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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Supplemental Materials

Supplemental Methods

Human samples

Blood samples were centrifuged, and erythrocytes were washed three times in phosphate buffered saline (PBS) and resuspended at 30-40% hematocrit in GVB^0 (gel veronal buffer without Ca^{++} and Mg^{++}, pH 7.3) supplemented with 10 mM MgEGTA (GVB^0/MgEGTA, pH 7.3). Blood from the three PNH patients was collected for serum and erythrocytes before scheduled eculizumab infusion (patients 1 and 2 on long-term treatment; patient 3, treatment naïve). For additional experiments, serum was also collected immediately after eculizumab infusion. Serum from the aHUS patients on eculizumab was collected immediately prior to dosing of eculizumab.

Binding Affinity to factor D

Binding kinetics and affinities of compounds to human factor D were characterized by surface plasmon resonance using a BiacoreTM 3000 system (GE Healthcare). Recombinant factor D protein (R&D Systems, Minneapolis, MN) was immobilized on a CM5 sensor chip (GE Healthcare) using standard amine coupling chemistry with HBS-N (10 mM HEPES, 0.15 M NaCl, pH 7.4) as the running buffer. Briefly, the surface was activated with a 1:1 ratio of 0.4 M 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride/0.1 M N-hydroxy succinimide at a flow rate of 10 μL/min. Protein was diluted to 0.02 mg/mL in 10 mM sodium acetate pH 6.0 was captured by injecting onto the activated chip surface. Residual activated groups were blocked with a 7 minute injection of 1:1 mixture of 1 M ethanolamine (pH 8.5) with HBS-N. Final surface density of protein was 4000 RU (resonance units). Compound binding data were collected in 50 mM TrisHCl pH 7.5, 1 M NaCl, 0.005%

Tween 20 supplemented with DMSO (1%) at 50 µL/min. Data were double referenced and corrected for DMSO-excluded volume effects and fitted to a 1:1 binding model, allowing for mass transport effects, using Scrubber software (BioLogic Software).

Factor D protease activity against a small synthetic substrate

Factor D protease activity was measured using a biochemical assay consisting of purified factor D (Complement Technology, Inc., Tyler, TX), the synthetic thioester substrate N-α-Cbz-L-lysine thiobenzyl ester (Z-Lys-SBzl; Sigma-Aldrich) and the colorimetric reagent 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB, Ellman's reagent). Factor D-mediated esterolysis of substrate generates free sulfhydryl groups that react with the colorimetric detection agent DTNB to produce yellow color. Compounds were dissolved in DMSO and diluted in DMSO in half-log dilution series to 50× final assay concentrations. Factor D was diluted in assay buffer (50 mM Tris, 1.0 M NaCl, pH 7.5) and added to compound in a polypropylene microtiter plate for a 5 minute room temperature preincubation at 2× final concentration. 50 µL factor D + compound mixture was then transferred with gentle mixing to 50 µL assay buffer with Z-Lys-SBzl and DTNB in a microtiter plate. Final concentrations were 2 µg/mL (80 nM) factor D, 100 µM Z-Lys-SBzl, and 100 µM DTNB. A_{405} was measured in a Molecular Devices Spectramax Plus 384 plate reader at 30-second intervals for 30 minutes. Reaction rates (mA_{450} min^{-1}) calculated by the Spectramax software were used to calculate IC_{50} values using nonlinear regression analysis with the four-parameter sigmoidal dose-response equation (Prism, GraphPad Software, La Jolla, CA).

Factor D proteolytic activity against its natural substrate C3bB

280 nM C3b, 400 nM factor B, and 0.8 nM factor D (Complement Tech) were incubated in assay buffer (PBS with 10 mM MgCl) in the absence or presence of

compound (in half-log dilution series from 0.000316 μM to 1 μM) at 37°C for 30 minutes. Factor B cleavage was visualized by non-reducing 4-20% Tris-glycine SDS-PAGE followed by Coomassie blue staining. Bb product was quantified in parallel by MicroVue Bb Plus ELISA (Quidel). Inhibitor IC_{50} values were calculated by nonlinear regression analysis using the four-parameter sigmoidal dose-response equation (Prism, GraphPad Software).

Inhibition of APC-mediated and CPC-mediated hemolysis

Alternative pathway of complement (APC) hemolytic assays with rabbit erythrocytes were performed using standard procedures as described previously with minor modifications. Specifically, a final concentration of 8% normal human serum (NHS) was used as this amount consistently led to nearly complete lysis of rabbit erythrocytes. Compounds were assayed in duplicate. Reactions consisted of 50 μL GVB^0 (gel veronal buffer without Ca^{++} and Mg^{++}, pH 7.3, Complement Technology) supplemented with 10 mM MgEGTA (GVB^0/MgEGTA, pH 7.3), 50μL 20% NHS (Complement Technology) in GVB^0/MgEGTA and 20μL erythrocytes (5×10^8/mL, Complement Technology) in GVB^0/MgEGTA in the absence or presence of compound (concentration range from 0.000316 to 1μM) or DMSO. A 0% hemolysis control consisted of 100μL GVB^0/MgEGTA plus 20μL erythrocytes and the 100% hemolysis control consisted of 100μL water plus 20μL erythrocytes. After 30 minutes incubation at 37°C, erythrocytes were pelleted and optical absorbance of supernatants was read at 405 nM. EC_{50} and EC_{90} values were determined by non-linear regression analysis using the four-parameter sigmoidal dose-response equation (Graphpad Prism, La Jolla, CA).

CPC hemolytic assays with antibody sensitized sheep erythrocytes were performed using standard procedures as described previously with minor

modifications [1]. Specifically, a final concentration of 0.5% normal human serum (NHS) was used as this amount consistently led to nearly complete lysis of erythrocytes. Compounds were assayed in duplicate. Reactions consisted of 100 µL GVB^{++} (gel veronal buffer with 0.15 mM Ca^{++} and 0.5 mM Mg^{++}, pH 7.3), Complement Technology), 0.6 µL NHS (Complement Technology) and 30 µL erythrocytes (5×10^8/mL, Complement Technology) in GVB^{++} in the absence or presence of compound (concentration range from 0.09 to 200 µM) or DMSO. A 0% hemolysis control consisted of 100µL GVB^{++} plus 30µL erythrocytes and the 100% hemolysis control consisted of 100µL water plus 30µL erythrocytes. After 60 minutes incubation at 37°C, erythrocytes were pelleted and optical absorbance of supernatants was read at 541 nM. EC_{50} and EC_{90} values t were determined by non-linear regression analysis using the four-parameter sigmoidal dose-response equation (Graphpad Prism, La Jolla, CA).

Inhibition of APC-mediated lysis in PNH (Ham test)

The hemolytic assay was performed with PNH erythrocytes at 1% hematocrit in GVB^0/MgEGTA (pH 6.4) and 20% acidified human serum, as previously described [2]. Briefly, ABO blood group-compatible normal human serum (blood group B serum for patient 1, blood group B; pooled serum for patients 2 and 3, blood group O; sera from Complement Tech) was preincubated for 10 minutes on ice with inhibitor solution in half-log dilution series in dimethyl sulfoxide (DMSO) (final concentrations: 0.001 µM to 1 µM inhibitor; 1% DMSO) prior to acidification. Samples containing serum and inhibitor were acidified by addition of 0.2 N HCl to GVB^0/MgEGTA (pH 6.4). 10 µL PNH erythrocytes were added per well and incubated at 37°C for 1 hour. Controls: "Buffer", GVB^0/MgEGTA (pH 6.4) alone; "Serum+EDTA"; acidified serum inactivated by 5 mM EDTA to determine 0% APC-mediated hemolysis; "H_2O", H_2O

in place of buffer for complete osmotic hemolysis; and “Serum”, DMSO in place of inhibitor to determine 100% APC-mediated hemolysis. Supernatants were collected by centrifugation and optical absorbance was measured at 405 nM (A_{405}) using an iMark Microplate reader (Bio-Rad). Hemolysis in each well was normalized to the 0% and 100% controls, and IC_{50} and IC_{90} values were determined from duplicate wells by nonlinear regression analysis using the four-parameter sigmoidal dose-response equation (Prism).

Inhibition of dysregulated APC in aHUS

The modified Ham test was performed to assess the efficacy of factor D inhibitors in aHUS patient serum[3]. Briefly, *PIGA*-null reagent cells were plated in 80 µL GVB^{++} (gel veronal buffer with 0.15 mM Ca^{++} and 0.5 mM Mg^{++}, pH 7.3), then 20% serum preincubated with DMSO or inhibitors was added, and reactions were incubated for 30 minutes at 37°C. Heat-inactivated serum was used as a negative control. Next, cells were washed and incubated with WST-1 (Roche) at 1:10 dilution for 2 hours at 37°C. Optical absorbance was measured in an iMark Microplate Absorbance Reader (Bio-Rad) at 490 nm with a reference wavelength at 595nm. Mean absorbance was calculated for each sample based on triplicates and background absorbance (from a blank containing WST-1) as previously described.

Inhibition of C3 fragment deposition

C5-depleted human serum (Complement Technology) was preincubated for 10 minutes at 37°C with DMSO or compound solution (half-log dilution series in DMSO; final concentrations 0.0001 µM to 1 µM compound, 1% DMSO). Serum was acidified by addition of 0.2 N HCl to GVB^0/MgEGTA (pH 6.4). 10 µL erythrocytes was added per well and reactions were incubated at 37°C for 24 hours. Negative control reactions

included: serum inactivated by 20 mM EDTA; and, opsonization of normal blood group O erythrocytes. After incubation, erythrocytes were collected by centrifugation and washed with 1% BSA/PBS. Erythrocytes were stained with 1:200 FITC-conjugated anti-C3c antibody (Abcam) and 1:100 allophycocyanin-conjugated anti-CD59 (Invitrogen) and analyzed by flow cytometry. The phenomenon was also examined with 1:50 FITC-conjugated anti-C3b (anti-Human C3/C3b/iC3b, Cedarlane, catalog # CL7632F) and 1:200 AlexaFluor488-conjugated anti-C3d antibodies (Novus Biologicals, catalog #NB100-65510AF488) with the same methodology. Ghost and intact erythrocytes were gated using forward and side scatter; analysis was limited to intact cells. Gating for FITC-conjugated (or AlexaFluor488-conjugated) and allophycocyanin-conjugated antibodies was based on isotypic control antibodies. Data were analyzed using FlowJo software (Tree Star, Inc.). IC_{50} and IC_{90} values were determined by nonlinear regression analysis using the four-parameter sigmoidal dose-response equation (Prism). C3 fragment deposition on PNH erythrocytes from patients 1 and 3 (obtained before eculizumab infusion) was similarly assessed using the patient's own serum collected immediately after eculizumab infusion.

Off-target serine proteases

Inhibition of purified serine proteases by ACH-3856 and ACH-4471 was determined by Cerep Laboratories (Eurofins Pharma Discovery Services) using purified enzymes paired with specific substrates under standardized reaction conditions. Reaction progress in duplicate reactions was measured following incubation by fluorometry or photometry. Percent inhibition was calculated from measured specific activity in the presence and absence of inhibitor. Reactions were validated with reference inhibitors in parallel reactions

Supplemental Results

Binding kinetics and affinities of ACH-3856 and ACH-4471 to recombinant factor D were assessed by surface plasmon resonance with immobilized recombinant protein: parameters are presented in the main manuscript (Table 3) and in Supplemental Figure 1. Inhibitory activities of these compounds against purified factor D enzyme with a small synthetic thioester substrate are presented in the main manuscript (Table 3) and Supplemental Figure 2. Inhibitory activities in APC and complement classical pathway hemolysis assays are presented in the main manuscript (Table 4).

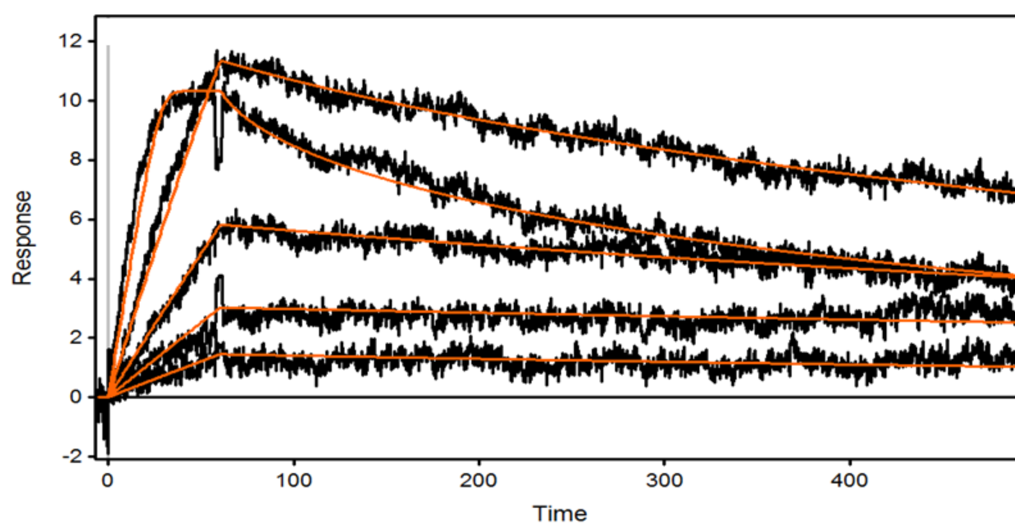
Inhibition of APC-mediated lysis of PNH erythrocytes (Ham test) is presented in the main manuscript (Figure 2). Inhibition of C3 fragment deposition on PNH erythrocytes as detected by anti-C3c staining is presented in the main manuscript (Figure 3); the staining of approximately 10% of cells by anti-C3d but not by anti-C3c when treated with serum in the presence of EDTA is presented in Supplemental Figure 3. Inhibitory activities against a panel of serine proteases are presented in Supplemental Table 1.

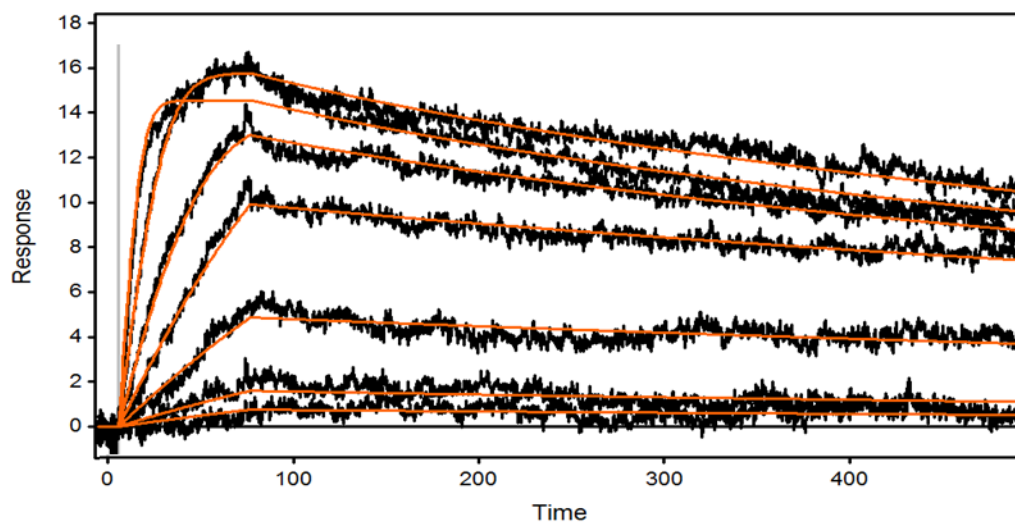
Supplemental Table 1. Inhibition of off-target serine proteases

Enzyme	Source	% inhibition at 10 µM	
		ACH-3856	**ACH-4471**
Thrombin	human plasma	3.3	1.2
Urokinase	human kidney	-7.6	ND
Chymase	human skin	27.6	-1.2
Elastase	human leukocytes	-3.5	0.7
cathepsin G	human leukocytes	7.6	0.4
dipeptidyl peptidase IV(DPP-IV)	human recombinant	-1.9	-1
HNE human neutrophil elastase)	human serum	-4.9	ND
Tryptase	human lung	-4.5	1.4
Trypsin	human pancreas	-6	0.1
Kallikrein	Human plasma	2.8	20.8
PLAU (Urokinase)	Human Urine	ND	2.5
ELA2 (Neutrophil Elastase 2)	Human neutrophils	ND	14.5

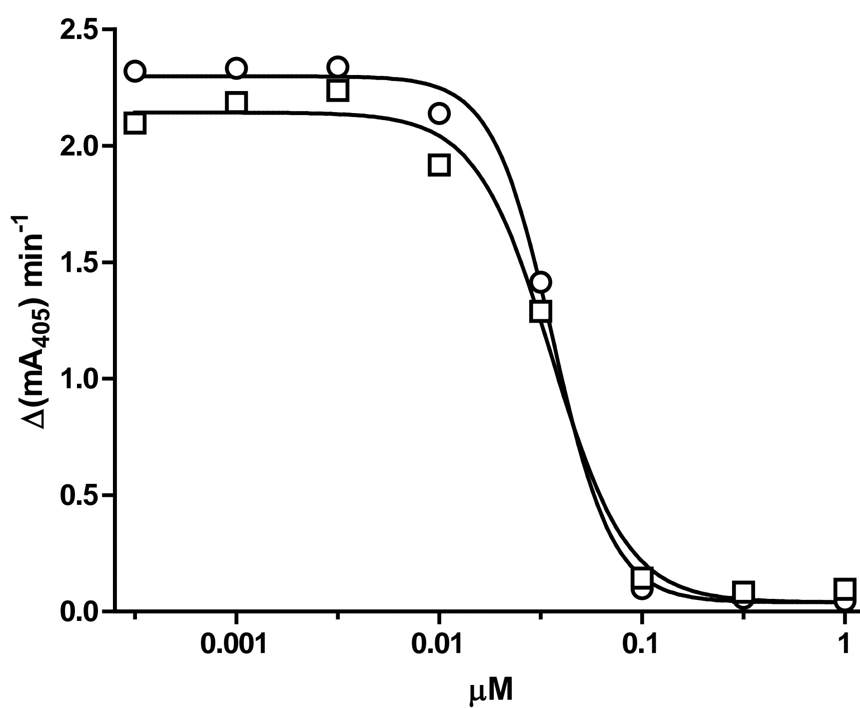
Supplemental Figure 1. Factor D inhibitor binding to factor D protein.

Representative surface plasmon resonance (SPR) sensorgrams with fitted curves are shown for compound association with (0 through 60 seconds) and dissociation from (60 seconds to end) immobilized factor D. ACH-3856 (top) or ACH-4471 (bottom) was added in two-fold dilution series up to 100 nM. SPR response is expressed in arbitrary units. Table 3 in the main manuscript presents kinetic parameters.



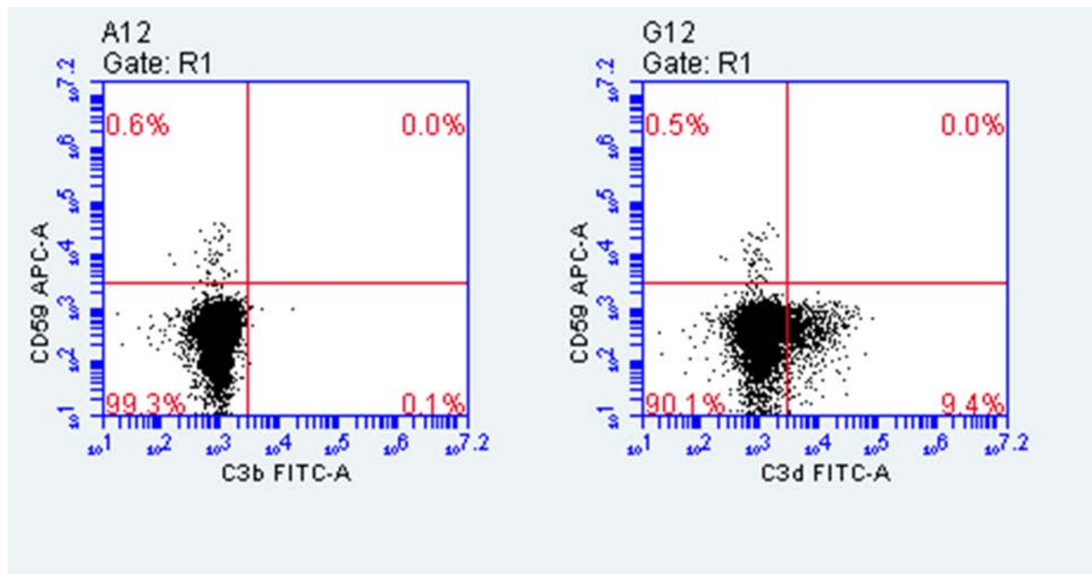


Supplemental Figure 2. fD esterolytic activity. Dose-response curves are shown from a representative experiment demonstrating enzymatic inhibition of purified factor D by ACH-3856 (○) and ACH-4471(□). Table 3 in the main manuscript presents IC_{50} values.



Supplemental Figure 3. Staining of a subset of erythrocytes recovered from PNH patient 2 with anti-C3d antibody but not anti-C3c antibody.

Flow cytometric scattergrams of erythrocytes from PNH patient 2 treated with acidified C5-depleted serum (EDTA treated). C3 fragment deposition was assessed using anti-human C3c or anti human C3b antibody (left panel, X-axis) or anti human C3d antibody (right panel, 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.



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