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Novel analogues of the therapeutic complement inhibitor compstatin with significantly improved affinity and potency

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ABSTRACT

Compstatin is a 13-residue disulfide-bridged peptide that inhibits a key step in the activation of the human complement system. Compstatin and its derivatives have shown great promise for the treatment of many clinical disorders associated with unbalanced complement activity. To obtain more potent compstatin analogues, we have now performed an N-methylation scan of the peptide backbone and amino acid substitutions at position 13. One analogue (Ac-I[CVW(Me)QDW-Sar-AHRC](NMe)I-NH_2) displayed a 1000-fold increase in both potency (IC_{50} = 62 nM) and binding affinity for C3b (K_D = 2.3 nM) over that of the original compstatin. Biophysical analysis using surface plasmon resonance and isothermal titration calorimetry suggests that the improved binding originates from more favorable free conformation and stronger hydrophobic interactions. This study provides a series of significantly improved drug leads for therapeutic applications in complement-related diseases, and offers new insights into the structure–activity relationships of compstatin analogues.

1. Introduction

The human complement system is an integral part of innate immunity that acts as a surveillance and clearance system of the human body (Mastellos et al., 2004; Ricklin et al., 2010). When complement recognizes foreign surfaces, its intricate network of membrane-bound and fluid-phase proteins is activated through several initiation pathways, which all result in the generation of strong opsonins, the lytic membrane attack complex, and pro-inflammatory anaphylatoxins. These processes are essential to the elimination of invading microorganisms, the clearance of immune complexes and apoptotic cells, and the stimulation of adaptive immune responses (Carroll, 2008; Sjoberg et al., 2009). However, inappropriate or uncontrolled activation of complement can cause damage to host cells or serious disturbance of homeostasis, and it has been associated with a wide array of autoimmune, inflammatory, and neurodegenerative disorders, including rheumatoid arthritis, systemic lupus erythematosus, age-related macular degeneration, sepsis, and Alzheimer's disease (Chen et al., 2010; Lachmann and Smith, 2009; Mollnes and Kirschfink, 2006). Excessive activation of complement has also been linked to ischemia-reperfusion injuries, as seen in stroke or during cardiopulmonary bypass surgery (Diepenhorst et al., 2009; Weisman et al., 1990). In addition, complement has recently been shown to contribute to tumor growth in mice (Markiewski et al., 2008).

Therapeutic inhibition of complement has been found to be highly beneficial in numerous disease studies involving both low molecular weight and biopharmaceutical complement inhibitors (Qu et al., 2009b; Ricklin and Lambris, 2007). The U.S. Food and Drug Administration has approved two complement-targeting drugs thus far, recombinant C1 esterase inhibitor (Cinryze, ViroPharma; Berinert, CSL Behring) for treating hereditary angioedema and the therapeutic antibody Eculizumab (Soliris, Alexion Pharmaceuticals) for paroxysmal nocturnal hemoglobinuria (Cocchio and Marzella, 2009; Inoue et al., 2003; Rother et al., 2007). In addition, a host of complement inhibitors are currently in clinical trials or in advanced pre-clinical development for various indications (Qu et al., 2009b; Ricklin and Lambris, 2007).

Owing to their excellent safety and efficacy profiles and their ability to block activation of complement regardless of the initiation pathway, compstatin derivatives are considered among the most

Abbreviations: Ac, acetyl group; DCM, dichloromethane; DIC, 1,3-diisopropylcarbodiimide; DIPEA, *N*,*N*-diisopropylethylamine; DMF, *N*,*N*-dimethylformamide; ESI, electrospray ionization; Fmoc, 9-fluorenylmethoxycarbonyl; HOAt, 1-hydroxy-7-aza-benzotriazole; ITC, isothermal titration calorimetry; Nle, L-norleucine; NMP, N-methylpyrrolidinone; SPR, surface plasmon resonance; TFA, trifluoroacetic acid; TIPS, triisopropylsilane.

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promising of candidate drugs for preventing undesirable effects of complement (Ricklin and Lambris, 2008). One compstatin analogue (POT-4; Potentia Pharmaceuticals & Alcon Inc.) has demonstrated beneficial results in a recently completed Phase I clinical trial for the treatment of age-related macular degeneration and is likely to be further developed for both wet and dry forms of the disease (Anon., 2009). In addition, compstatin has shown highly promising effects in a number of other diseases, as very recently in the case of sepsis (Silasi-Mansat et al., 2010) and complement-related adverse effects during hemodialysis (Kourtzelis et al., 2010). Finally, compstatin is widely used as a valuable tool in immunological research for investigating the effect of the complement cascade in both physiological and pathophysiological processes.

Compstatin was originally identified as a 13-residue disulfide-bridged peptide (H-Ile-[Cys-Val-Val-Gln-Asp-Trp-Gly-His-His-Arg-Cys]-Thr-NH_2) that selectively binds to human and primate forms of the central complement component C3 and its active fragment, C3b (Sahu et al., 1996). It thereby prevents the essential conversion of C3 to C3b and simultaneously impairs all initiation, amplification, and terminal pathways of complement. Over the past decade, extensive structure–activity relationship studies of compstatin have been conducted with the aid of computational molecular modeling and biophysical analysis (Klepeis et al., 2003; Magotti et al., 2009; Ricklin and Lambris, 2007, 2008; Soulika et al., 2003). This work has led to the development of [Trp(Me)^4]-Ac-compstatin (Ac-Ile-[Cys-Val-Trp(Me)-Gln-Asp-Trp-Gly-Ala-His-Arg-Cys]-Thr-NH_2), which displays the highest inhibitory activity reported thus far: a 264-fold increase in potency (IC_{50} = 205 nM) over the original compstatin in inhibiting complement activation (Katragadda et al., 2006).

Despite these impressive improvements, the development of more potent and stable compstatin analogues is still desirable, given the high plasma concentration of C3 (0.75–1.35 mg/mL) and the limited half-life of compstatin in vivo (Qu et al., 2009a; Soulika et al., 2000). Such analogues would provide greater therapeutic value and allow broader clinical applications. In the present study, we began by analyzing the previously reported data from surface plasmon resonance (SPR) and isothermal titration calorimetry (ITC) assays, which are very useful guides for rational drug development effects (Carbonell and Freire, 2005; Huber, 2005; Sarver et al., 2007; Zhu et al., 2009). We noticed that the binding of compstatin analogues to C3 is predominantly enthalpy-driven and features large entropic penalties, a situation that is in sharp contrast to that observed for compounds such as statins or HIV protease inhibitors, which generally show favorable entropy terms (Freire, 2008). In the case of [Trp(Me)^4]-Ac-compstatin, for example, the highly favorable enthalpy of −17.6 kcal/mol is largely compensated by an unfavorable entropy of 6.9 kcal/mol (Katragadda et al., 2006). These studies clearly indicated that there is still room for further improvement in terms of decreasing entropy.

One of the ways to decrease the binding-related entropy of peptides is backbone N-methylation, which has been shown to provide local constraints to the peptide backbone and thereby affect both secondary structure and side chain orientation (Fairlie et al., 1995; Laufer et al., 2009; Moretto et al., 2006). Such modifications have been shown to offer several potential benefits, including enhanced binding or receptor selectivity, increased half-life in plasma, and improved cell membrane penetration (Chatterjee et al., 2008; Rovero et al., 1989; Weltrowska et al., 2010). Therefore, we performed a mono-N-methylation scan on the [Tyr^4]-Ac-compstatin template (Klepeis et al., 2003). Based on the ELISA results for these analogues, selective N-methylation and amino acid substitutions were then applied to the more potent [Trp(Me)^4]-Ac-compstatin. The most active analogues were further characterized using SPR and ITC. Using this integrated approach, we were able to generate compstatin analogues with significantly improved efficacy and affinity when compared to the previous lead compound.

2. Materials and methods

2.1. Chemicals

Low-loading Rink amide MBHA resin and the following Fmoc-amino acids were obtained from Novabiochem (San Diego, CA): Ile, Cys(Acm), Val, Tyr(tBu), Gln(Trt), Asp(OtBu), Trp(Boc), Gly, Sar, Ala, MeAla, His(Trt), Arg(Pmc), MeIle, Nle, Phe, and Thr(tBu). DIC and Fmoc-Trp(Me)-OH were purchased from AnaSpec (San Jose, CA). HOAt was purchased from Advanced ChemTech (Louisville, KY). NMP and DCM were obtained from Fisher Scientific (Pittsburgh, PA). All other chemical reagents for synthesis were purchased from Sigma–Aldrich (St. Louis, MO) and used without further purification.

2.2. Peptide synthesis and purification

All peptides in this study were synthesized manually by Fmoc solid-phase methodology using DIC and HOAt as coupling reagents. When N-methylated amino acids were not commercially available, N-methylation was performed by using the optimized methodology reported by Biron et al. (2006). The following procedures were used for the synthesis of the linear peptides: Rink amide MBHA resin (294 mg, 0.34 mmol/g) was placed into a 10 mL HSW polypropylene syringe with frits on the bottom (Torviq, Niles, MI) and swollen in DCM (5 mL) for 30 min. After removal of the Fmoc protecting group (25% piperidine in NMP, 5 mL, 5 and 10 min), the resin was washed four times with NMP (5 mL per wash) and DCM (5 mL per wash), and the individual amino acids were coupled to the resin. For each coupling, 3 equivalents (0.3 mmol) of the amino acid, HOAt, and DIC were used, with 10 min preactivation in NMP. All couplings were performed for 1 h and monitored by either the Kaiser test or the chloranil test. In case of a positive test result, the coupling was repeated until a negative test result was observed.

The N-terminal amino group was acetylated with 20 equivalents of acetic anhydride and 2 equivalents of DIPEA in 5 mL of DCM for 30 min. Linear peptides containing Cyc(Acm) residues were cyclized on resin using thallium trifluoroacetate in DMF/anisole (19:1) at ambient temperature for 3 h. The resin was washed four times with DMF, DCM, and DCM/diethylether (1:1) (each 5 mL per wash), and dried under vacuum for 4 h. The peptides were cleaved from the resin with a mixture of 95% TFA, 2.5% water, and 2.5% TIPS for 3 h. After evaporation of the TFA under vacuum, the peptides were precipitated and washed three times with 30 mL of cold diethyl ether per wash. The liquid was separated from the solid by centrifugation and decanted. The crude peptides were dried in air and dissolved in acetonitrile and 0.1% TFA in water (1:3) before purification by preparative RP-HPLC (Vydac C_{18} 218TP152022 column, Western Analytical Products, Murrieta, CA) and elution with a linear gradient of 15–50% acetonitrile in aqueous 0.1% TFA solution over 35 min at a flow rate of 15 mL/min. Fractions containing the desired products were collected, concentrated, and lyophilized. The purified peptides were isolated in 10–15% overall yields and were >95% pure as determined by analytical RP-HPLC (Phenomenex 00G-4041-E0 Luna 5 μm C_{18} 100 Å column, 250 mm × 4.60 mm; Phenomenex, Torrance, CA). The mass of each peptide was confirmed using ThermoQuest Finnigan LCQ Duo and Waters MALDI micro MX instruments.

2.3. Purification of C3

C3 was purified from fresh human plasma obtained from the blood bank of the Hospital of the University of Pennsylvania as described previously (Hammer et al., 1981). In brief, the plasma was