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Structure–activity relationships for the binding of polymyxins with human α-1-acid glycoprotein

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ABSTRACT

Here, for the first time, we have characterized binding properties of the polymyxin class of antibiotics for human α-1-acid glycoprotein (AGP) using a combination of biophysical techniques. The binding affinity of colistin, polymyxin B, polymyxin B_3, colistin methansulfonate, and colistin *nona*-peptide was determined by isothermal titration calorimetry (ITC), surface plasma resonance (SPR) and fluorometric assay methods. All assay techniques indicated colistin, polymyxin B and polymyxin B_3 display a moderate binding affinity for AGP. ITC and SPR showed there was no detectable binding affinity for colistin methansulfonate and colistin *nona*-peptide, suggesting both the positive charges of the diaminobutyric acid (Dab) side chains and the *N*-terminal fatty acyl chain of the polymyxin molecule are required to drive binding to AGP. In addition, the ITC and fluorometric data suggested that endogenous lipidic substances bound to AGP provide part of the polymyxin binding surface. A molecular model of the polymyxin B_3–AGP F1*S complex was presented that illustrates the pivotal role of the *N*-terminal fatty acyl chain and the D-Phe6-L-Leu7 hydrophobic motif of polymyxin B_3 for binding to the cleft-like ligand binding cavity of AGP F1*S variant. The model conforms with the entropy driven binding interaction characterized by ITC which suggests hydrophobic interactions coupled to desolvation events and conformational changes are the primary driving force for polymyxins binding to AGP. Collectively, the data are consistent with a role of this acute-phase reactant protein in the transport of polymyxins in plasma.

1. Introduction

The lipocalin superfamily is a ubiquitous class of extra-cellular transporters of small hydrophobic molecules around the body [1–3]. α_1-Acid glycoprotein (AGP; *syn.* orosomucoid), an acute-phase protein, is the principal extracellular lipocalin with high concentrations in the blood plasma [4,5]. One of the major physiological roles of AGP involves the binding and transportation of a range of endogenous (*e.g.* lysophospholipids and biliverdin) and exogenous (*e.g.* drugs) compounds [6–10]. AGP–drug interactions are a focus of great importance in the pharmaceutical sciences as this interaction is a major factor in drug transport to tissue receptors, storage sites and clearing organs but in the latter may also limit elimination [7]. AGP is largely selective for basic and neutral drugs [6,7]; however, certain acidic drugs also bind to AGP, albeit with lower affinities [6,7]. In healthy individuals, the basal plasma concentration of AGP is approximately 20 μM; whereas in disease states associated with stress, such as sepsis, it can increase up to 5-fold [7,8,11,12]. Therefore, the effect of AGP binding on the pharmacological activity of highly bound drugs (*e.g.* certain antibiotics) can be significant during acute-phase reactions (*e.g.* sepsis) that warrant their use [13–20].

Human plasma AGP exists as three genetic variants, the A variant and the F1 and S variants [7,21–25]. The expression of human AGP is under the control of two adjacent genes ORM1 (*syn.* AAG-A) and ORM2 (*syn.* AAG-B/B′), situated on chromosome 9 [22,23]. The more active of the two, ORM1, that is induced during acute-phase reactions, encodes the F1 and S variants, and ORM2

Abbreviations: ANS, 1-anilino-8-naphthalene sulfonic acid; AO, Auramine O; AGP, human α-1-acid glycoprotein; CAPs, cationic antimicrobial peptides; CMS, colistin methanesulfonate; Fmoc, fluorenylmethyloxycarbonyl; Dab, diaminobutyric-acid; ITC, isothermal titration calorimetry; LPS, lipopolysaccharide; OM, outer-membrane; colistin NP, colistin *nona*-peptide; PmB, polymyxin B; NR, Nile Red; NDM-1, New Delhi metallo-beta-lactamase 1; SAR, structure–activity relationships; SPR, surface plasmon resonance.

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encodes the A variant [7,21–25]. The precursor product of the ORM1 gene is a 201 amino acid polypeptide with an 18 residue *N*-terminal secretory peptide that is cleaved [7,21–25]. The F1 and S variants, encoded by two alleles of the ORM1 gene differ only in a single amino acid codon (Gln20 → Arg), and hereon in shall be referred to collectively as the F1*S variant. The ORM2 gene displays 22 base substitutions, which translates into 21 amino acid substitutions between the F1*S and A protein variants [21–23].

The recent elucidation of the three dimensional crystal structure of the F1*S and A variants of human AGP revealed that AGP, not unlike other lipocalins, possesses a structural fold consisting of eight anti-parallel β-strands connected by four loops arranged into a β-barrel with three flanking α-helices (*cf.* Fig. 7A) [3,26–28]. Within the β-barrel motif lies the ligand binding pocket [26,27]. On the primary level, AGP is composed of a single polypeptide chain of 183 amino acids [4,5,7]. The polypeptide component only contributes about a half of its total molecular mass of approximately 41 kDa, the rest of its mass derives from the five *N*-linked sialyl-glycans which confer AGP with a net negative charge at physiological pH [29–31]. These features also render AGP very soluble and acidic (pI ∼ 2.8–3.8) [4,5,7].

Plasma protein binding has been implicated as a major factor limiting the active free concentration of many clinically important antibiotics [13,14,16,18–20]. This in turn translates into reduced antibacterial activity, the need for dose escalation and in certain cases where the antibacterial agent is highly bound, limits its intravenous use [15,17,18]. The polymyxin class of antibiotics (colistin and polymyxin B, PmB) are important last-line therapeutic agents against many multidrug-resistant (MDR) Gram-negative bacteria, in particular the emergent NDM-1 phenotypes [32–36]. The structure of polymyxins consists of an *N*-terminal fatty acid side chain that is attached to a poly-cationic *deca*-peptide backbone (Fig. 1) [37,38]. These structural features confer amphipathicity, which is a key feature of many cationic antimicrobial peptides (CAPs) [37–39]. Plasma protein binding of 55–57% was reported for colistin in rats [41]. Equilibrium dialysis studies with human plasma have indicated colistin and PmB are over 50% bound to plasma, representing a significant fraction of the circulating drug [42]. However, the actual plasma components, albumin, AGP, lipoproteins, or globulins that bind polymyxins remain to be fully elucidated. Therefore, an understanding of the structure–activity relationships (SAR) that drive the binding of polymyxins to important plasma drug transporters such as AGP is of great clinical relevance. Despite the wealth of literature on drug–AGP binding interactions, to the best of our knowledge, no study to date has examined the SAR for the binding of the polymyxin class of antibiotics to human AGP. This study is the first to utilize ITC, SPR and fluorometric and binding assays, together with molecular docking techniques to characterize the microscopic thermodynamic parameters that drive polymyxin binding to AGP and correlate these with structural information inferred from the docking results. Taken together, these principal findings provide a molecular-level understanding of the energetics of polymyxin–AGP binding interactions.

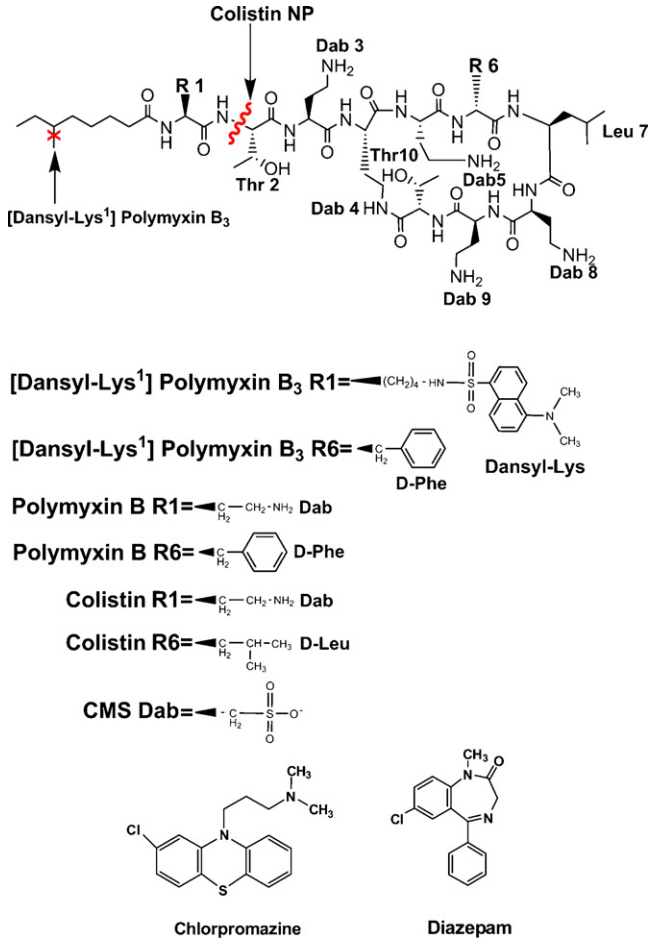

Fig. 1. Chemical structures of the test compounds used in this study. Colistin NP; [Dansyl-Lys]¹polymyxin B_3 ($DPmB_3$); colistin methansulfonate (CMS).

2. Materials and methods

2.1. Materials

Polymyxin B (lot # 453306, ≥6000 USP units per mg) was purchased from Fluka (Castle Hill, NSW, Australia). Human AGP (lot # 018K7535), colistin (lot # 036K1374, 15,000 units per mg), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES; 99.5%), 1-anilino-8-naphthalene sulfonic acid (ANS) (lot # 20K2523), Auramine O (lot # 01801EH), and Nile Red (NR) (lot # BCBC7818), were obtained from Sigma–Aldrich (Sydney, NSW, Australia). Phospholipids 2-(4,4-difluoro-5-methyl-4-bora-3a,4a-diaza-s-indacene-3-dodecanoyl)-1-hexadecanoyl-*sn*-glycero-3-phosphocholine (β-BODIPY® 500/510C_{12}-HPC, lot # 423878) and *N*-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-propionyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine, triethylammonium salt (BODIPY® FL DHPE, lot # 799190)) were from Invitrogen Molecular Probes (Melbourne, Victoria, Australia). Colistin *nona*-peptide (colistin NP) was prepared by papain digestion of colistin as previously described [43] and purified by reversed-phase high-performance liquid chromatography (RP-HPLC). The purity was ascertained by analytical liquid chromatography mass spectrometry (LC–MS). [Dansyl-Lys¹]PmB_3 was synthesized as previously described [44]. All other reagents were of analytical grade or better.

2.2. Delipidation of AGP

Delipidation of AGP was performed as previously reported [45] using lipidex 1000 (a hydroxyalkylpropyl (HAP) derivative of Sephadex G25 substituted 10% with alkyl chains of C_{15}–C_{18} in length *syn.* (HAP)-dextran type VI *syn.* Sephadex LH 20-100; Sigma–Aldrich Cat # H-6258). The sample was applied to a 15-mL column of lipidex 1000 pre-equilibrated with 20 mM HEPES pH 7.4 at 37 °C, and eluted at a flow rate of 15 mL/h. The column temperature and all solutions were maintained at 37 °C throughout the procedure.

2.3. Synthesis of polymyxin B_3 (PmB_3)

Synthesis of the partially protected linear PmB_3 was carried out employing Fmoc solid-phase peptide synthesis on a CEM Liberty