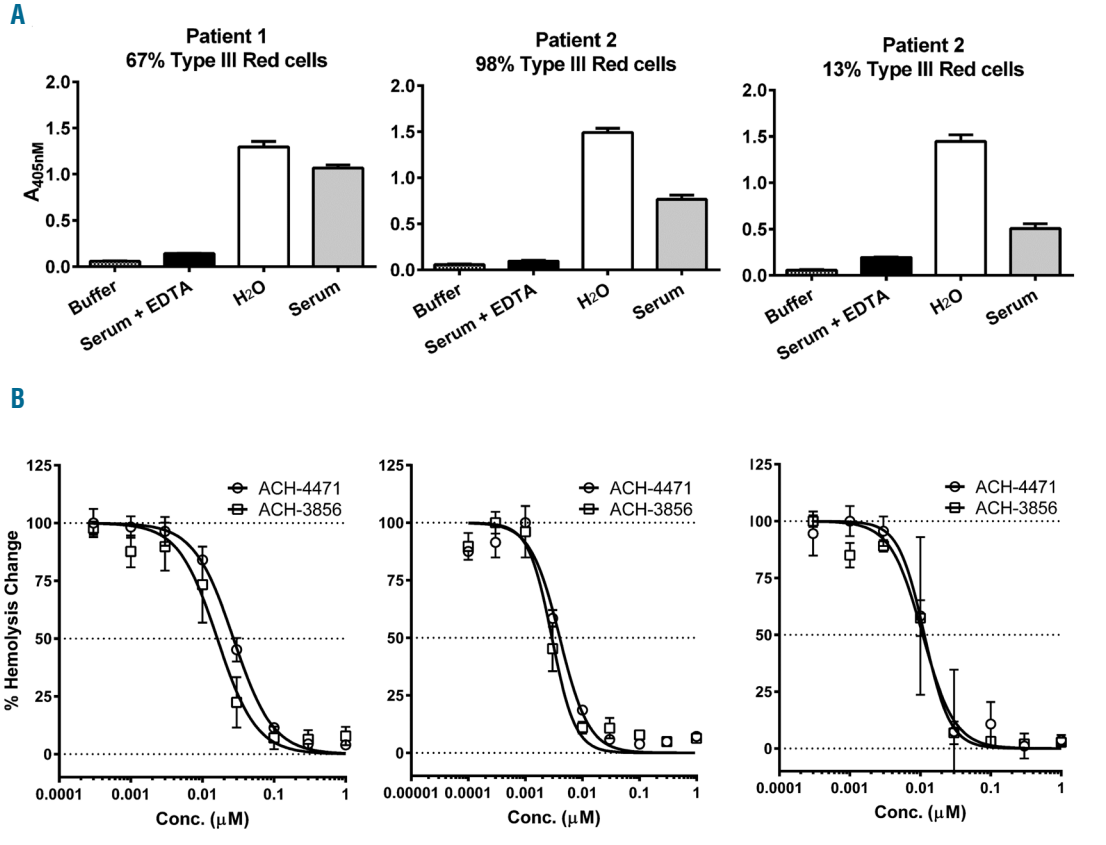
from 3 PNH patients: 2 on long-term eculizumab treatment (Patients 1 and 2) and 1 treatment-naïve (Patient 3), and were used for the hemolytic assay and the C3 fragment deposition assay. Serum was also recovered from PNH Patient 1 and Patient 3 (this time after Patient 3 was started on eculizumab) following eculizumab administration for use in the C3 fragment deposition assay. Five serum samples used in the modified Ham assay were recovered from 4 aHUS patients: 1 in acute phase before treatment and later in remission while on eculizumab treatment (Patient 2, 2 samples), 1 in remission while on eculizumab treatment (Patient 3, 1 sample), and 2 in remission after discontinuation of eculizumab (Patients 1 and 4, one sample each).

Inhibitory effects on hemolysis of PNH erythrocytes

The Ham test identifies PNH erythrocytes by their hemolytic susceptibility to acidified serum. We evaluated ACH-3856 and ACH-4471 by incubating erythrocytes from PNH patients with acidified ABO blood group-compatible normal human serum containing the inhibitors in half-log dilution series (Figure 2). As C3 convertase formation is Mg^{++}-dependent, EDTA inhibition was used to quantitate complement-independent background in each experiment. Above this background, the acidified serum in the presence of Mg^{++} mediated the complement-dependent hemolysis of 80% (Patient 1), 48% (Patient 2), and 25% (Patient 3) of patient erythrocytes (Figure 2A). The Factor D inhibitors potently inhibited hemolysis with IC50 values ranging from 0.0029 μM to 0.016 μM for ACH-3856 (IC90 values from 0.0082 μM to 0.064 μM) and from 0.0040 μM to 0.027 μM for ACH-4471 (IC_{90} values from 0.015 μM to 0.11 μM) (Figure 2B and C). The Factor D inhibitors, therefore, have the potential to inhibit the APC-mediated hemolysis that underlies much of the morbidity and mortality in PNH patients.

C3 fragment deposition on PNH erythrocytes

As eculizumab blocks terminal complement activity only, the continued accumulation of C3 fragments on



C

Compound	IC_{50} (μM)	IC_{90} (μM)
ACH-4471	0.027	0.110
ACH-3856	0.016	0.064

Compound	IC_{50} (μM)	IC_{90} (μM)
ACH-4471	0.004	0.015
ACH-3856	0.0029	0.0082

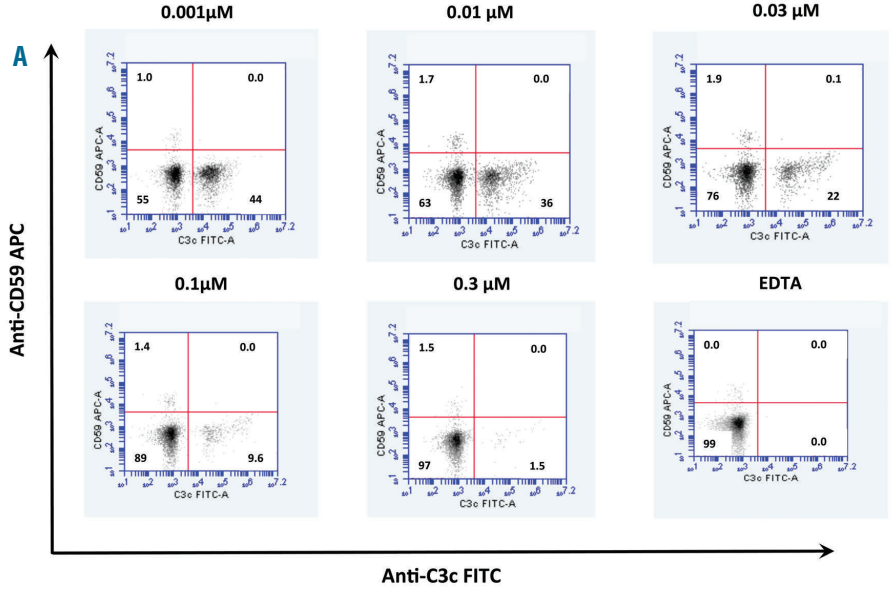
Compound	IC_{50} (μM)	IC_{90} (μM)
ACH-4471	0.011	0.029
ACH-3856	0.011	0.034

Figure 2. Factor D inhibitors block hemolysis of erythrocytes from paroxymal nocturnal hemoglobinuria (PNH) patients. (A) Hemolysis of erythrocytes obtained from the 3 PNH patients in 20% acidified normal human serum (Ham test). The control samples "Buffer", "Serum + EDTA", and "H_2O", and the patient-specific samples without inhibition, "Serum", were as defined in the Methods section. (B) Inhibition by ACH-4471 (○) and ACH-3856 (□) of hemolysis reactions using erythrocytes from the 3 PNH patients. Each inhibitor concentration was tested in duplicate with mean±standard deviation (SD) shown. (C) IC_{50} and IC_{90} values of ACH-4471 and ACH-3856 from the hemolysis reactions in (B).

mutant erythrocytes in treated patients often results in extravascular hemolysis. As Factor D inhibitors should inhibit this opsonization, we tested the ability of ACH-4471 to prevent C3 fragment deposition on PNH erythrocytes as detected by flow cytometry. Erythrocytes from PNH Patient 2 were incubated with acidified human serum depleted of C5 to prevent hemolysis, yet allow continued C3 fragment deposition during the incubation period (Figure 3). While serum acidification led to C3 fragment deposition on CD59-negative PNH erythrocytes, EDTA inhibited this event, confirming that it was a complement-specific activity. ACH-4471 inhibited this deposition in a dose-dependent manner, with IC50 and IC90 values of 0.031 μM and 0.089 μM, respectively. Inhibition was also observed with erythrocytes from PNH Patients 1 and 3 when assessed using their own serum collected immediately after eculizumab infusion; the percentage of C3-positive erythrocytes was 37.6% (Patient 1) and 5.5% (Patient 3) without ACH-4471, decreasing to 11.9% (Patient 1) and 1.8% (Patient 3) with 0.1 μM ACH-4471 (*data not shown*). Furthermore, in addition to the anti-C3c antibody used above, we also examined the phenomenon with anti-C3b and anti-C3d antibodies. We observed similar results on erythrocytes from PNH Patient 2 except that approximately 10% of erythrocytes stained positive for C3d in the presence of EDTA, indicating that these C3 fragments were deposited *in vivo* before harvesting from patients (*Online Supplementary Figure S3*). These results suggest that the extravascular hemolysis that occurs in PNH patients on eculizumab treatment might be blocked by Factor D inhibition.

Factor D inhibitors block dysregulated APC in aHUS patient serum

An important distinction between aHUS and other TMAs is the underlying APC dysregulation observed in aHUS. The modified Ham test can distinguish aHUS from other TMAs based on the sensitivity of a *PIGA*-null reagent cell line to APC activation; cell killing greater than 20% by patient serum has been shown to serve as a sen-



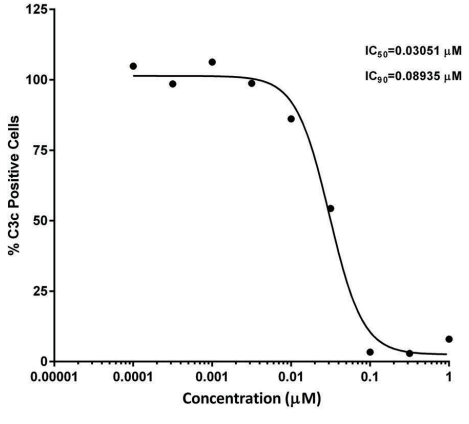


Figure 3. Factor D inhibitor ACH-4471 blocks C3 fragment deposition on erythrocytes from a paroxymal nocturnal hemoglobinuria (PNH) patient. (A) Representative flow cytometric scattergrams of erythrocytes from PNH Patient 2 treated with acidified C5-depleted serum (ACH-4471-treated or EDTA-treated). C3 fragment deposition was assessed using anti-human C3c antibody (x-axis). PNH mutant erythrocytes were identified by the absence of cell-surface CD59 expression (y-axis). Respective gating thresholds are indicated by vertical and horizontal lines. Note, a fraction of CD59-negative cells (approx. 10%) were positive when stained with anti-C3d antibody despite staining negative for anti-C3b and anti-C3c antibodies when tested in the presence of EDTA to prevent *in vitro* complement activation; this result indicates that some CD59-negative cells had been coated with C3 fragments but were transformed into the terminal opsonin C3dg *in vivo* before harvesting, a process known to be rather rapid (below and *Online Supplementary Figure S3*).[43] (B) ACH-4471 dose-response curve from the experiment in (A); IC50 and IC90 values were 0.031 μM and 0.089 μM, respectively.

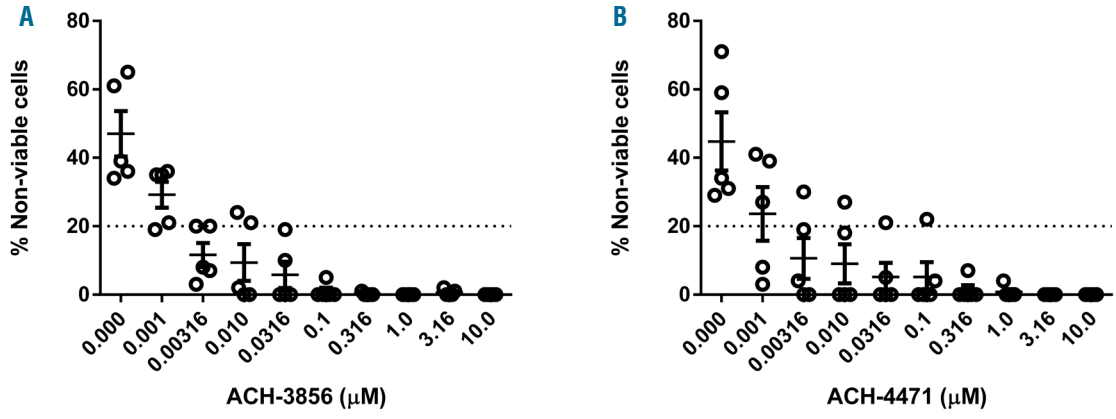


Figure 4. Factor D inhibitors block cell killing by sera from atypical hemolytic uremic syndrome (aHUS) patients. Significant killing of *PIGA*-null reagent cells by the 5 aHUS patients' sera in the modified Ham test is shown (0.000 μM ACH-3856, left; 0.000 μM ACH-4471, right). The addition of Factor D inhibitors caused a dose-dependent reduction of cell killing at the indicated concentrations (ACH-3856, left; ACH-4471, right). Activity was also abrogated by heat inactivation of serum complement (*data not shown*). Horizontal dashed line indicates the 20% threshold that is considered indicative of cell killing due to alternative pathway of complement (APC) dysregulation in aHUS serum. The 5 patient samples are shown for each condition with mean ± standard deviation.

sitive and specific indicator of aHUS.[5,20] We evaluated ACH-3856 and ACH-4471 in the modified Ham test by incubating *PIGA*-null cells with aHUS patient sera containing the Factor D inhibitors (Figure 4). In the absence of inhibitor (0.000 μM), all 5 aHUS serum samples promoted a degree of cell killing similar to previously described levels.[5,20] Notably, the presence of therapeutic eculizumab in 2 of the 5 sera (Patient 2, sample b, and Patient 3) did not minimize the observed killing; this finding is consistent with previous observations and has been attributed to the eculizumab dilution that accompanies the serum dilution necessary for appropriate assay conditions.[20] *PIGA*-null cell killing activity in aHUS patient sera was heat-sensitive and therefore complement dependent. ACH-3856 (Figure 4A) and ACH-4471 (Figure 4B) both blocked the APC-mediated killing by aHUS patient sera at sub-micromolar concentrations. These results suggest that small-molecule Factor D inhibitors have the potential to mitigate complement-mediated damage in aHUS patients, and support the utility of the modified Ham assay for preclinical aHUS drug assessment.

Efficacy of ACH-4471 following oral delivery in non-human primates

To profile the effects of continuous Factor D inhibition over a period of at least 24 hours *in vivo*, we administered ACH-4471 orally to 3 monkeys in two serial 200 mg/kg doses separated by 12 hours. This deliberately high dose was chosen based on an initial assessment of pharmacokinetic properties in monkeys which revealed lower oral exposure due to higher clearance than in other animal species, including rats and dogs (*data not shown*). The monkeys tolerated the compound well and showed no clinical abnormalities. Figure 5A shows ACH-4471 concentrations in plasma at time points from 0 hours (pre-dosing) to 30 hours (18 hours after the second dose). In parallel, serum was collected for determination of APC activity by Wieslab assay and of Factor D concentrations. APC activity was suppressed continuously by more than 95% continuously through the 30-hour time period (Figure 5B) with no significant increase in Factor D concentrations (Figure 5C). The observed stability of serum Factor D concentrations was expected because, as a small molecule, ACH-4471 should not interfere with renal Factor D clearance, in marked contrast to the effect of systemic delivery of the humanized monoclonal anti-Factor D antibody lampalizumab.[29] These results demonstrate the potential utility of oral delivery of ACH-4471 for therapeutic inhibition of APC activation.

Discussion

The present study describes the novel compounds ACH-3856 and ACH-4471 that potently inhibit Factor D proteolytic activity. Factor D is a highly specific serine protease with Factor B as its only natural substrate,[30] making it a favorable target for the largely APC-driven manifestations of both PNH and aHUS. We demonstrate that these inhibitors effectively block APC-mediated cell killing using erythrocytes from PNH patients (Ham test) or serum from aHUS patients (modified Ham test). Furthermore, ACH-4471 inhibited the elevated deposition of C3 fragments that proceeds on PNH cells when terminal complement activity is blocked, such as by therapeutic eculizumab. In addition, ACH-4471 demonstrated good oral bioavailability and inhibited APC activity in serum samples recovered following oral administration to non-human primates.

Eculizumab improves life for PNH patients by eliminating intravascular hemolysis, decreasing the need for blood transfusions, and reducing the risk of thrombosis.[10,11] Yet, the Factor D inhibitors present a potential advantage as some eculizumab-treated PNH patients experience symptomatic extravascular hemolysis due to dysregulated C3 fragment deposition that proceeds even with inhibition of terminal complement activity.[31] Several upstream complement inhibitors under investigation may address this limitation, including the engineered complement receptor 2/Factor H fusion protein TT[30,32] peptidic C3 inhibitors (such as Cp40 and APL-2),[33] and the C1 esterase inhibitor C1INH (Cinryze).[27] Suitable systemic exposure, however, may be difficult to achieve with some of these investiga-

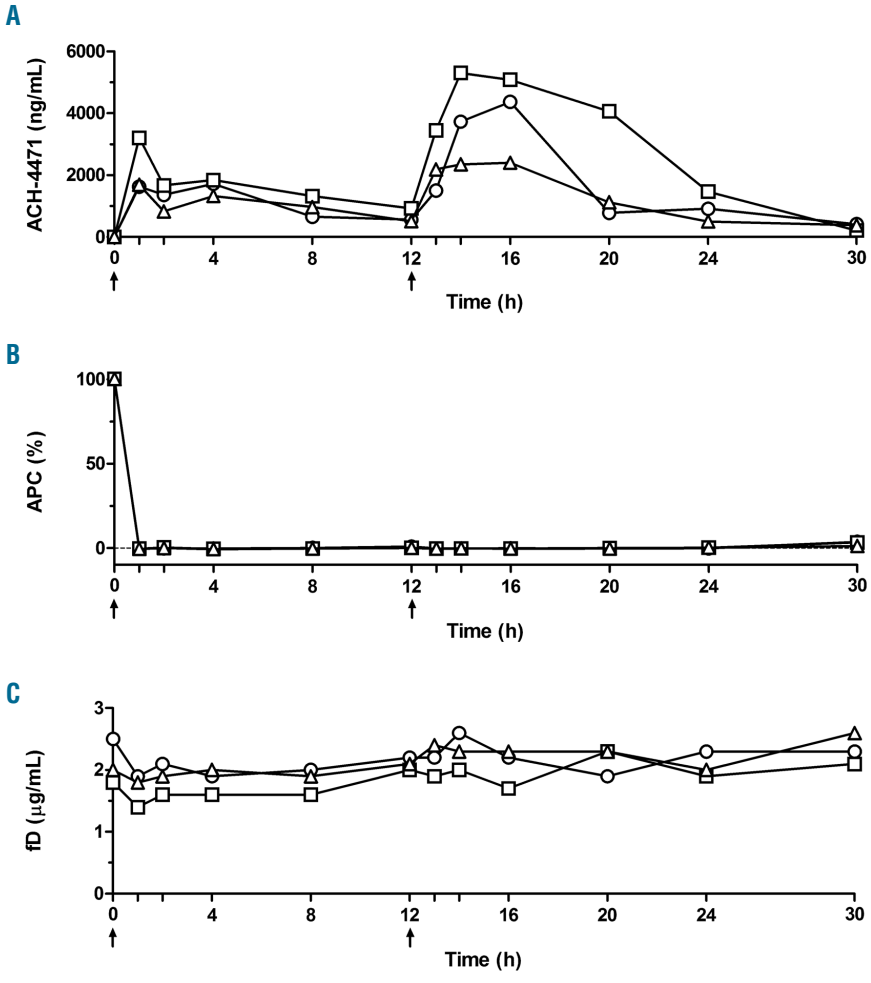


Figure 5. Factor D inhibitor inhibits alternative pathway of complement (APC) activity following oral dosing in cynomolgus monkeys. (A) ACH-4471 concentrations at the indicated time points in plasma samples collected from 3 cynomolgus monkeys following oral dosing with ACH-4471 at 200 mg/kg at 0 and 12 hours (arrows). Key pharmacokinetic parameters were calculated as follows: maximum concentration (C_{max}), 4020±1480 ng/mL; time at C_{max} (T_{max}), 15.3±1.2 hours (3.3±1.2 hours following the second dose), and 30-hour exposure level (AUC0-30), 48,300±19,100 ng/mL/h. Parameters were calculated as mean ± standard deviation (SD) from the 3 animals. (B) APC activity assessed by Wieslab assay in serum samples collected at the indicated time points. Activity in each sample was normalized to the activity in the same animal at 0 h (pre-dosing). Mean±SD are shown from duplicate assay values. (C) Serum FD concentrations at the indicated time points. Circle, square, and triangle shapes; animals 1, 2, and 3, respectively.

Table 4. Alternative pathway of complement-specific inhibition of complement hemolytic activity.

Compound	APC hemolysis IC_{50} (μM)	APC hemolysis IC_{90} (μM)	N	CPC hemolysis IC_{50} (μM)	N
ACH-3856	0.0087 ± 0.0039	0.022 ± 0.0089	40	>200	1
ACH-4471	0.017 ± 0.011	0.070 ± 0.023	6	>200	2

APC: alternative pathway of complement; CPC: classical pathway of complement; N: number. Mean ± standard deviation from N independent experiments.

tional compounds, and unlike the Factor D inhibitors these molecules will not be available in oral formulation.

APC-mediated hemolysis of erythrocytes from all 3 PNH patients was sensitive to Factor D inhibitors, albeit over an approximately 7-fold range in IC50 values. The reason for this variation remains unknown, yet the inhibitors showed excellent potency against even the least susceptible PNH patient cells. Also of note, C3 fragment opsonization proved less susceptible than hemolysis, with respective IC50 values of 0.031 μM and 0.004 μM for ACH-4471 against cells from Patient 2. This distinction is likely derived in part from operational differences between the assays, as PNH cell opsonization requires substantially more serum (60%) than hemolysis (20%); moreover, the detection thresholds for APC activity could differ widely if, for example, less activity is required for hemolysis than for C3 fragment detection.

Terminal complement inhibition with eculizumab is also highly effective for treating aHUS. Until recently, there was no functional assay with adequate sensitivity and specificity to distinguish aHUS from other TMA syndromes, or to evaluate potential therapeutic compounds. The experiments presented here use the recently reported modified Ham test to demonstrate that Factor D inhibitors effectively overcome the characteristic APC activation on target membranes by serum of aHUS patients. Moreover, although the modified Ham test does not replicate the complex pathophysiology of aHUS observed *in vivo*, this assay may serve as a useful pre-clinical system for testing novel complement inhibitors in aHUS.

Importantly for systemic delivery, Factor D levels remained unchanged in the monkeys following ACH-4471 administration. The Factor D inhibitors thus differ from the humanized monoclonal anti-Factor D antibody lampalizumab, which is in clinical study as an intravitreal treatment for age-related macular degeneration.[34]

Lampalizumab delivered intravenously to cynomolgus monkeys elicited nearly 10-fold increases in serum Factor D levels within five hours, effectively neutralizing its inhibitory activity.[29] Similar increases have been reported for other therapeutic antibodies against proteins that, like Factor D, have high turnover rates mediated by processes including renal filtration.[35] In contrast, the stability of serum Factor D levels in the present study indicates that the small molecule inhibitors will likely maintain their potency following systemic delivery.

A significant concern with complement inhibition is the potential for increased susceptibility to certain bacterial infections. Complete or partial Factor D deficiency in humans is associated with reduced bacterial phagocytosis *in vitro*,[36,37] and complete Factor D deficiency is associated with increased risk for recurrent infections with Neisseria or other encapsulated bacteria which is comparable to the lifetime risk observed in the setting of terminal complement deficiencies.[36-41] Hence, vaccination for subjects on a Factor D inhibitor will be warranted as it is for subjects on eculizumab. We anticipate that immunological protection following vaccination against Neisseria will be better preserved by Factor D inhibitors than by eculizumab. Studies have demonstrated, for example, that opsonophagocytosis of Neisseria by granulocytes depends on antibody-mediated activation of the complement classical pathway, and that serum bactericidal activity depends additionally on the complement terminal pathway.[42] As Factor D inhibitors selectively target the APC and preserve the classical, lectin, and terminal pathways, the protection elicited by antibodies through vaccination should be at least partially preserved in the presence of Factor D inhibition. In contrast, eculizumab is expected to block the serum bactericidal activity conferred by vaccination, leading to diminished protection. Furthermore, the higher clearance of small molecules relative to therapeutic biologics will allow for rapid restoration of complement activity if dosing needs to be ceased in the event of infection. Nevertheless, the safety of ACH-4471 will need to be monitored closely, especially given the contribution of the APC-dependent amplification loop, which by one estimate may account for up to 80% of complement classical pathway activation.[6]

In conclusion, Factor D is a promising target for oral therapy of diseases driven by APC dysregulation. Based on results presented here, and guided by additional assessments of its pharmacology, pharmacokinetic properties, and safety and toxicology, ACH-4471 has been selected for clinical development in PNH and is currently in phase I clinical study.

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