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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 B₃, colistin methanesulfonate, 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 B₃ display a moderate binding affinity for AGP. ITC and SPR showed there was no detectable binding affinity for colistin methanesulfonate 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 B₃–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 B₃ 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.

Keywords

Human α -1-acid glycoprotein; Binding affinity; Polymyxin; Colistin

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 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 $\rightarrow$ 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 sialylglycans which confer AGP with a net negative charge at physiological pH [29–31]. These features also render AGP very soluble and acidic ($\text{pI} \sim 2.8\text{--}3.8$) [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.

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), Aur-amine O (lot # 01801EH), and Nile Red (NR) (lot # BCBC7818), were obtained from Sigma–Aldrich (Sydney, NSW, Australia). Phospho-lipids 2-(4,4-difluoro-5-methyl-4-bora-3a,4a-diaza-s-indacene-3-dodecanoyl)-1-hexadecanoyl-*sn*-glycero-3-phosphocholine (β -BODIPY® 500/510C₁₂-HPC, lot # 423878) and *N*-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-propionyl)-1,2-dihexa-decanoyl-*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]PmB₃ 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 C₁₅–C₁₈ 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 B₃ (PmB₃)

Synthesis of the partially protected linear PmB₃ was carried out employing Fmoc solid-phase peptide synthesis on a CEM Liberty Microwave Automated Peptide Synthesizer (CEM, NC, USA), using chloro-trityl resin (PepChem, Reutlingen, Germany). Selective removal of the 1-(4,4-dimethyl-2,6-dioxo-cyclohexylidene)-3-methyl-butyl (ivDde) group from diaminobutyric-acid4 (Dab) was achieved using 2% hydrazine in dimethylformamide (DMF). Cleavage of the partially protected linear PmB₃ from the resin was carried out using 1% trifluoroacetic acid in dichloromethane. Cyclization of the linear PmB₃ was performed in DMF overnight using the coupling reagent diphenylphosphoryl azide (DPPA) in the presence of diisopropylethylamine (DIPEA). Removal of the remaining side chain protecting groups was achieved using 5% triisopropylsilane in trifluoroacetic acid to give the crude PmB₃. Crude PmB₃ was first de-salted using a Vari-Pure™ IPE strong anion exchange column, then purified by RP-HPLC using conventional gradients of acetonitrile:water:trifluoroacetic acid. The product was dried by lyophilization to give the PmB₃-trifluoro salt. Conversion of PmB₃-trifluoro- to the PmB₃-acetate was performed using a Vari-Pure™ IPE strong anion exchange column to remove the trifluoro followed by lyophilization from an acetic acid:acetonitrile:water solution. The purity, as estimated by

RP-HPLC, was >95%. Analytical LC–MS was performed on a Shimadzu 2020 LCMS system, incorporating a photodiode array detector (214 nm) coupled directly to an electrospray ionization source and a single quadrupole mass analyzer. RP-HPLC was carried out employing a Phenomenex column (Luna C₈(2), 100 mm × 2.0 mm ID) eluting with a gradient of 0–80% acetonitrile in 0.05% aqueous trifluoroacetic acid, over 10 min at a flow rate of 0.2 mL/min. Mass spectra were acquired in positive ion mode with a scan range of 200–2000 *m/z*.

2.4. Fluorometric assay of binding of polymyxins to human AGP

Purified human AGP, PmB, colistin, ANS and Auramine O were prepared in phosphate buffered saline (PBS, pH 7.4). Chlorpromazine, diazepam, phospholipids and Nile Red were freshly prepared in DMSO. The binding of each fluorescent probe to AGP was measured by titrating a 1-mL volume of AGP (1.5 μM) in a quartz cuvette with aliquots of probe at 2-min intervals until fluorescence intensity reached a plateau. All spectra were corrected for background fluorescence determined from probe only into buffer titrations. The optical density of the solutions was below 0.1 at the excitation and emission wavelengths, so as to avoid the inner-filter effect. Fluorescence was measured using a Cary Eclipse Fluorescence spectrophotometer (Varian, Mulgrave, Victoria, Australia) set at an excitation wavelength (Exλ) specific for each probe, as follows: ANS (420 nm), Auramine O (428 nm), Nile Red (550 nm), [Dansyl-Lys¹]PmB₃ (340 nm) and BODIPY phospholipids (500 nm for fatty acyl BODIPY labeled phosphocholine (BODIPY-PL2) and 505 nm for head group BODIPY labeled phosphoethanolamine (BODIPY-PL1)). The emission spectrum for each probe was collected across the following wavelengths (Emλ): ANS (420–600 nm), Auramine O (460–660 nm), Nile Red (590–750 nm), [Dansyl-Lys¹]PmB₃ (400–650 nm) and BODIPY phospholipids (510–665 nm). Slit widths were set to 5 nm for both the excitation and emission monochromators. Fluorescence enhancement was determined from the integrated area under the emission spectrum. The background-corrected binding fluorescence from the probe was fitted to a one-site binding model (Eq. (1)):

$$\Delta F = \frac{\Delta F_{\max} \times [L]}{K_d + [L]} \quad (1)$$

where ΔF represents the specific fluorescence enhancement upon the addition of probe to the fixed concentration of AGP, $\Delta F_{\max}$ is the maximum specific fluorescence enhancement at saturation, K_d represents the dissociation constant for the probe–AGP complex at a probe concentration equivalent to half $\Delta F_{\max}$, and $[L]$ represents the total concentration of probe (it was assumed that ligand depletion was not in effect and only a small fraction of the total probe was bound). Model parameters, including K_d , were determined by non-linear least square regression analysis of the binding isotherms using GraphPad Prism V5.0 software (GraphPad software, San Diego, CA, USA).

For probe displacement experiments, drug stock solutions of colistin, PmB, CMS, and drugs known to bind AGP (chlorpromazine and diazepam, as experimental controls) were titrated into a quartz cuvette containing a 1-mL volume of AGP–probe complex at a saturating concentration necessary to obtain the maximum fluorescence ($\Delta F_{\max}$) when bound. Displacement of probe was measured as the corresponding decrease in fluorescence upon the progressive titration of aliquots of drug solution at 2-min intervals, until no further decrease in fluorescence was observed. Fluorescence was corrected for dilution before plotting the fraction of probe bound as a function of the concentration of the displacing drug. The concentration of drug required to displace 50% of the bound probe (I_{50}) was determined by fitting to the fluorescence data a sigmoidal dose-response model which assumed a single class of binding sites (Eq. (2)):

$$\% \text{Initial Fluorescence} = \frac{F_{\min} + (F_{\max} - F_{\min})}{1 + 10^{((\log I_{50} - \log [L]) \times \text{Hill Slope})}} \quad (2)$$

where I_{50} is the midpoint of competition and is defined as the concentration of displacer at which the maximum fluorescence intensity ($F_{\max}$) of the probe-saturated complex is reduced to 50% of the initial value. $F_{\min}$ represents the maximum probe displacement produced by the competitor and is defined by the bottom plateau of the displacement curve; $[L]$ represents the concentration of the competitor. The inhibition constant (K_i) was determined from the following equation (Eq. (3)):

$$K_i = \frac{[I_{50}]}{1 + [\text{Drug}]_{\text{free}} / K_{\text{d Probe}}} \quad (3)$$

where $K_{\text{d Probe}}$ represents the dissociation constant for the AGP–probe complex. As noted above, GraphPad Prism V5.0 software was employed for the modeling.

2.5. Isothermal titration calorimetry (ITC) assay of binding of polymyxins to human AGP

Microcalorimetric measurements of polymyxin binding to AGP were performed on a VP-ITC isothermal titration calorimeter (Microcal, Northampton, MA, USA). The sample was thoroughly degassed beforehand. AGP (63 μM) dissolved in 20 mM HEPES (pH 7.0) was filled into the microcalorimetric cell (volume, 1.3 mL) and titrated with $80 \times 3\text{-}\mu\text{L}$ injections of 10–13 mM colistin sulfate, PmB sulfate, PmB₃, colistin NP and CMS at 240-s intervals from a 250- μL injection syringe. The cell contents were stirred constantly at 307 rpm. The ITC titrations were performed at 37 °C. In order to determine the heat capacity for the binding reaction, the colistin–AGP titration was also performed at 15 °C, 20 °C, 25 °C and 30 °C under the same buffer conditions. In all cases, the system was allowed to equilibrate and a stable baseline was recorded before initiating an automated titration. The heat of interaction after each injection measured by the ITC instrument was plotted versus time. The total heat signal from each injection was determined as the area under the individual peaks and plotted versus the polymyxin/AGP molar concentration ratio. As a control for all ITC experiments, drug solution was titrated into buffer under the same injection conditions and the heat of dilution was subtracted from the polymyxin–AGP titration data. The corrected data were analyzed to determine the number of binding sites (n) and molar change in enthalpy of binding (ΔH) by nonlinear least square regression analysis using an equation series that defines one set of binding sites [44,46].

2.6. Surface plasmon resonance (SPR) assay of binding of polymyxins to human AGP

SPR experiments were performed in PBS pH 7.4 using a Biacore 3000 instrument. AGP was immobilized on a CM5 sensor chip surface using thiol-coupling method. In brief, 0.5 mg AGP was dissolved in 0.5 mL of 0.1 M morpholino-ethanesulfonic acid (MES) buffer pH 5.0. Then 250 μL of 15 mg/mL 2-(2-pyridinyldithiol)ethaneamine hydrochloride (PDEA) and 25 μL of 0.4 M 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) were added and incubated at room temperature for 10 min. After the reaction, excess reagents were removed using a desalting column. The PDEA-modified AGP was then used for thiol coupling. After the introduction and reduction of disulfide group on the CM5 chip, PDEA-modified AGP was injected across the surface at a flow rate of 10 $\mu\text{L}/\text{min}$ for 7 min. Deactivated flow cell 1 was used as a blank reference. The immobilization level of AGP was 5000 response units (RU). Polymyxin antibiotics (10 mM stock concentrations) were prepared in SPR PBS running buffer. The concentration range used in the experiments was 3 mM–0.12 μM , in 3-fold dilutions. Samples were injected across the 4 flow cells on CM5 chip at a flow rate of 50 $\mu\text{L}/\text{min}$. Association period was set up for 30 s followed by 60 s

dissociation. Binding responses were recorded 10 s before the end of the association period. Glycine (10 mM, pH 1.7) was used for regeneration of the chip surface. The assays were repeated at least 3 times at 25 °C. The reference flow cell response was subtracted from all data generated in the analysis. For comparison of compounds of variant molecular weight (MW), the responses were normalized using Eq. (4):

$$RU_{MW \text{ adjusted}} = 100 \times \frac{RU}{MW} \quad (4)$$

The dissociation constant K_d was determined by Eq. (5):

$$\frac{RU_{eq}}{MW} = \frac{C \cdot RU_{max}}{C + K_d} \quad (5)$$

where RU_{eq} is the measured response at each different test compound concentration (C); while RU_{max} is the response at saturation.

2.7. Molecular modeling of the PmB₃–AGP complex

A docking model of PmB₃ in complex with human AGP F1*S variant was constructed using Accelrys Discovery Studio V2.1 CDOCKER algorithm as per the standard protocol in the manufacturer's instructions (Accelrys, San Diego, CA, USA). The coordinates of the NMR solution structure of PmB were obtained from Pristovsek and Kidric [47]. The crystallographic coordinates of *apo*-human AGP F1*S variant were retrieved from the protein data bank (PDB ID: 3KQ0) [27].

3. Results

3.1. Fluorometric assay of binding interaction of polymyxins with human AGP

The use of fluorescent probes with differing chemical properties to examine the various drug binding sites on AGP is not uncommon in the literature [48–52]. Here we have used the three most commonly employed fluorophores, ANS (consisting of three hydrophobic phenyl groups, a secondary amine and an acidic sulfonate group), Auramine O (consisting of a bridged bi-phenyl structure with a tertiary amine on each phenyl and a central basic group) and Nile Red (consisting of a hydrophobic multi-cyclic structure and a tertiary amine) (Fig. 2, insets). The calculated pKa of the tertiary amine on Nile Red is 3.6, so at the experimental pH 7.4 this group will be largely unionized. Together, these three fluorophores have been employed to purportedly probe the acidic (ANS), basic (Auramine O) and hydrophobic (Nile Red) drug binding sites on AGP [48–54]. Firstly, we determined the binding affinity of human AGP for each probe in PBS buffer by measuring the increase in fluorescence intensity upon probe–AGP complex formation (Fig. 2). The rise in fluorescence at the specific emission wavelengths of each probe was monitored with each successive addition of probe until no further increase was detected, indicating all binding sites were occupied. A one-site binding model was fit to the binding isotherms to derive the dissociation constant for each probe–AGP complex. ANS and Nile Red exhibited a similar low micromolar binding affinity for human AGP, whereas Auramine O displayed a 16-fold lower affinity (Table 1). These values were consistent with affinity constants reported for each probe by a number of independent studies [48–52]. With the knowledge of the dissociation constants for each fluorescent binding site probe, we could now proceed to determine the binding inhibitory constants (K_i) for the test polymyxin compounds. The ability of the polymyxins to compete with each probe binding to AGP was examined by incremental titration with each test polymyxin until no reduction in fluorescence intensity was detected upon further addition (Fig. 3). The concentration of polymyxins required to achieve 50% probe

displacement (I_{50}) was used to calculate K_i values as a measure of affinity of the displacing compound (Table 1). The positive control test compounds, chlorpromazine and diazepam, displayed a comparable ability to displace each probe with low micromolar K_i values, consistent with those reported in the literature [6]. Displacement titrations with Nile Red and diazepam led to an unexpected increase in Nile Red fluorescence emission due to what appeared to be direct drug-probe interactions; therefore, a K_i for diazepam could not faithfully be determined by Nile Red displacement. Titration of probe-AGP solutions with colistin or PmB resulted in a moderate to weak displacement ability for each polymyxin, with K_i values in the high micromolar range ($>200 \mu\text{M}$) (Table 1); titrations with CMS indicated no ability to displace any of the probes. In order to exclude the possibility that the poor ability of the polymyxins to displace the probes was because they do not compete for the same binding sites as the fluorescent probes, we have performed direct fluorometric measurements using a fully synthetic fluorescent polymyxin analog, [Dansyl-Lys¹]PmB₃ (SI Fig. 1) [44]. Titration of AGP solutions with [Dansyl-Lys¹]PmB₃ produced an increased emission maxima at ~520 nm upon binding to AGP (SI Fig. 1), however the emission did not plateau with continued addition of [Dansyl-Lys¹]PmB₃. We attribute this observation to the non-specific emission from the intrinsic fluorescence of the dansyl fluorophore substituent, which potentially provides a greater contribution to emission than from the binding of [Dansyl-Lys¹]PmB₃ to AGP. We could not correct for this intrinsic fluorescence by subtracting the emission from [Dansyl-Lys¹]PmB₃ into buffer titrations, as the weak AGP affinity for [Dansyl-Lys¹]PmB₃ meant that titrations required very high concentrations of [Dansyl-Lys¹]PmB₃ to reach saturation. To examine if polymyxins can compete with native *in vivo* AGP substrates such as phospholipids (PL), we measured the binding of two fluorescent PL analogs labeled with the BODIPY fluorophore on the head group (BODIPY-PL1) and fatty acyl chain (BODIPY-PL2) (SI Fig. 2). There was no fluorescence emission observed upon titration of AGP with the BODIPY-PL1, indicating the head group segment of the molecule with the fluorescent label does not bind to AGP (SI Fig. 2). In contrast, titration of AGP with the BODIPY-PL2, resulted in fluorescence emission in the range characteristic for the BODIPY label, suggesting the fatty acyl segment of the molecule with the fluorescent label is responsible for binding to AGP (SI Fig. 2). However, similar to the [Dansyl-Lys¹]PmB₃ titrations, the continued addition of BODIPY-PL2 did not result in a plateau of emission due to what appeared to be the intrinsic fluorescence emission of the BODIPY fluorophore surmounting the specific emission from AGP binding. Once again, we were unable to correct for this non-specific intrinsic fluorescence due to the apparent weak affinity and the large concentration of BODIPY-PL2 required to reach saturation. Finally, we examined the ability of our test compounds to displace BODIPY-PL2. Both chlorpromazine and diazepam were able to compete with BODIPY-PL2, as indicated by the decrease in fluorescence emission upon addition of each compound (SI Fig. 2). In comparison, colistin or PmB were unable to produce any discernible displacement at similar concentrations; instead, the addition of polymyxin produced an increase in BODIPY-PL2 fluorescence emission (SI Fig. 2).

3.2. Isothermal titration calorimetry (ITC) assay of polymyxin binding to human AGP

In order to examine the energetics that drives polymyxin-AGP binding we have characterized the interaction thermodynamically using ITC. Typical microcalorimetric titrations of AGP (0.063 mM) with colistin, PmB, PmB₃, colistin NP, and CMS performed at 37 °C are shown in Fig. 4. Titration curves corresponding to an endothermic reaction were observed for colistin, PmB, PmB₃ and colistin NP (Fig. 4A–C). In contrast, the titration with CMS largely exhibited an exothermic reaction, with a small superimposing endothermic process (Fig. 4D). This process is most likely attributable to the presence in the CMS titrant of colistin that formed from CMS during the 180 min time course of the titration [55]. The enthalpy changes versus the polymyxin:AGP ratio for colistin, PmB and PmB₃ were well fit

by a one-site interaction model (Fig. 4). The calculated microscopic thermodynamic parameters from the ITC binding curves are documented in SI Table 1 and in graphical form (Fig. 4, insets in lower sections of panels A–C). The colistin binding interaction at 37 °C revealed an approximately 1:1 [colistin:AGP] binding stoichiometry with a moderate binding affinity. In comparison, the PmB and PmB₃ binding interactions at 37 °C revealed an approximately 2.5:1 [PmB:AGP] binding stoichiometry with a higher binding affinity than for colistin. A PmB titration was also performed using 130 µM AGP to reflect the elevated plasma AGP levels observed during infection (SI Table 1). All of the binding affinity values determined by ITC correlated well with the binding affinities determined fluorometrically and by SPR. The colistin, PmB and PmB₃ binding reactions at 37 °C were entropically driven with an accompanying unfavorable (positive) enthalpy (Fig. 4A–C, insets and SI Table 1). In order to examine the effect of endogenous lipidic substances that remain bound to AGP throughout the purification process [8], a sample of AGP was subjected to a delipidation process [45] and compared to a sample from the same preparation for PmB binding at 25 °C (Fig. 4F and SI Table 1). The comparison of the binding isotherms clearly shows that the non-delipidated sample displays a higher binding affinity ($K_d = 103$ µM), compared to the delipidated sample ($K_d = 210$ µM). An examination of the microscopic thermodynamic binding parameters indicates the higher binding affinity of the non-delipidated sample is driven by a more favorable binding enthalpy (Fig. 4F insets). In contrast, the PmB binding reaction for the delipidated sample is associated with a larger favorable entropy; however, this is offset by the unfavorable enthalpic component which reduces the binding affinity overall. The free energy for both samples remained largely unchanged. The non-delipidated sample displayed a 2.5:1 (PmB:AGP) binding stoichiometry, whereas the delipidated sample displayed a 2:1 (PmB:AGP) binding stoichiometry, suggesting the bound endogenous lipidic substances contribute 1/2 site toward the PmB binding surface.

To examine the temperature dependence and in an attempt to determine the heat capacity (ΔC_p) changes associated with the colistin–AGP interaction, we performed titrations at a number of different temperatures (Fig. 5 and SI Table 1). Interestingly, at 15 °C and 20 °C a second colistin binding site was observed, which may reflect differential binding affinities for the F1*S and A AGP variants at lower temperatures. With titrations at 25 °C, 30 °C and 37 °C only one colistin binding site was observed, suggesting that at higher temperatures the binding affinity of colistin for both AGP variants is comparable. A plot of the enthalpy (ΔH°) at each temperature as a function of the entropy ($T\Delta S^\circ$) and the free energy (ΔG°), reveals that the ΔG° change for the interaction remained largely unchanged with increasing temperature (Fig. 5E and SI Table 1). A favorable (positive) increase in entropy was evident with increasing temperature, which was accompanied by an unfavorable (positive) increase in enthalpy (Fig. 5E and SI Table 1). Although, the plot of the binding enthalpy as a function of the experimental temperature at first glance suggests that entropy–enthalpy compensation is at play, a closer examination reveals that the binding enthalpy does not markedly vary over the 15–25 °C temperature range (Fig. 5F and SI Table 1). Moreover, the plot deviates from linearity due to the large enthalpy changes at the higher temperatures (30–37 °C), suggesting that perhaps complex rearrangements in the system are taking place at these temperatures, further complicating the determination of ΔC_p by linear regression analysis of the data. However, the positive slope of the plot is consistent with the hydrophobic effect from the hydration of apolar groups [56].

3.3. Surface plasmon resonance assay of polymyxin binding to human AGP

The binding of the polymyxins was examined by SPR using AGP immobilized to a chip surface and titrated with the compounds at a concentration series from 3 mM to 1.3 µM (Fig. 6A). PmB, colistin and colistin *nona*-peptide gave linear concentration-response curves

between 0 and 333 μM , and started to saturate the surface around 1 mM. The rank order of affinity determined from the K_d estimates at steady state binding in order of decreasing affinity was $\text{PmB} > \text{colistin} > \text{colistin NP} > \text{CMS}$ (Fig. 6B). The binding of CMS to AGP was not concentration dependent, which is consistent with CMS not binding to the surface-immobilized AGP. Ranking of polymyxin binding to AGP was also investigated at single, non-saturating, concentrations of 100 μM and 300 μM of each compound (Fig. 6C). Chlorpromazine served as a positive control to test the immobilized surface. PmB gave the highest response, followed by colistin whereas CMS and colistin NP gave very poor responses, giving a rank order of affinity similar to that determined from the K_d estimates.

3.4. Molecular modeling of the DPmB₃–AGP complex

In an attempt to understand how polymyxins bind AGP we have constructed a molecular docking model of the polymyxin–AGP complex using the crystallographic structure of human AGP [27] as the receptor and the NMR structure of PmB₁ as the ligand [47] (Fig. 7A and B). In order to allow for useful correlations between the modeling and ITC results, we have modified the *N*-terminal fatty acyl chain of the PmB₁ NMR structure to that of PmB₃ (PmB₁ = 6-methyloctanoyl fatty acyl $\rightarrow$ PmB₃ = octanoyl fatty acyl), as we have employed the pure, fully synthetic form of the latter for the binding studies. The F1*S cavity has three distinct lobes (Fig. 7C) [27]. The model suggests the PmB₃–AGP complex is primarily stabilized through contacts between the *N*-terminal fatty acyl chain of PmB₃ and a set of non-polar side-chains within lobe 3 of the cavity (Fig. 7B). The cavity of lobe 3 is formed by secondary structural elements $\alpha 2$, βE , βF , βG and the $\beta\text{A} \rightarrow \alpha 2$ loop (Fig. 7A). The major contact points with the *N*-terminal fatty acyl chain of PmB₃ involve the side chains of Phe32, Tyr37, Val92, His97, Ala99, Leu112 and Phe114 (Fig. 7B). The complex is further stabilized through contacts with the D-Phe6-L-Leu7 hydrophobic motif of PmB₃ which docks within lobes 1 and 2 of the cavity. The side chain of D-Phe6 penetrates into the apolar central lobe 1, whereas the L-Leu7 side chain makes fewer contacts and is largely situated within lobe 2. Although, the polar and cationic side chains of the PmB₃ molecule consisting of the Dab residues at positions 1, 3, 4, 5, 8, 9 and Thr at positions 2, 10 are proximal to α -helix 2, the model shows they largely do not contact the AGP protein surface (Fig. 7A and B). AGP displays five *N*-linked sialyloligosaccharides attached to the side chain of Asn residues found at positions 15, 38, 54, 75 and 85 in its amino acid sequence [29–31]. The Asn residues at positions 15, 54 and 85 are situated at the closed end of the β -barrel on the opposite side of the molecule from the entrance of the ligand binding cavity and therefore, are unlikely to interfere with ligand entry [27]; Asn 38 and 75 line the entrance to the ligand binding pocket [27,29–31]. Therefore, it is tenable to propose that the polar and cationic side chains of the polymyxin molecule interact with the complementary negative charge of the *N*-linked sialyl-oligosaccharides attached to Asn 38 and 75 that decorate the entrance of the cavity.

4. Discussion

Antibiotic binding to drug-transport plasma proteins such as AGP can play a significant role in determining its pharmacological efficacy and pharmacokinetic profile *in vivo* [7]. This study focuses on elucidating the molecular level mechanisms and SAR that drive the binding of polymyxins to native human AGP derived from plasma.

4.1. Molecular mechanism of polymyxin–AGP complexation

Mass spectrometric analysis of organic extracts from AGP preparations derived from human plasma suggested it is mostly bound to lysophospholipids [8]. Moreover, AGP was shown to bind lysophospholipids with high affinity (nanomolar K_d) *in vitro* using ANS displacement fluorometric measurements [8]. Accordingly, it has been suggested that AGP complements

the lysophospholipid buffering activity of human serum albumin (HSA) [8]. In an attempt to emulate the situation *in vivo* where AGP is largely complexed with endogenous lipidic substances such as lysophospholipids and biliverdin [8,9], we performed most of the binding experiments with non-delipidated AGP preparations. So it follows, the binding data should be reflective of the ability of the polymyxins to compete with endogenous ligands for AGP binding sites. Most interestingly, when the AGP preparation was stripped of bound endogenous lipids, the PmB binding affinity and stoichiometry determined by ITC, actually decreased. This would suggest that lipidic substances, such as phospholipids (PL), bound to AGP may actually provide part of the binding surface for polymyxin binding to AGP. This presents a possible scenario *in vivo* where polymyxins preferentially bind to the PL–AGP complex as opposed to competing with endogenous lipids for AGP binding sites. Such an unorthodox PL co-binding mechanism is plausible given that the large polymyxin molecule cannot enter the cavity to form a tight complex, and consequently its binding affinity is too low to effectively compete with PL which can bind AGP with a nanomolar K_d . In order to overcome this limitation, the polymyxin molecule would appear to simply “piggy-back” onto the PL–AGP complex.

Furthermore, we have attempted to directly probe the ability of polymyxins to interact with the phospholipid binding site on AGP, by performing fluorometric displacement experiments with BODIPY-labeled PL. The head group BODIPY labeled PL did not exhibit any fluorescence emission when titrated into a solution of AGP, whereas the titrations with the fatty acyl BODIPY labeled PL produced a strong fluorescence emission (SI Fig. 2). Taken together, these data would suggest that only the fatty acyl portion of the PL enters the apolar environment of the AGP binding cavity. An alternative explanation is that the head group BODIPY label negates the positive head group charge which may be required for AGP binding interactions. Titration of the fatty acyl BODIPY–AGP solution with colistin or PmB produced an enhancement of BODIPY fluorescence. This may represent the binding of the polymyxin molecule to the BODIPY PL while it is bound to AGP, a scenario that would be consistent with the ITC experiments with the delipidated AGP preparation. Alternatively, the fluorescence enhancement may represent incorporation of the BODIPY-PL into polymyxin aggregates once the critical micelle concentration had been reached in the titration series [57]. In contrast, titrations with the control drugs, chlorpromazine and diazepam, produced a displacement of the fatty acyl BODIPY-PL from AGP seen as a dose-dependent decrease in BODIPY fluorescence. This would suggest that the binding mechanism of smaller molecule drugs (*e.g.* chlorpromazine and diazepam) *in vivo* may involve competing with PL for AGP binding sites, as opposed to the unorthodox PL co-binding mechanism invoked above for the polymyxins.

In a further attempt to dissect the potential polymyxin binding sites on AGP, we employed fluorometric displacement experiments with acidic (ANS), basic (Auramine O) and neutral (Nile Red) binding cavity probes. Although colistin and PmB were capable of competing with ANS and Nile Red, they did so with a low binding affinity (Table 1). This would suggest the probe binding surfaces are not entirely coincident with the polymyxin binding surface on AGP which would be coincident with the BODIPY-PL data. Accordingly, the molecular docking model of the PmB₃–AGP complex illustrates the potential of the PmB₃ molecule to interact with a large surface area on AGP by virtue of the large size of polymyxin, as compared to the small molecule probes, which most likely penetrate deep into the binding cavity in an area not entirely accessible to the large polymyxin molecule (Fig. 7).

4.2. SAR for polymyxin–AGP binding

The resolution of the crystallographic structures of the human F1*S and A AGP variants revealed the drug binding cavity of each variant is sub-divided into lobes [26,27]. This

intricate cavity organization suggests the AGP–drug binding site consists of partially overlapping sub-sites as opposed to the existence of distinct binding sites for acidic, basic and non-polar ligands [26,27,53]. This is consistent with our fluorometric results that show AGP is capable of binding non-polar, acidic and basic binding site probes. Although our binding data suggest that bound PL may form part of the polymyxin binding surface on AGP, it would be difficult to accurately model the ternary complex given that there are no available structural data describing the nature of the PL–AGP complex. Alternatively, we have employed available experimental structural data to construct a model of the PmB₃–AGP F1*S complex (Fig. 7). The model suggests the *N*-terminal fatty acyl chain and D-Phe6-L-Leu7 hydrophobic motif of the polymyxin molecule are responsible for a series of stabilizing non-polar contacts with side chains lining lobes 1 and 3 of the AGP cavity (Fig. 7). The model is consistent with the SAR inferred from the binding experiments which showed that AGP does not bind colistin NP which lacks the *N*-terminal fatty acyl chain, demonstrating its pivotal role for the AGP binding interaction. Although, the complex appears to be largely stabilized by hydrophobic interactions, the observation that CMS, a colistin analog with negative sulfonate groups blocking its Dab amines (Fig. 1), does not bind AGP, also suggests a key role for the positive charge of the Dab side chains. If one considers that at the pH of plasma (7.35–7.45) [58], the existence of the strong net negative charge at the AGP surface (conferred largely by its five surface sialyloligosaccharides) and the complementary positive charge of the polymyxin Dab side chains, it is certainly plausible that the initial attraction between the two species is partially driven by electrostatic forces. In addition, the observation that PmB and PmB₃ displayed a ~2-fold higher affinity for AGP compared to colistin, suggests the D-Phe6 position (D-Leu6 in colistin) is more favorable for binding. This is coincident with the PmB₃–AGP F1*S model which suggests the D-Phe6 penetrates into the apolar lobe 1 of the cavity; thus, it is understandable how having a more hydrophobic side chain (D-Phe6 versus D-Leu6) may enhance the binding affinity.

In vitro mechanistic studies of the ability of polymyxins to destroy bacteria suggest their antibacterial action involves an initial electrostatic attraction between the cationic Dab side chains of the polymyxin molecule and the negatively charged lipid A phosphate moieties on the lipopolysaccharide (LPS) on Gram-negative outer membrane (OM) [38]. Once the polymyxin molecule is in close proximity to the OM, it is believed the *N*-terminal fatty acyl chain inserts into the lipid A fatty acyl layer of the OM leading to a degree of membrane disruption, the polymyxin molecule then further penetrates to the cytoplasmic membrane, resulting in permeabilization, leakage of cellular contents and cell death [38]. This two-stage ‘self-promoted uptake’ model demonstrates the pivotal role played by both the hydrophobic and polar domains of the polymyxin molecule and accounts for the absolute requirement of structural amphipathicity for CAPs [37–39]. By analogy, we propose a two-step model wherein the association of the polymyxin molecule with AGP appears to initially rely on an electrostatic attraction between the positive Dab side chains of the polymyxins and the negative sialyloligosaccharides proximal to the binding cavity entrance. The complex is then stabilized by insertion of the *N*-terminal fatty acyl chain and Phe6-L-Leu7 hydrophobic motif of the polymyxin molecule into the hydrophobic ligand binding cavity of AGP, and/or co-binding to bound lipidic substances such as PL. The importance of both the complementary charge and hydrophobic interactions is exemplified by the fact that neither CMS nor colistin NP, where these properties are abrogated bind to AGP.

4.3. Energetics of polymyxin–AGP binding

Hydrophobic interactions are often dominated by large favorable (positive) entropy and smaller (frequently positive) unfavorable enthalpy contributions to the binding free energy [59–61]. These observations are consistent with our ITC data which revealed that the

binding of colistin, PmB and PmB₃ to AGP is largely driven by a favorable entropic contribution to the free energy that becomes more dominating with increasing temperature [62], providing evidence for the role of hydrophobic interactions as a major driving force for the association of polymyxins with AGP. The large positive entropy could be associated with hydration effects, where the release of ordered water from the binding surfaces into the bulk solvent occurs upon complex formation. This is also enthalpically unfavorable as the stronger hydrogen bonding network within the solvation shell surrounding the apolar surfaces on the polymyxin and AGP cavity is disrupted and replaced with weaker bonds within the bulk solvent. The favorable entropic contribution could also be related to conformational changes upon complexation, as a plot of the enthalpy as a function of the experimental temperature showed a large deviation from linearity at higher temperatures, suggesting a change in the conformational dynamics or solvation of the system at higher temperatures takes place (Fig. 5F). Notwithstanding, the observed thermodynamic parameters for the polymyxin–AGP interaction cannot solely be rationalized on the basis of hydrophobic interactions. Consistent with our data, the binding of charged groups to proteins is often entropically driven, with small enthalpic contributions to the free energy [59]. This has been explained in terms of the chelate effect wherein the penalty to the translational entropy upon co-ordination of charged groups (*i.e.* the five Dab amines of polymyxins) with a poly-dentate protein (*i.e.* AGP) is much less compared to co-ordination with mono-dentate water molecules [63,64].

The commercial AGP preparation derived from human plasma that we have employed throughout this study consists of proportions of the F1, S, and A variants in a nearly constant ratio of 40:30:30 (F1:S:A) [25,65,66]. Investigations into the individual drug binding selectivity of the A and F1*S variants has revealed the A variant displays a high drug binding selectivity and affinity [66–74]. In contrast, the F1*S variant generally displayed a broader drug binding selectivity, with an overall lower drug binding affinity [66–74]. Our results show that at higher temperatures (25–37 °C) colistin displays a ~1:1 (colistin:AGP) binding stoichiometry, suggesting it binds comparably to both the A and F1*S AGP variants. Whereas, at lower temperatures (20–15 °C) colistin displayed a ~2:1 (colistin:AGP) binding stoichiometry with a higher binding affinity. This would indicate that the complex is much more stable at lower temperatures and colistin is able to bind to both variants. In comparison, PmB and PmB₃ displayed a ~2.5:1 (PmB:AGP) binding stoichiometry at 25 °C, suggesting they are capable of binding to both variants at higher temperatures, perhaps by virtue of their overall higher AGP binding affinity.

4.4. Possible physiological significance of polymyxin–AGP interactions

Fluorometric binding studies demonstrated polymyxins can bind the bacterial LPS with a high affinity [44]. Given the moderate binding affinity of polymyxins for AGP, it may be perceived that *in vivo*, the affinity for LPS on the bacterial surface is strong enough to dissociate and sequester the polymyxin from AGP. So it follows, AGP may actually facilitate the antibacterial action of polymyxins by serving as a transporter to sites of infection. Interestingly, plasma protein binding of PmB and PmB₁ has been reported to exceed more than 90% in critically ill septic patients compared to around 56% in healthy subjects [75,76]. Coincidentally, the level of plasma protein binding of colistin was reported to be greater in neutropenic infected mice compared to healthy mice [42]. Under basal conditions the HSA concentration is ~33-fold greater (~660 μM) than that of AGP (~20 μM) [7,8,11,12,24,77]. During infection the plasma levels of AGP can increase up to 5-fold (~120 μM), relative to that of HSA which may decrease to ~50% of basal levels [7,8,11,12,24]. The PmB binding energetics and affinity constant derived from the ITC data collected using a higher AGP concentration (130 μM; reflective of the elevated levels observed during infection), were similar to the affinity parameters derived from the ITC data

collected at approximately half this concentration (63 μ M) (SI Table 1). This would suggest that *in vivo*, the elevated levels of AGP more likely serve to increase the capacity (as opposed to the affinity) of the plasma pool to bind to exogenous compounds, such as polymyxin antibiotics that are administered for treatment of infections.

4.5. Conclusions

This is the first study to investigate the SAR of the binding of polymyxin antibiotics to AGP. In addition to the critical roles played by complementary charge–charge and hydrophobic interactions, a potential ‘piggy-back’ mechanism which appears to involve co-binding of the polymyxin molecule to the PL–AGP complex was revealed. Collectively, the data suggest polymyxin–AGP interactions may play a role in the transport and disposition of these important drugs of last resort *in vivo*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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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 <i>nona</i> -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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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.bcp.2012.05.004>.

Fig. 1. Chemical structures of the test compounds used in this study. Colistin NP; [Dansyl-Lys]¹ polymyxin B₃ (DPmB₃); colistin methansulfonate (CMS).

Fig. 2. Binding isotherms for fluorescent probe binding to human AGP in PBS pH 7.4. (A) ANS. (B) Auramine O. (C) Nile Red. Data points are the mean of three independent measurements $\pm$ standard deviation. The solid lines represent the non-linear least squares regression fit of a one-site binding model to the data (R^2 values for the fits ranged between 0.93 and 0.98). Insets show the chemical structure of each probe. The right panels show the fluorescence emission spectra for each probe, illustrating the enhanced (arrow) fluorescence emission upon probe binding to human AGP.

Fig. 3.

Fluorometric assay of probe displacement from human AGP by polymyxins and positive control compounds, chlorpromazine and diazepam. Data points are the mean of three independent measurements $\pm$ standard deviation. Solid lines represent a one-site displacement model fit by non-linear least squares regression to the data. (A) Displacement of ANS. (B) Displacement of Auramine O. (C) Displacement of Nile Red. (∇) Colistin; ($\square$) PmB; ($\blacktriangle$) diazepam; ($\circ$) chlorpromazine. The right panels show the fluorescence emission spectrum for each probe, illustrating the decrease (arrow) in fluorescence upon displacement of probe from AGP. The PmB displacement emission series for each probe is given as the example data.

Fig. 4.

(A–E) Isothermal titration calorimetry measurement of human AGP–polymyxin binding interactions. Top section within each panel (A–E) shows the heat in $\mu\text{cal/s}$ for the respective injectants. Bottom sections within the panels (A–E) show the enthalpy (kcal/mol) as a function of the polymyxin–AGP molar ratio. All titrations were performed at 37°C . The insets show the stoichiometry (n), binding affinity (K_d) and thermodynamic parameters (ΔH , enthalpy; $T\Delta S$, entropy; ΔG° , free energy) for each interaction. (F) The effect of delipidation of AGP on the thermodynamic parameters for PmB binding to AGP at 25°C .

Fig. 5. (A–D) Isothermal titration calorimetry measurement of human AGP–colistin binding interactions at four different temperatures. Top section within each panel (A–D) shows the heat in $\mu\text{cal/s}$ for the respective injectants. Bottom sections within the panels (A–D) show the enthalpy (kcal/mol) as a function of the polymyxin–AGP molar ratio. The insets show the stoichiometry (n), binding affinity (K_d) and thermodynamic parameters (ΔH° , enthalpy; $T\Delta S^\circ$, entropy; ΔG° , free energy) for each interaction. (E) Plot of ΔH° measured at each temperature as a function of $-\Delta G^\circ$ (■) and ΔH° (○). (F) Temperature dependence of ΔH° for colistin AGP binding.

Fig. 6. SPR assay of the binding of polymyxins to immobilized AGP. (A) SPR sensorgrams of polymyxins binding to AGP. (B) Determination of binding affinity constants (K_d) by non-linear regression fitting of a one-site binding model to the SPR data. NB = No binding. (C) Binding response at 100 μ M and 300 μ M polymyxins.

Fig. 7. Molecular model of the PmB₃-AGP complex. (A) Two perspectives of the complex rotated 120° about the *Y*-axis. AGP is shown in ribbon representation with secondary structural elements numbered. PmB₃ is shown in stick representation with transparent space filling representation. (B) The complex with AGP in surface representation with lobe 3 of the binding cavity highlighted in red. The magnified expansion of the cavity shows the lobe 3 side chains that contact with the *N*-terminal C₈ fatty acyl chain of PmB₃. (C) Surface representations depicting the amino acids that constitute the three structural lobes of the F1*S (PDB code: 3KQ0) variant of human AGP.

Table 1

Binding parameters for polymyxins to human AGP at 20°C determined with the fluorometric assay. SE, standard error.

	ANS	AO	NR
Fluorescent probe	K_d (μ M) mean $\pm$ SE, $n = 3$ unless otherwise indicated		
	0.87 ± 0.07	14.70 ± 1.60	0.91 ± 0.20
Displacing drug	ANS	AO	NR
	K_i (μ M) mean $\pm$ SE, $n = 3$ unless otherwise indicated		
PmB	225 ± 49	ND ^a	1471 ($n = 1$)
Colistin	351 ± 188	ND ^a	265 ± 81
CMS	ND ^a	ND ^a	ND ^a
Chlorpromazine	0.14 ± 0.01	2.80 ± 0.20	0.67 ± 0.05
Diazepam	3.13 ± 0.06	8.24 ± 0.99	ND ^a

^aND, not detected.