Assessment of Complement-Mediated Bacterial Killing and the Effect of a Small Molecule Factor D Inhibitor in Vitro

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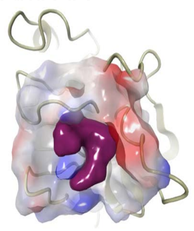
INTRODUCTION

- The complement system is an enzymatic cascade of more than 30 proteins that is activated via the classical pathway (CP), lectin pathway (LP) or alternative pathway (AP) upon pathogen associated molecular pattern recognition.
- Activation of complement leads to the generation of biologically active fragments that play a critical role in host defense. Complement fragments opsonize pathogens, recruit and activate inflammatory cells at the site of complement activation and act as the nidus for assembly of the terminal lytic attack complex on the surface of pathogens.
- Complement activation is a well coordinated physiological response that is highly regulated by various fluid phase as well as membrane bound regulatory proteins to prevent host tissue damage. The advancement of complement-directed therapies has proven to be a viable therapeutic approach for diseases associated with dysregulated complement (either genetic or acquired).
- Strategies aimed at complement blockade could be accompanied with increased risk of bacterial infection as has been reported in humans with the C5 inhibitor, eculizumab. Moreover, the contribution of individual complement pathways to elimination/clearance of pathogens is not completely understood.
- Using normal human serum, complement depleted serum and purified complement components for reconstitution studies we undertook the study herein to define the mechanism(s) by which complement enhances bactericidal activity and opsonophagocytosis of *E. coli*. Moreover we evaluated the effects of factor D (fD) inhibitor ACH-4471 in the context of these experimental conditions.
- ACH-4471 is a novel, highly-potent and specific orally-administered small molecule inhibitor of fD, a serine protease within the AP.
- ACH-4471 prevents factor B cleavage into Ba and Bb in the AP, leading to blockade of C3 convertase production and the Amplification loop upon activation of either the CP or LP.
- ACH-4471 is currently in phase 1 clinical study.



Inhibitor: CFD complex at 0.8 Å

OBJECTIVES

- Assess the contribution of individual complement pathways to bactericidal activity and opsonophagocytosis *in vitro*
- Evaluate the effect of ACH-4471 on bactericidal activity and opsonophagocytosis *in vitro*

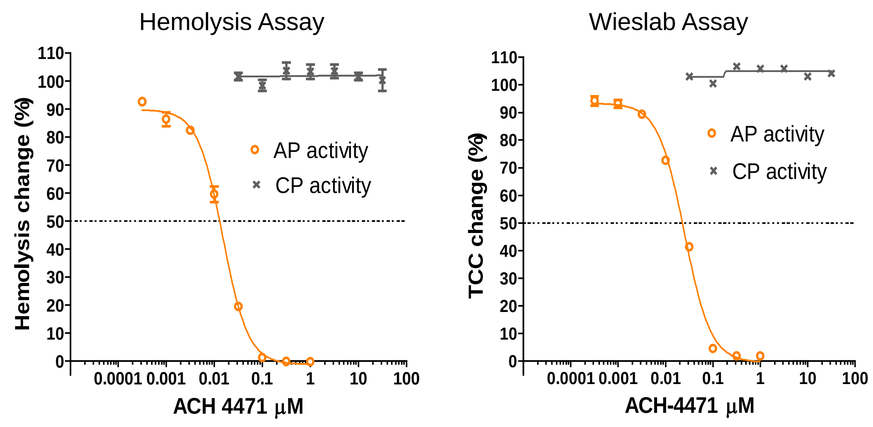
METHODS

- **Reagents:** Human whole blood was obtained with informed consent. Normal human serum (NHS), complement-depleted NHS, complement proteins, rabbit erythrocytes, GVB^{++} buffer were purchased from Complement Technology, Inc. (Tyler, Texas USA). Wieslab assay was purchased from (Euro Diagnostica, Sweden). *Escherichia coli* (*E. coli*) strain DH5α was purchased from ThermoFisher Scientific (Waltham, MA USA). Phagotest kit was purchased from Glycotope Biotechnology (Heidelberg, Germany). PE Mouse Anti-Human CD11b and PE-Cy™7 Mouse Anti-Human CD14 were purchased from BD Biosciences (San Jose, CA USA).
- **Complement assays:** Briefly, NHS pre-incubated with ACH-4471 or vehicle control (DMSO) for 10 minutes was incubated with rabbit erythrocytes or in Wieslab assay microtiter strips according to the manufacturer's recommendations for hemolysis activity or terminal complement complex formation, respectively. The degree of complement activation directly correlates with color intensity as measured by optical density.
- **Bactericidal activity:** Briefly, NHS or complement depleted serum was pre-incubated with ACH-4471 or vehicle control (DMSO) for 10 minutes. *E. coli* was added and then the reactions were incubated at 37 ºC for 30 minutes. At the end of the incubation period, the reaction mixtures were stained with SYBR® Green I Nucleic Acid Gel Stain and Propidium Iodide and viable bacteria analyzed on BD Accuri C6 flow cytometer.
- **Monocyte and granulocyte isolation:** Heparinized blood samples were used for isolation of leukocytes via density gradient centrifugation. Cells were resuspended at 1x10^8 per ml in GVB^{++} buffer for phagocytosis assays.
- **Phagocytosis assay:** Briefly, NHS, complement-depleted NHS or complement- depleted NHS reconstituted with the depleted complement protein(s) was pre-incubated with ACH-4471 or vehicle control (DMSO) for 10 minutes. Next leukocytes and FITC-labeled *E. coli* were added and the reactions were incubated in a 37ºC water bath for 15 minutes; in parallel a negative control reaction was incubated on ice. The final reactions consisted of 5 × 10^6 leukocytes and 40 × 10^6 FITC-labeled *E. coli*. Cells were labeled with PE Mouse Anti-Human CD11b antibody and PE-Cy™7 Mouse Anti-Human CD14 antibody and analyzed on BD Accuri C6 flow cytometer for gating and identification of cell types.

RESULTS

Effect on Serum Complement Activities

Fig 1: AP Activity as Measured by Hemolysis and Wieslab



Compound	AP Hemolysis Assay IC$_{50}$ ± SD (µM)	N	AP Wieslab Assay IC$_{50}$ ± SD (µM)	N
ACH-4471	0.027 ± 0.013	16	0.023 ± 0.014	4
Anti-C5 mAb	2.5 ± 1.1	2	0.054 ± 0.023	5

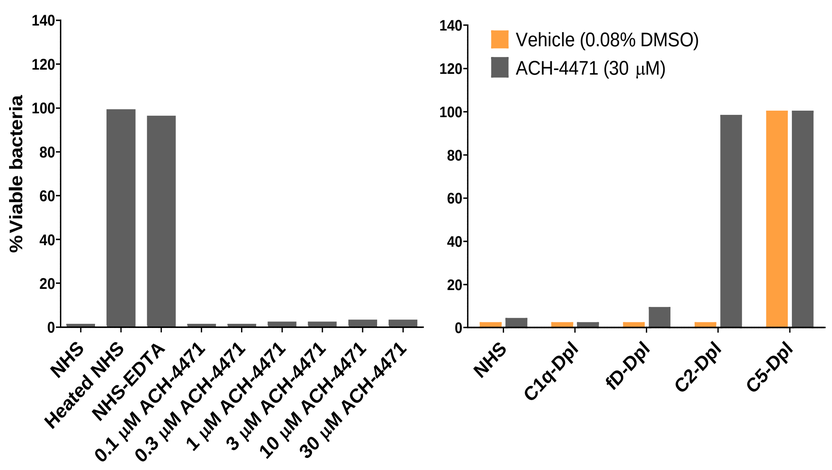
Compound	CP Hemolysis Assay IC$_{50}$ ± SD (µM)	N	CP Wieslab Assay IC$_{50}$ ± SD (µM)	N
ACH-4471	>100	2	>100	3
Anti-C5 mAb	0.0047 ± 0.0038	2	0.010 ± 0.003	2

ACH-4471, fD inhibitor
Anti-C5 mAb, C5 terminal pathway inhibitor
TCC: Terminal Complement Complex

- ACH-4471 potently and selectively inhibits the complement alternative pathway (AP) in both assays
- While able to inhibit both the complement classical pathway (CP) and AP, a monoclonal C5 antibody displays much weaker potency in the AP hemolysis assay (see Abstract **86** for details)

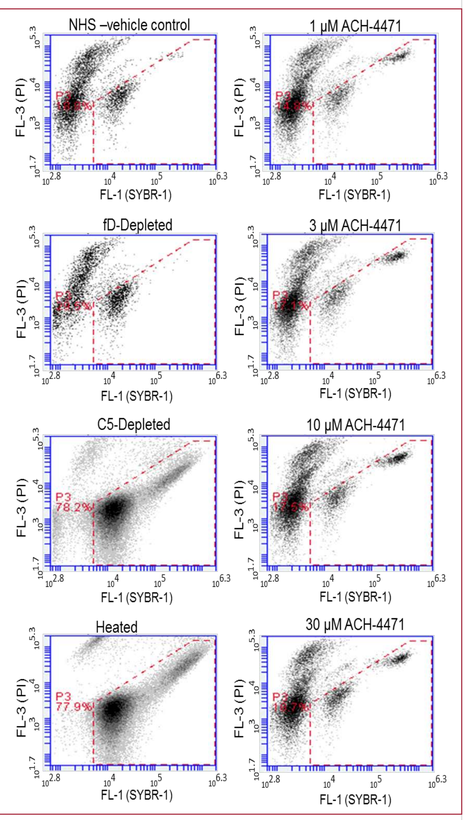
Effect on Serum Bactericidal Activities

Fig 2: Bacterial Viability Measured in the Presence of NHS or Complement Depleted NHS



	% Viable Bacteria
NHS	2
NHS-Heated	100
0.1 µM ACH-4471	2
0.3 µM ACH-4471	2
1 µM ACH- 4471	3
3 µM ACH-4471	3
10 µM ACH-4471	4
30 µM ACH-4471	4
C1q -DpI	2
fD-DpI	2
C2-DpI	2
C2-DpI + 30 µM ACH-4471	98
C5-DpI	100

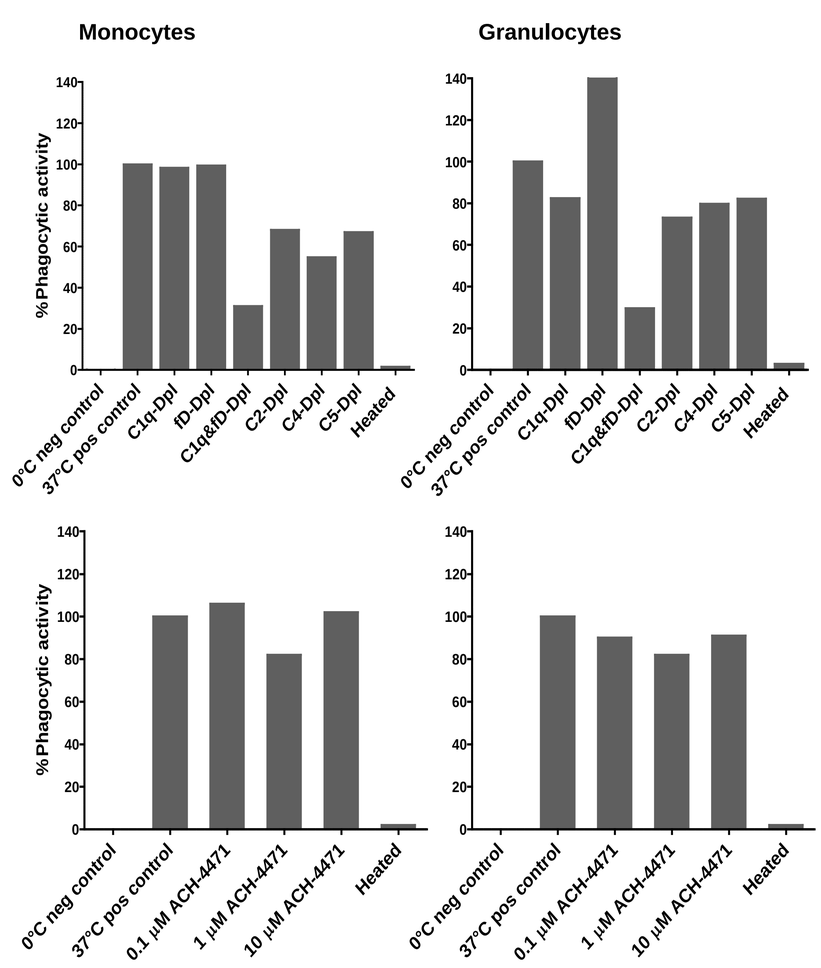
ACH-4471, fD inhibitor
NHS, Normal Human Serum
Dpl, Depleted
PI, Propidium Iodide
FL, Fluorescence



- ACH-4471 did not alter the bactericidal activity of normal human serum (NHS)
- Sera depleted of C1q (CP), C2 (CP & LP) or fD (AP) retained complete bactericidal activity
- Blockade of all complement pathways (C2-DpI + ACH-4471) or the terminal pathway (C5-DpI) completely inhibited serum bactericidal activity

Effect on Opsonophagocytosis

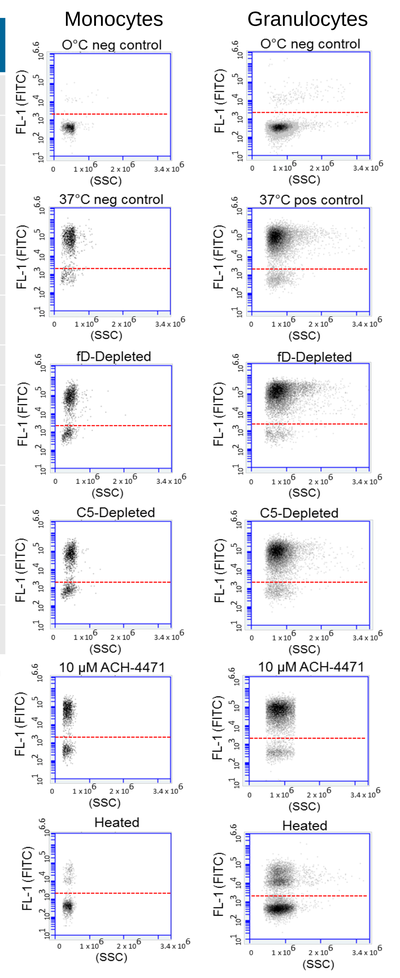
Fig 3: Opsonophagocytosis Measured in the Presence of NHS or Complement Depleted NHS



Serum/ Treatment	% Phagocytic Activity	
	Monocyte	Granulocyte
0°C Neg control	0[a]	0[a]
37°C Pos control	100[a]	100[a]
C1q-DpI	98[b]	82[b]
fD-DpI	99[b]	144[b]
C1q&fD-DpI	31[b]	30[b]
C2-DpI	68[b]	73[b]
C4-DpI	55[b]	80[b]
C5-DpI	67[b]	82[b]
Heated	2[b]	3[b]
0.1 µM ACH-4471	106[a]	90[a]
1 µM ACH-4471	82[a]	82[a]
10 µM ACH-4471	102[a]	91[a]

[a.] Percentages were calculated relative to NHS incubated at 37°C
[b.] Percentages were calculated relative to the depleted serum fully reconstituted with the missing component(s) at 37°C

ACH-4471, fD inhibitor
NHS, normal human serum
Dpl, depleted serum
Neg, negative
Pos, positive
Heated, NHS heated at 56ºC for 30 min



- Serum complement enhanced phagocytosis of *E. coli* by monocytes and granulocytes
- Serum depleted of C1q or fD retained near complete phagocytic activity (<20% reduction) whereas depletion of both (C1q & fD-DpI) exhibited a marked defect in phagocytic activity (~70% reduction)
- Serum depleted of C2, C4 and C5 exhibited reduced phagocytic activity of monocytes and/or granulocytes (30 - 50% reduction)
- Serum ACH-4471 did not significantly alter the phagocytic activity of normal human serum (NHS)

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CONCLUSIONS

- The terminal complement pathway is indispensable for bactericidal activity against *E. coli*
- There is a significant degree of redundancy in the contribution of complement pathways for bactericidal activity and opsonophagocytosis
- Inhibition of fD by ACH-4471 did not significantly alter the bactericidal activity as well as monocyte/granulocyte opsonophagocytosis against *E. coli* at the concentrations that completely inhibited AP activity
- Clinical use of a fD inhibitor might present reduced infection risk compared to terminal complement inhibitors, especially in the population who have developed antibodies towards pathogens naturally and/or via vaccination
- Future studies are aimed at investigating other pathogens including bacteria that cause meningococcal disease

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