Supplemental Methods

Characterization of C3 binding by surface plasmon resonance
Surface plasmon resonance (SPR) was used to determine binding affinities and kinetic rate constants based on previously described protocols. All experiments were performed on a Biacore 3000 instrument (GE Healthcare) at 25 °C in 10 mM PBS pH 7.4 containing 0.005% Tween-20. C3 was immobilized on a CM5 sensor chip (GE Healthcare) to a density of 5000 resonance units by amine coupling. A series of five increasing concentrations of each compstatin analog (1.25, 2.5, 5, 10, and 20 nM for Cp40; 6.25, 12.5, 25, 50, and 100 nM for PEG-Cp40 and Cp40-K-PEG) was injected consecutively for 2 min at a flow rate of 30 µl/min with a final dissociation step of 40 min. All sensorgrams were processed using Scrubber and analyzed in BIAevaluation using a single-cycle kinetics protocol for 1:1 interactions (GE Healthcare).

Determination of plasma inhibitor levels by mass spectrometry
Monitoring of inhibitor levels in monkey plasma was performed using a combination of solid-phase extraction (SPE), ultra-performance liquid chromatography (UPLC) and high-resolution mass spectrometry (MS) essentially as described before. Briefly, plasma samples (50 µL) were mixed with 4% H$_3$PO$_4$ (50 µL), and 200 µL acetonitrile (ACN) was added to precipitate proteins. After centrifugation (10,000 rpm, 10 min), the supernatant was evaporated and the residue was resuspended in PBS. In the case of PEG-Cp40, subtilisin-A (0.4 mg/ml final concentration) was added to plasma samples and incubated for 1 h at 37 °C prior to SPE in order to generate fragments suitable for MS analysis. Calibration curves were prepared on the day of the analysis by spiking compstatin analogs (final concentrations of 1, 2, 4, 8, and 16 µM) into freshly thawed plasma from untreated animals. All calibration samples were subjected to enzymatic treatment (in the case of PEG-Cp40), SPE and measured using UPLC-MS as described below. SPE was performed using 96-well HLB oasis plates (30 µm, 10 mg; Waters) after conditioning with methanol, ACN and water. Each sample was loaded, washed with water and with 10% ACN containing 0.1% formic acid, and eluted into LoBind tubes (Eppendorf) with 60% ACN 0.1% formic acid at 4 °C. MS analysis was performed using a Waters Synapt G2-S instrument equipped with an ESI source controlled by MassLynx 4.1 software (Waters). Each sample was injected in quadruplicates. A online UPLC (ACQUITY, Waters) system was used for peptide separation by reversed-phase liquid chromatography (1.7 µm UPLC BEH130 C18 column; 1.0 µm x 100 mm; Waters) using a gradient of 15-55% B (0.1% formic acid in acetonitrile) over 7 min. The capillary voltage was 3.2 kV, the cone voltage was 30 V and the source temperature was 120 °C. Leucine enkephalin was used for lock-mass correction with a sampling rate of 30 s. Mass spectra were acquired in positive mode over an m/z range 100-1800 Da at a scan rate of 1 s. The presence of the analyte was confirmed by retention time and mass. MS peak areas were determined by integration and plotted against the concentration, resulting in calibration curves that showed good linearity ($R^2 > 0.99$). For the pharmacokinetic analysis, the plasma concentration (Cp) at each time point was calculated from the extracted peak area of each peptide using the corresponding standard curve. The elimination constant ($k_e$) and plasma half-life ($t_{1/2}$) were determined from the slope of the terminal elimination phase using the following equation: \( \ln(Cp) = \ln(Cp0) - k_e \times t \), and \( t_{1/2} = 0.693/k_e \).
Measurement of ex vivo hemolytic activity in non-human primate plasma

Although the lack of in vivo models of PNH in NHP restricts acquisition of true pharmacodynamic data, a relevant ex vivo model was developed that allows the simulation and measurement of anti-hemolytic activity during inhibitor treatment in the plasma of the same animals that were used for the pharmacokinetic studies. Plasma samples before injection of compstatin analogs (time 0; T0) were passed through a Micro Bio-Spin 6 column (Bio-Rad) to remove EDTA, which had been added during sample collection to prevent coagulation and plastic-induced complement activation; a selective thrombin inhibitor (lepirudin; 50 µg/ml) was added during this exchange step to maintain anticoagulant status without blocking complement activity. Cp40 was added to complement-restored plasma samples at concentrations measured by LC-MS at each time point during the pharmacokinetic studies; please note that this extrinsic addition of Cp40 to pre-treated plasma was required as direct filtration of treatment samples through the Micro Bio-Spin 6 column would partially remove unbound Cp40 and shift the target-inhibitor ratio. To assess hemolytic activity, rabbit erythrocytes (Lampire Biological Laboratories) were incubated in each Cp40-treated plasma sample for 60 min, and inhibition of hemolysis was measured as a decrease of absorbance at 405 nm compared to the untreated full-hemolysis sample at T0.

References used in the Supplemental Materials

Supplemental Figures

Supplemental Figure S1. Compstatin analogs mentioned in this study. (A) Structure of compstatin with analog-specific modifications marked as R₁-R₃. Blue numbers (1-3) represent residue numbers based on the sequence of original compstatin. (B) Overview of compstatin analogs with their synonyms used in clinical development, peptide sequence, complement inhibition activity (IC₅₀), target binding affinity (Kₑ) and terminal elimination half-lifes (Plasma t½).

Supplemental Figure S2. Binding of compstatin Cp40 and its PEGylated derivatives to immobilized C3. Surface plasmon resonance (SPR) analysis of compstatin analogs Cp40 (red), PEG-Cp40 (blue), and Ac-Cp40-K-PEG (green) binding to C3 that was covalently immobilized to a carboxymethyl-dextran sensor chip. Single cycle kinetic analysis using five consecutive injections of each compound was performed. The table on the right side lists association rate (kₐ) and dissociation rate (kₜ) constants and binding affinity (Kₑ = kₜ/kₐ) after fitting to a Langmuir binding model based on 1:1 binding to a single binding site. RU, resonance units.
Supplemental Figure S3. *Ex vivo* hemolysis assay in Cp40-treated non-human primate (NHP) plasma. The anti-hemolytic potency of compstatin Cp40 at inhibitor levels measured during the subcutaneous injection regimen was assessed by an *ex vivo* hemolysis assay. (A) Schematic overview of the assay procedure. Plasma samples from one of the animals used in the repetitive subcutaneous treatment were used for the assay. The plasma concentrations of Cp40 were determined using solid-phase extraction (SPE) and liquid chromatography-coupled mass spectrometry (LC-MS) for all 17 time points collected during treatment period; the pre-treatment plasma sample (T0) was used to generate the standard curve. The same T0 sample was also used to determine the baseline C3 concentration by ELISA. As all samples has been collected in EDTA tubes to prevent coagulation and inadvert complement activation, the T0 was passed through a Micro Bio-Spin size exclusion column to remove EDTA and restore complement activity; lepirudin was added to maintain prevention of coagulation. Cp40 was added to the complement-restored plasma at the concentrations measured by LC-MS before incubation with rabbit red blood cells (RBC). (B) Inhibition of rabbit RBC lysis in complement-restored NHP plasma at Cp40 concentrations observed during repetitive subcutaneous treatment. The percent inhibition of hemolysis compared to the Cp40-free control (red data points) is shown alongside the Cp40 concentrations (green data points) and the baseline C3 level (blue dashed line) for all time points of the subcutaneous treatment study. Strong inhibition was observed for all time points, in which the Cp40 concentration exceeded the C3 level.