Outer membrane interaction kinetics of new polymyxin B analogs in Gram-negative bacilli

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Abstract

Infections caused by drug resistant Gram-negative bacilli are a severe global health threat, limiting effective drug choices for treatment. In this study polymyxin analogs designed to have reduced nephrotoxicity, direct activity and potentiating activity, were assessed for inhibition and outer membrane interaction kinetics against wild type (WT) and polymyxin or multi-drug resistant (MDR) *Escherichia coli*, *Pseudomonas aeruginosa*, *Acinetobacter baumannii* and *Klebsiella pneumoniae*. In minimal inhibitory concentration (MIC) assays, two polymyxin B (PMB) analogs (SPR1205 and SPR206) and a polymyxin E analog (SPR946), with shortened peptide side chains and branched aminobutyryl N-termini, exhibited promising activity compared to PMB and previously-tested control polymyxin analogs SPR741 and polymyxin B nonapeptide (PMBN). Using dansyl-polymyxin (DPX) binding to assess the affinity of interaction with lipopolysaccharide (LPS), purified or in the context of intact cells, SPR206 exhibited similar affinities to PMB, but higher affinities when compared to the other SPR analogs. Outer membrane permeabilization measured by the 1-N-phenyl-napthylamine (NPN) assay did not differ significantly between the polymyxin analogs. Moreover, Hill numbers were

greater than 1 for most of the compounds tested on *E. coli* and *P. aeruginosa* strains which indicates that the disruption of the outer membrane by one molecule of compound cooperatively enhance the subsequent interactions of other molecules against WT and MDR strains. The high activity demonstrated by SPR206 as well as its ability to displace LPS and permeabilize the outer membrane of multiple strains of Gram-negative *bacilli*, while showing cooperative potential with other membrane disrupting compounds, supports further research with this polymyxin analog.

Introduction

Infections caused by Gram-negative bacilli are a serious obstacle in hospital settings as multidrug resistance (MDR) is leading to limited or non-existent means of treatment (1). Reminiscent to the pre-antibiotic era, what were minor injuries or routine surgeries have the potential to become serious threats to human health (1). Of particular interest in health care settings are the Gram-negative opportunistic pathogens *Klebsiella pneumoniae, Acinetobacter baumannii and Pseudomonas aeruginosa* which have been declared some of the most critical pathogens to target for novel drug research and development by the World Health Organization (2, 3).

Lipopeptide polymyxin antibiotics, polymyxin B (PMB) and polymyxin E (colistin) exhibit potent activity and can effectively treat many infections caused by Gram-negative bacilli (4). Unfortunately, the toxicity of polymyxins has limited their clinical use and can lead to complications in treatment, thus use of polymyxins is limited to last resort treatments (5). Dosage studies have been performed on polymyxins to assess the pharmacokinetics and pharmacodynamics of these drugs (4, 6, 7), yet optimized, safe and effective dosage regimens have not been established for clinical treatment of many opportunistic pathogens. A recently reported clinical study using colistin as a comparator used doses of 300 mg loading, followed by 5mg/kg/day divided into treatments every 8 or 12 hours. In this study, after 28 days of treatment with colistin, 25% of patients had drug related serious adverse events, all related to renal function (8).

Polymyxins are lipopeptide antibiotics comprised of a polycationic peptide ring and a tripeptide side chain with a fatty acyl tail (9). Analogs of polymyxin tested in this study have been synthesized with shorter peptide chains (as observed for the deacylated polymyxin B nonapeptide, PMBN), modified acyl chains and substituted amino acid residues (**Figure 1**; 10–13). These modifications were made to reduce nephrotoxicity and allow for treatment at more

effective concentrations. The analog SPR741 has the same cyclic core as PMB, but with a shorter N-terminus, and a D-serine residue at the position adjacent to the cyclic core. Though it does not have any meaningful antimicrobial activity on its own, this drug was shown to have decreased toxicity against renal proximal tubular cells, and synergy with antibiotics against multiple strains of Gram-negative bacilli *in vitro* and *in vivo* (10–14). Novel, next-generation polymyxins have been designed to have direct-acting as well as potentiating activity. These compounds include SPR1205, SPR206, and SPR946, which have shortened nonapeptide structures with diaminopropionate (L-Dap) residues adjacent to the peptide ring and β-substituted aminobutyryl N-termini. In pre-clinical nephrotoxicity models in mice and monkeys, they were shown to have reduced toxicity compared to PMB, while exhibiting efficacy against Gram-negative bacilli *in vitro* and in animal models (P. Brown, S. Boakes, E. Duperchy, D. Rivers, J. Singh and M. J. Dawson. Poster presented at ASM/ESCMID, Lisbon, Portugal, 4-7 September 2018, T. Lister, L. Utley, and M. Bleavins, A GLP 14 Day Repeat Dose Toxicology Study of SPR206 in Monkeys, Poster presented at ASM/ESCMID, Lisbon, Portugal, 4-7 September 2018, and L. Grosser, K. Heang, J. Teague, P. Warn, D. Corbett, M. J. Dawson and A. Rubio, Poster presented at ASM/ESCMID, Lisbon, Portugal, 4-7 September 2018).

Previously, we have demonstrated that polycationic aminoglycoside and polymyxin antibiotics are taken up across the Gram-negative outer membrane by a fundamentally different route to other antibiotics, termed “self-promoted uptake” (15). According to this hypothesis, polycationic antimicrobials interact with bacterial cell surfaces at divalent cation binding sites on lipopolysaccharide (LPS). The polycationic antibiotics are bulkier and have higher affinity for the LPS than the divalent cations that they competitively displace. This allows them to perturb and ultimately pass through the outer membrane. Thus, these polycationic antibiotics promote their own uptake. We have developed a range of biochemical assays for assessing this mechanism (including those utilized here) and demonstrated that compounds that were effective in these assays often show synergy with conventional antibiotics (16–18).

Mono-N-dansyl-polymyxin B (DPX) is a dansylated derivative of PMB (19). When it interacts with isolated LPS associated with intact outer membranes of Gram-negative bacterial cell, the dansyl group penetrates into the fatty acyl region of LPS and there is an increased fluorescent yield due to the enhanced mobility of this hydrophobic moiety. The addition of PMB or cationic compounds such as cationic antimicrobial peptides, azithromycin or aminoglycosides,

leads to displacement of DPX from LPS and reduces fluorescence, thus providing a relative assessment of binding affinity.

The N-phenyl-napthylamine (NPN) assay assesses the ability of cationic compounds to permeabilize the outer membrane to the hydrophobic fluorophore NPN. The resultant increase in NPN fluorescence as this fluorophore enters the hydrophobic interior of the outer and cytoplasmic membranes is concentration dependent and can be used to assess the kinetics and relative permeabilization efficiency of the interacting polycation.

The DPX displacement assay and NPN uptake assay enable an assessment of the relative ability of cationic compounds to bind to LPS and permeabilize Gram-negative bacterial outer membranes. In this study polymyxin analogs were assessed for their relative antimicrobial activity against *Escherichia coli*, *P. aeruginosa*, *A. baumannii* and *K. pneumoniae*. Interaction kinetics assays were used to investigate whether altered antimicrobial activities of novel polymyxin compounds could be explained by altered interaction with the outer membranes of these Gram-negative bacilli.

Results

Antimicrobial activity of Polymyxin Analogs

Using the microtiter broth dilution MIC assay (20), we tested the susceptibility of wild type (WT) and drug resistant strains of *E. coli*, *P. aeruginosa*, *A. baumannii* and *K. pneumoniae* to PMB and SPR-compounds in LB without salt. The polymyxin resistance phenotypes of the polymyxin resistant mutants were confirmed and we also identified a polymyxin resistant *Klebsiella pneumoniae* isolate termed ATCC 13883. This strain was supposed to be polymyxin susceptible but was actually found to be considerably PMB resistant, a phenomenon that has also been shown to occur in a previously published derivative of this isolate (21). Because of the polymyxin resistant phenotype, this isolate has been termed 13883-PXR. Overall these isolates demonstrated greatest susceptibility to SPR206>SPR1205>SPR946, with SPR206 being generally as effective as PMB. The highest MICs were observed for PMBN and SPR741 that were up to 1000-fold less active than the other compounds studied (**Table 1**). Similar results were found in standardized MIC assay conditions using Meuller Hinton Broth (MHB) (**Table S1**).

LPS displacement

The first stage of self-promoted uptake of polycations such as PMB involves association

with the divalent cation binding sites on polyanionic LPS on the surface of the bacterial outer membrane, which helps to determine Gram-negative selectivity of these compounds. DPX is a fluorescent probe that shows a dramatic increase in fluorescence intensity as it binds to LPS. Here we tested the ability of PMB analogs to displace DPX from both extracted LPS and whole cells, assessed by the reduction in DPX fluorescence. Overall, DPX displacement was greater for all compounds using isolated LPS from *E. coli* and *P. aeruginosa* when compared to whole cells. This is likely because whole cells had secondary binding sites other than LPS and some of these sites would be less accessible to the displacing PMB analogs. Little difference in fluorescence reduction was observed between LPS isolated from WT and MDR strains (**Table 2**), indicating that any change in affinity between WT and MDR strains similarly affected the binding of DPX (a modified version of PMB) and the SPR-analogs. In addition to demonstrating the effects of polymyxin analog concentration on DPX fluorescence/displacement, double reciprocal (equivalent of Lineweaver-Burk often called Benesi-Hildebrand) plots were constructed by plotting 1/[compound concentration] on the X-axis and 1/% reduction in fluorescence on the Y-axis (not shown). Based on these double reciprocal plots, maximal inhibition and I_{50} (a measure of relative affinity) values for the SPR-compounds were calculated (**Table 2**).

Three SPR-compounds, SPR206, SPR1205 and SPR946 demonstrated very similar DPX displacement from *E. coli* SC9251 (WT) whole cells (~60% of that of PMB, i.e. ~ 40% total fluorescence reduction) (**Figure 2A, Table 2**). PMBN, and SPR741 however, could not displace any DPX from *E. coli* SC9251 whole cells at the same concentration that was used for the other compounds. In whole cells of MCR-1-expressing polymyxin-resistant *E. coli* NCTC 13846 (**Figure 2B)**, the SPR-compounds had a 2- to 7-fold lower affinity (as judged by I_{50} values; **Table 2**) to displace DPX than observed with WT *E. coli* SC9251 cells. The maximal inhibitory activity of all SPR-compounds and PMB with polymyxin-resistant *E. coli* NCTC 13846 whole cells was very low (<35% fluorescence reduction) (**Figure 2B, Table 2**).

In whole cell DPX displacement assays using *P. aeruginosa* (**Figure 2C and D, Table 3**), the compounds tested had similar effects for both WT and MDR strains. PMB showed a slightly greater maximal ability to displace DPX from WT whole cells (53% fluorescence reduction) than from polymyxin-resistant whole cells (48% fluorescence reduction), consistent with the greater arabinosaminylation of the latter. Of the novel PMB analogs, the two SPR-compounds SPR206 and SPR1205 had the greatest DPX displacement (30-33% fluorescence reduction).

DPX displacement experiments were also performed on whole cells (but not isolated LPS) of WT and MDR *Acinetobacter* and *Klebsiella* strains (**Figure 2E** to **H, Table 4** and **Table 5**). As for the other tested strains, PMB demonstrated the highest ability to compete with DPX for binding to the outer membrane of *A. baumannii* and *K. pneumoniae* strains, at ~35-50% reduction in fluorescence compared to untreated for each of them. SPR741 showed the lowest ability to displace DPX at <10% reduction in fluorescence, and the three SPR-compounds SPR206, SPR946 and SPR1205 placing in between with 20-30% reduction in fluorescence.

Outer membrane permeabilization

The hydrophobic fluorophore NPN provides a sensitive probe for outer membrane barrier function because it is excluded from the outer membrane of intact cells. If the outer membrane is permeabilized, e.g. by a polycationic compound, NPN is taken up by cells in a time- and permeabilizer concentration-dependent fashion and becomes fluorescent in the hydrophobic interior of the membrane, until the cell membranes become saturated (23, 24).

The addition of NPN to intact bacterial cells resulted in only a small increase in fluorescence, while the addition of the polymyxin analogs resulted in a rise in NPN fluorescence until membranes became saturated. Serial concentrations of SPR-compounds were tested to find the concentration needed for complete permeabilization. There was no significant increase in NPN uptake at concentrations higher than 6.25 µg/mL of SPR-compounds (**Figure S1**).

SPR-compounds (0.1-6.25 µg/mL) were added in increasing concentrations to evaluate their abilities to permeabilize the outer membranes of WT and polymyxin/multidrug resistant test strains, and % fluorescence was normalized to the maximum fluorescence achieved by PMB. Increasing fluorescence with increasing concentrations of SPR206 for all strains is shown in **Figure 3**. This compound was chosen because it exhibited the highest antimicrobial activity, and comparable LPS binding and NPN uptake to other SPR-compounds. Results for the other SPR-compounds can be found in Supplementary information (**Figure S2-S5**).

SPR206 showed very similar ability to promote NPN uptake across the outer membrane of WT *E. coli* SC9251 and polymyxin-resistant *E. coli* NCTC 13846 at concentrations lower than 1.6 µg/mL. Modest reductions were observed for the resistant isolate at concentrations above 3.1 µg/mL (**Figure 3A**). Promotion of NPN uptake into polymyxin-resistant *P. aeruginosa* (9BR) and *A. baumannii* (C4) was substantially reduced compared to that of the WT strains (**Figure 3B, C**). For both the *K. pneumoniae* strains, lower concentrations of SPR206 were

required to result in saturation of the membranes with NPN (0.8 to 3.1 µg/mL or less) than those required for the other tested species (**Figure 3D**). Especially at lower concentrations, SPR206 promoted similar uptake of NPN into the polymyxin-susceptible MDR isolate *K. pneumoniae* VA360, when compared to the polymyxin-resistant *K. pneumoniae* 13883-PXR. Analogous results were observed for all strains for the other polymyxin SPR-analogs and PMBN.

The NPN uptake data was also redrawn as Hill plots (**Figure 3, S2-5**) to assess cooperativity among interactions (since self-promoted uptake is a cooperative phenomenon) and estimate relative affinity. The slope of the Hill plot, referred to as Hill number (25), was calculated (**Table 6**), and can be interpreted as the approximate number of binding sites. For example, the Hill number of SPR206 was 1.59 for *E. coli* SC9251 and 1.38 for *E. coli* NCTC 13846, which indicated a cooperative interaction between SPR206 and the cell surface of both strains, with ~2 interaction sites (**Table 6**). The derived Hill numbers were greater than 1 for most of the SPR-compounds tested on *E. coli* and *P. aeruginosa* strains, indicating that the interaction of one molecule with the outer membrane cooperatively enhanced the subsequent interactions of other molecules, thus permeabilizing the cell to NPN in both wild-type and drug-resistant strains. The exceptions were PMBN and SPR741 which did not appear to show cooperativity with PMB-resistant *P. aeruginosa* 9BR cell surfaces and marginal cooperativity with PMB-resistant *E. coli* NCTC 13846 (**Table 6**). Conversely, while SPR206, SPR1205, and SPR946 showed Hill numbers greater than 1, the Hill numbers were lower for PMB-resistant *E. coli* NCTC 13846. Furthermore these analogs demonstrated excellent cooperativity for polymyxin susceptible *K. pneumoniae* VA360, but marginal or no cooperativity for polymyxin-resistant *K. pneumoniae* 13883-PXR (**Table 6**).

Measuring the concentration of compound leading to a 50% increase in NPN uptake activity (basically the relative ability to permeabilize the outer membrane) led to the results reported in **Table 7**. In contrast to the case for polymyxin B-resistant *E. coli* and *K. pneumoniae*, the resistant isolates of *P. aeruginosa* and *A. baumannii* demonstrated markedly higher concentrations (~1.5 to 3-fold) of analogs required to cause 50% NPN uptake when compared to the susceptible isolates, indicating that there was a markedly decreased ability to permeabilize the membranes of polymyxin resistant isolates.

Discussion

Here we investigated a series of polymyxin B analogs that demonstrate antimicrobial activity

ranging from superior to PMB to very weakly active. . The compounds tested were modified PMB analogs with truncated or heavily modified N-terminal groups. They were designed to have lower nephrotoxicity than PMB, along with direct-acting and potentiating antimicrobial activity. Similar design strategies were described previously for development of SPR741, which was previously shown to enhance the translocation of co-administered compounds such as macrolides across the outer membrane (10, 11, 13). Pre-screening enabled the selection of polymyxin derivatives with low *in vitro* kidney cell cytotoxicity relative to PMB. These molecules were assayed for their ability to interact with and permeabilize the outer membrane of Gram-negative bacilli to determine if activities superior to PMB could be explained by interactions with the outer membrane.

As expected, the polymyxin-resistant phenotype of the polymyxin-resistant mutants was confirmed against all of the polymyxin analogs with the MCR-1 plasmid-based phosphoethanolamine transferase increasing MICs by 10- to 40-fold. The chromosomally encoded arabinosamine and phosphoethanolamine transfer systems of *Pseudomonas* and *Acinetobacter*, respectively increased MICs by 5- to 10-fold (except in the case of SPR946 that was 50-fold worse). Intriguingly, *K. pneumoniae* 13883-PXR was considerably more resistant to SPR-compounds than the multidrug resistant (but polymyxin susceptible) strain *K. pneumoniae* VA360.

The results demonstrated here are consistent with all analogs being taken up across the outer membrane by self-promoted uptake. Thus, all analogs demonstrated an ability to bind to polyanionic LPS and to permeabilize the outer membrane. Additionally, the results demonstrate that disruption of outer membrane uptake is the likely mechanism of polymyxin resistance in all species but the manner in which these resistance mechanisms affected our functional assays was different in the different species. Thus, only the plasmid mediated MCR-1 mechanism in *E. coli* had a substantial effect on relative binding affinity for whole cells, with resistance leading to a substantial (~2-3 fold) increase in I_{50} values (consistent with reduced affinity) and reduced maximal inhibition values. Conversely, the chromosomally encoded resistance mechanisms in *P. aeruginosa* and *A. baumannii* reduced the ability of the analogs to permeabilize the outer membrane to NPN (i.e. increased the concentration required to give rise to 50% NPN uptake by about 2-3 fold).

Of the novel polymyxin analogs, SPR206 was identified as the most active with the lowest

MIC against all strains except for *K. pneumoniae* 13883-PXR, and often the highest LPS binding and permeabilization capability. This PMB analog is composed of the nonapeptide cyclic core with an L-Dap residue directly adjacent and an aminobutyryl N-terminus substituted with a chloroaryl group. Despite increased MICs of all compounds against MDR strains, MIC values of SPR206 tended to remain relatively low, with values comparable to PMB for most polymyxin resistant strains. SPR206 also had 2- to 6-fold lower MICs than PMB against all polymyxin susceptible strains. Intriguingly, despite having equal or lower MICs, this SPR-analog had somewhat lower DPX displacement when compared to PMB, potentially implicating that it accessed fewer binding sites on LPS than did PMB, possibly due to the shorter hydrophobic tail in the R1 position (**Figure 1**). Furthermore, in NPN assays, SPR206 (**Figure 3A**) displayed similar but not better outer membrane permeabilization compared to PMB. Additionally, Hill numbers derived from Hill plots were greater than 1 for most of the SPR-compounds, including SPR206, against *E. coli* and *Pseudomonas* strains. This indicates that SPR206 can cooperatively enhance the subsequent interactions of other molecules. Thus the enhanced efficacy of SPR206 was not due to improved outer membrane interaction but likely pertained to some other feature of its mechanism of action which for polymyxin B has been proposed to be either permeabilization of the cytoplasmic membrane or an extra-membranous target (19, 26).

SPR206 has been selected for ongoing continued development based on pre-clinical studies that indicate low renal toxicity, and potent, broad-spectrum efficacy against key Gram-negative pathogens *in vitro* and *in vivo* (P. Brown, S. Boakes, E. Duperchy, D. Rivers, J. Singh and M. J. Dawson. Poster presented at ASM/ESCMID, Lisbon, Portugal, 4-7 September 2018, L. Grosser, K. Heang, J. Teague, P. Warn, D. Corbett, M. J. Dawson and A. Rubio, Poster presented at ASM/ESCMID, Lisbon, Portugal, 4-7 September 2018, and L. Grosser, K. Heang and A. Rubio, Poster presented at ASM/ESCMID, Lisbon, Portugal, 4-7 September 2018). Results showed that in a murine lung model, subcutaneous administration of SPR206 reduced the *P aeruginosa* strain PA14 burden by up to 3.6 log CFU/mL after 24 hours, which was twice as effective as the maximum dose of PMB. Similarly in the murine lung model, SPR206 was found to reduce the burden of *A. baumannii* NCTC 13301 by up to 4.6 log CFU/mL after 16 hours of infection, whereas PMB only reduced bacterial burden by up to 2.8 log CFU/mL. The superior *in vitro* (Table 1) and *in vivo* activity of SPR206, in the light of similar or more modest LPS binding and permeabilization, compared to PMB, indicates that factors other than outer membrane

permeability are very influential, allowing it to become more effective as a treatment. One potential secondary mechanism might relate to the aryl chloride group at the N-terminus of the compound. Aryl chloride groups have been linked to antimicrobial activity for the glycopeptide antibiotic vancomycin (27). Vancomycin is a gram-positive antibiotic with a primary mechanism of action involving inhibition of cell wall synthesis, though research has also suggested it can alter the permeability of cell membranes and inhibit ribonucleic acid synthesis (28). Removal either of the aryl chloride groups in vancomycin has been shown to diminish antimicrobial activity by reducing ligand binding specificity or destabilizing in situ dimerization (27).

The previously described compound SPR741, which acts as a sensitizer rather than an antibiotic (11), was used as a baseline comparison for susceptibility tests and interaction kinetics in this study. This derivative displayed the least activity against almost all Gram-negative bacilli used in this study which is consistent with previous research (10, 11, 14). Despite low direct antimicrobial activity however, SPR741 has been shown to synergize with antibiotics at concentrations between 2 and 8 µg/mL *in vitro* (10, 13). For example, in a neutropenic murine thigh infection model, SPR741 at 400 mg every 8 hours over 24 hours, when combined with azithromycin treatment against *Enterobacteriaceae*, reduced bacterial burden by ~ 0.53 $\log_{10}$ CFU/thigh, whereby each individual treatment resulted in net growth of bacteria (11). Against *A. baumannii*, 60 mg/kg SPR741 synergized with 5 mg/kg rifampin in murine pulmonary model, reducing bacterial load by 6 $\log_{10}$ CFU/g (14). These synergistic effects were proposed to be due to increased permeabilization of the outer membrane of bacteria to the second antibiotic (10, 11, 13). Intriguingly we showed here that SPR206 was more than twice as effective as SPR741 in LPS binding and permeabilization studies, while demonstrating similar potential cooperativity (based on Hill numbers), indicating that it might be highly suited for use in combination therapy which is commonly used in complicated infections. In this regard we observed overall superior synergy with clarithromycin (FIC $\leq$ 0.5) in the *P. aeruginosa* polymyxin susceptible and resistant isolates utilized here (C.R. Belanger, unpublished data).

In summary we have demonstrated that substantially redesigned polymyxin analogs have improved MICs, retain their outer membrane interaction properties and likely have considerable potential as combination drugs.

Materials and Methods

Bacterial strains, growth conditions and antimicrobial compounds

Wild type (WT) strains used in this study were *E. coli* SC9251, *P. aeruginosa* PAO1 strain H103 (29), *A. baumannii* ATCC 17978 (31), and the PMB susceptible, but multiple drug resistant *K. pneumoniae* VA360 (obtained from BEI Resources, Manassas, VA). PMB resistant strains used were *E. coli* NCTC 13846 with mobile colistin resistance (MCR-1) (obtained from Public Health England), clinical isolate *P. aeruginosa* 9BR which has aminoarabanose addition to Lipid A (30), *A. baumannii* C4 which has a *pmrB* mutation leading to phosphatidylethanolamine addition to lipid A (31), and polymyxin resistant *K. pneumoniae* 13883-PXR. These strains were grown in LB broth at 37°C overnight. Fresh LB broth with no added sodium chloride (10 g tryptone and 5 g of yeast extract per liter) were inoculated with the overnight cultures at a final dilution of 1:100, and the resulting cultures were grown with vigorous aeration at 37°C to mid exponential growth phase for subsequent assays.

Polymyxin B (PMB) and its derivatives SPR206, SPR1205, SPR946, PMBN and SPR741 were all provided by Spero Therapeutics Inc.

Minimal inhibitory concentration (MIC) assays

MICs were determined using a standard microtitre dilution method in LB no salt broth in order to verify antimicrobial susceptibility of each bacterial strain in conditions relevant to the NPN/DPX assays(20). Briefly, cells were grown overnight at 37 °C in LB no salt broth and diluted in the same medium. Serial dilutions of SPR-compounds were added to the 96 well plates in a volume of 10 µl followed by addition of 90 µl of bacteria to give a final inoculum concentration of 1 x $l0^6$ CFU/mL. Plates were incubated at 37°C overnight and MICs determined as the lowest concentration of compound that inhibited growth (32). MIC assays were also repeated in MHB to verify the values were representative to standard conditions.

LPS isolation and quantitation

LPS was isolated from *P. aeruginosa* and *E.coli*, using the Darveau-Hancock method (33). The LPS concentration was determined by measuring the LPS-specific saccharide 2-keto-3-deoxyoctonate (KDO, 34, modified by the Hancock lab: http://cmdr.ubc.ca/bobh/method/kdo-assay). To calculate the concentration of LPS, two reactive 2-keto-3-deoxyoctonate residues were assumed for each *P. aeruginosa* and *E. coli* LPS molecule and the molecular weight of *E. coli* and *P. aeruginosa* was considered to average 4500 g/mol and 5000 g/mol, respectively (25,

35–37). SDS-PAGE and LPS silver staining were performed as described by Tsai and Frasch (38). A modified Laemmli SDS-PAGE system was used with the Mini-Protean TGX gel apparatus (Bio-Rad, 12% stain-free gels) and 2X Laemmli sample buffer (Bio-Rad).

Dansyl-polymyxin binding experiments

Dansyl-polymyxin (DPX) was prepared according to the modified method of Schindler and Teuber (19). The synthesized DPX was dissolved in 3 ml of 5 mM HEPES buffer (pH 7.0), purified using a Sephadex G-25 column and quantitated using the dinitropphenylation assay (39). The concentration of DPX was calculated as 1.54 mM relative to a triplicate standard curve from a 1 mg/mL stock solution of PMB.

The fluorescence of dansyl-polymyxin bound to LPS was measured by using a fluorescence spectrofluorimeter (Perkin-Elmer LS-55), set at an excitation wavelength of 340 nm and an emission wavelength of 485 nm. The amount of DPX needed for LPS saturation was measured by recording the fluorescence after the addition of aliquots of DPX (5 µL of 100 µM DPX) into cuvettes containing LPS (20 µL of 300 µg/mL LPS) in 2 ml of 5 mM HEPES buffer (PH 7.2). The background DPX fluorescence was measured by adding aliquots of DPX to cuvettes containing 2 ml of HEPES buffer.

For binding of dansyl-polymyxin to the intact cells, the cells at an optical density at 600 nm of 0.5, were washed one time in 5 mM HEPES buffer/5 mM sodium azide (pH 7.2). The fluorescence was recorded after the addition of portions of DPX to cuvettes containing 10 µl of cells suspended in 990 µL of the same buffer to an optical density at 600 nm of 0.5. The amount of DPX needed to reach the maximum fluorescence level was considered the amount needed for maximum LPS saturation by DPX (25, 40).

Dansyl-polymyxin binding inhibition experiments

The amount of DPX required to give 90% saturation based on the values measured in binding experiments was calculated and added to the cuvettes containing 3 µg/mL isolated LPS in 2 ml of 5 mM HEPES buffer (PH 7.2). For the whole cell samples, the amount of DPX required to give 90% saturation was added to cuvettes containing 990 µl of 5 mM HEPES and 5 mM sodium azide buffer (pH 7.2) containing 10 µl of cells suspended in the same buffer to an optical density at 600 nm of 0.5 (final optical density = 0.005). Inhibitors of dansyl-polymyxin binding (SPR-compounds) were titrated into the cuvettes, and the decrease in the observed fluorescence was recorded. The percentage fluorescence reduction was calculated as [(F-

f)/F*100] where F was the maximum initial fluorescence (measured fluorescence level when LPS was 90% saturated with DPX) and f was the measured fluorescence level in the presence of a specific concentration of test compound. The inhibition graphs were drawn by plotting the percentage of fluorescence reduction on the Y-axis and the concentration of SPR-compounds on the X-axis. The maximum inhibition by any given compound was calculated as the extrapolated Y-axis intercept of a plot of the reciprocal of percent inhibition as a function of the reciprocal of the inhibitor concentration. The X-axis intercept provided the value for $-1/I_{50}$ where I_{50} was the concentration which resulted in 50% of maximal inhibition at the LPS and dansyl-polymyxin concentrations used (25, 40).

Permeabilization of whole cells to NPN

NPN uptake assays were performed as previously described (32). Briefly, cells were grown to an optical density of 0.4-0.6 at 600 nm, centrifuged at 7000 rpm for 10 min. at 20 °C. Cells were then washed and resuspended to the same optical density in 5 mM HEPES buffer (pH 7.2) containing 5 mM glucose and 5 mM CCCP. Serial dilutions of SPR-compounds in distilled water were made at 100 fold the desired final concentrations (from 2.5 l to 0.01 mg/mL). Fluorescence measurements were made using the fluorescence spectrofluorimeter (Perkin-Elmer LS-55) set at an excitation wavelength of 350nm with a slit width of 5; and an emission wavelength of 420nm with a slit width of 5 or 6. Twenty µl of NPN (obtained from Sigma) from a 500 µM NPN stock solution in acetone was added to the cuvettes contained 1 ml of cells suspended in the buffer to give a final concentration of 10 µM. After the background fluorescence was recorded, 10 µl of diluted SPR-compounds were added to the cuvettes to a final concentration of 25l to 0.1 µg/mL. A fresh cuvette of cells with NPN was used for each concentration of each SPR-compound, and the fluorescence recorded as a function of time until there was no further increase in fluorescence. Control experiments were performed to demonstrate that enhanced fluorescence was due to the uptake of NPN into cells, as described previously (32).

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Professorship.

Conflicts of interest

This research was sponsored by Spero Therapeutics who have a commercial interest in the described polymyxin analogs (SPR-compounds).

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Tables and Figures

Table 1. Minimal inhibitory concentration (MIC) assay results of SPR analogs tested against *Escherichia coli*, *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, and *Klebsiella pneumoniae* wild type (WT) and polymyxin/multi drug resistant strains in no salt LB.

Strain	Strain phenotype	MIC (μg/mL)					
		PMB	SPR206	SPR1205	SPR946	PMBN	SPR741
E. coli SC9251	WT	0.3	0.05	0.3	0.15	100	12.5
E. coli NCTC 13846	pEtN[a]	1.56	1.56	3.1	6.25	500	500
P. aeruginosa PAO1	WT	0.6	0.3	0.6	0.6	200	100
P. aeruginosa 9BR	MDR, Ara4N[b]	3.1	3.1	3.1	31.25	1000	1000
A. baumannii ATCC 17978	WT	0.3	0.05	0.3	0.6	800	25
A. baumannii C4	(pEtN)[c]	25	25	25	400	>2000	>2000
K. pneumoniae VA360	MDR	0.5	0.25	0.25	0.5	500	250
K. pneumoniae 13883-PXR	PMB resistant	4	32	25	250	500	>2000

[a] *E. coli* MCR-1-containing isolate NCTC 13846 expresses phosphatidylethanolamine transferase which transfers phosphatidylethanolamine residue to lipid A (pEtN). [b] Brazilian polymyxin and multi-drug resistant clinical isolate *P. aeruginosa* 9BR exhibits dysregulation of *phoPQ* and *arn* operons leading to aminoarabinose modification of lipid A (ara4N) (30). [c] Polymyxin and extremely drug resistant *A. baumannii* C4 *pmrB* mutation is linked to the addition of phosphoethanolamine to lipid A (31). [d] Polymyxin susceptible but multidrug resistant. [e] *K. pneumoniae* 13883-PXR exhibits polymyxin-resistance by an unknown mechanism.

Table 2. Displacement by SPR-compounds of DPX bound to WT *E. coli* SC9251 and polymyxin resistant *E. coli* NCTC 13846 LPS and whole cells. The I_{50} values (concentration of compound giving rise to 50% decrease in fluorescence) and Maximal inhibition were derived from double reciprocal plots using values averaged between replicates. Standard error values were below 3% for all compounds tested and standard error for each compound at each concentration is shown in Figure 2.

Compound	*E. coli* SC9251				*E. coli* NCTC 13846			
	Purified LPS		Whole cells		Purified LPS		Whole cells	
	I_{50} (μg/mL)	Maximal inhibition (%)	I_{50} (μg/mL)	Maximal inhibition (%)	I_{50} (μg/mL)	Maximal inhibition (%)	I_{50} (μg/mL)	Maximal inhibition (%)
PMB	4.6	80.0	11.8	100	4.1	86.5	21.1	30.2
SPR206	6.5	54.7	12.4	59.1	6	66.4	31.4	28.1
SPR1205	5.7	58.6	12.1	59.9	4.9	65.3	37.1	35.7
SPR946	6.9	49.7	13.3	63.4	8	64.2	26.3	21.5
PMBN	7.8	56.8	-*	-	5.6	57.3	31.9	19.7
SPR741	29.7	35.8	-	-	23.2	35.3	-	-

* - The ability of the compound to inhibit DPX-binding to LPS or whole cells was very low,

542 rendering the calculation meaningless.

Table 3. Displacement by SPR-compounds of DPX bound to *P. aeruginosa* PAO1 (WT) and polymyxin-resistant *P. aeruginosa* 9BR LPS and whole cells. The I_{50} values (concentration of compound giving rise to 50% decrease in fluorescence) and Maximal inhibition were derived from double reciprocal plots using values averaged between replicates. Standard error values were below 3% for all compounds tested and standard error for each compound at each concentration is shown in Figure 2.

Compound	*P. aeruginosa* PAO1				*P. aeruginosa* 9BR			
	Purified LPS		Whole cells		Purified LPS		Whole cells	
	I_{50} (µg/mL)	Maximal inhibition (%)	I_{50} (µg/mL)	Maximal inhibition (%)	I_{50} (µg/mL)	Maximal inhibition (%)	I_{50} (µg/mL)	Maximal inhibition (%)
PMB	5.8	85.7	10.5	52.8	3.4	83.0	14.5	47.9
SPR206	9.5	47.5	9.5	30.9	7.6	55.0	15.8	33.4
SPR1205	9.8	50.7	13.3	32.1	5.9	56.6	10.1	31.4
SPR946	14.1	48.5	6.1	18.5	10.1	54.8	13	18.2
PMBN	10.2	54.6	7.7	20.0	7.5	49.3	22.6	24.1
SPR741	-	14.9	24.2	7.9	15.5	28.3	-	8

Table 4. Displacement by SPR-compounds of DPX bound to WT *A. baumannii* ATCC 17978 and polymyxin-resistant *A. baumannii* C4 whole cells. The I_{50} values (concentration of compound giving rise to 50% decrease in fluorescence) and Maximal inhibition were derived from double reciprocal plots using values averaged between replicates. Standard error values were below 3% for all compounds tested and standard error for each compound at each concentration is shown in Figure 2.

Compound	***A. baumannii* ATCC 17978**		***A. baumannii* C4**	
	I_{50} (µg/mL)	Maximal inhibition (%)	I_{50} (µg/mL)	Maximal inhibition (%)
PMB	18.7	46.3	10.0	35.8
SPR206	18.9	29.2	19.7	31.0
SPR1205	19.3	27.6	18.8	33.4
SPR946	30.5	18.7	17.9	16.8
PMBN	19.8	24.5	18.2	15.5
SPR741	32.2	14.5	27.0	8.1

Table 5. Displacement by SPR-compounds of DPX bound to *K. pneumoniae* VA360 and polymyxin-resistant *K. pneumoniae* 13883-PXR whole cells. The I_{50} values (concentration of compound giving rise to 50% decrease in fluorescence) and Maximal inhibition were derived from double reciprocal plots using values averaged between replicates. Standard error values were below 3% for all compounds tested and standard error for each compound at each concentration is shown in Figure 2.

Compound	*K. pneumoniae* VA360 whole cells	*K. pneumoniae 13883-PXR whole cells*

	I_{50} (µg/mL)	Maximal inhibition (%)	I_{50} (µg/mL)	Maximal inhibition (%)
PMB	10.6	40.0	7.4	50.1
SPR206	15.8	21.4	8.4	31.7
SPR1205	10.3	22.2	9.5	28.2
SPR946	19.6	20.6	10.6	21.6
PMBN	28.8	17.2	15.2	28.1
SPR741	32.3	11.1	35.0	17.4

562

563 **Table 6. Hill number of the SPR-compounds for NPN uptake into *E. coli, P. aeruginosa, A.***
564 ***baumannii,* and *K. pneumoniae* wild type and polymyxin or multi drug resistant strains**

Strain	Hill Number				
	SPR206	**SPR1205**	**SPR946**	**PMBN**	**SPR741**
E. coli SC95271	1.59	1.65	1.35	1.36	1.64
E. coli NCTC 13846	1.38	1.26	1.2	1.07	1.07
P. aeruginosa PAO1	1.6	1.59	1.45	1.89	1.18
P. aeruginosa 9BR	1.42	1.68	1.56	0.64	0.47
A. baumannii ATCC 17978	1.31	1.11	1.06	1.17	0.88
A. baumannii C4	1.37	2.01	1.54	1.57	1.59
K. pneumoniae VA360	2.02	1.56	1.85	2.02	1.51
K. pneumoniae 13883-PXR	0.77	1.02	0.81	1.32	1.2

565 **Table 7. Concentration of SPR-compounds leading to 50% NPN uptake into *E. coli, P.***
566 ***aeruginosa, A. baumannii,* and *K. pneumoniae* wild type and polymyxin or multi drug**
567 **resistant strains.**

Strain	[SPR] leading to 50% NPN uptake				
	SPR206	**SPR1205**	**SPR946**	**PMBN**	**SPR741**
E. coli SC95271	1.5	1.26	1.36	1.44	0.72
E. coli NCTC 13846	1.5	1.44	2.23	2.61	2.77
P. aeruginosa PAO1	0.83	0.67	0.72	2.11	3.00
P. aeruginosa 9BR	2.17	1.89	2.17	5.33	25
A. baumannii ATCC 17978	0.44	0.50	0.47	1.12	0.86
A. baumannii C4	1.25	1.13	0.69	2.14	1.50
K. pneumoniae VA360	0.52	0.62	0.29	0.55	0.32
K. pneumoniae 13883-PXR	0.41	0.36	0.17	0.18	0.16

568

Figure 1. **Polymyxin analogs tested in this study.** (A) Each analog shared the same polymyxin cyclic core. The substituents R^1 and R^2 shown on the core structure in part A were substituted as follows, where R^1 is shown in the left hand box and R^2 in the right hand box: (B) Polymyxin B (PMB). (C) Deacylated polymyxin nonapeptide (PMBN) (D) SPR741 has the same cyclic core as PMB, a shorter N-terminus and a D-serine residue at the position adjacent to the cyclic core. (E) SPR1205 and (F) SPR206 have a diaminopropionate (L-Dap) residue adjacent to the cyclic core in place of diaminobutyrate (L-Dab) in PMB, and β-branched aminobutyryl N-termini. (G) SPR946 is based on the polymyxin E scaffold with L-Dap adjacent to the cyclic core and with a shorter N-terminal side chain.

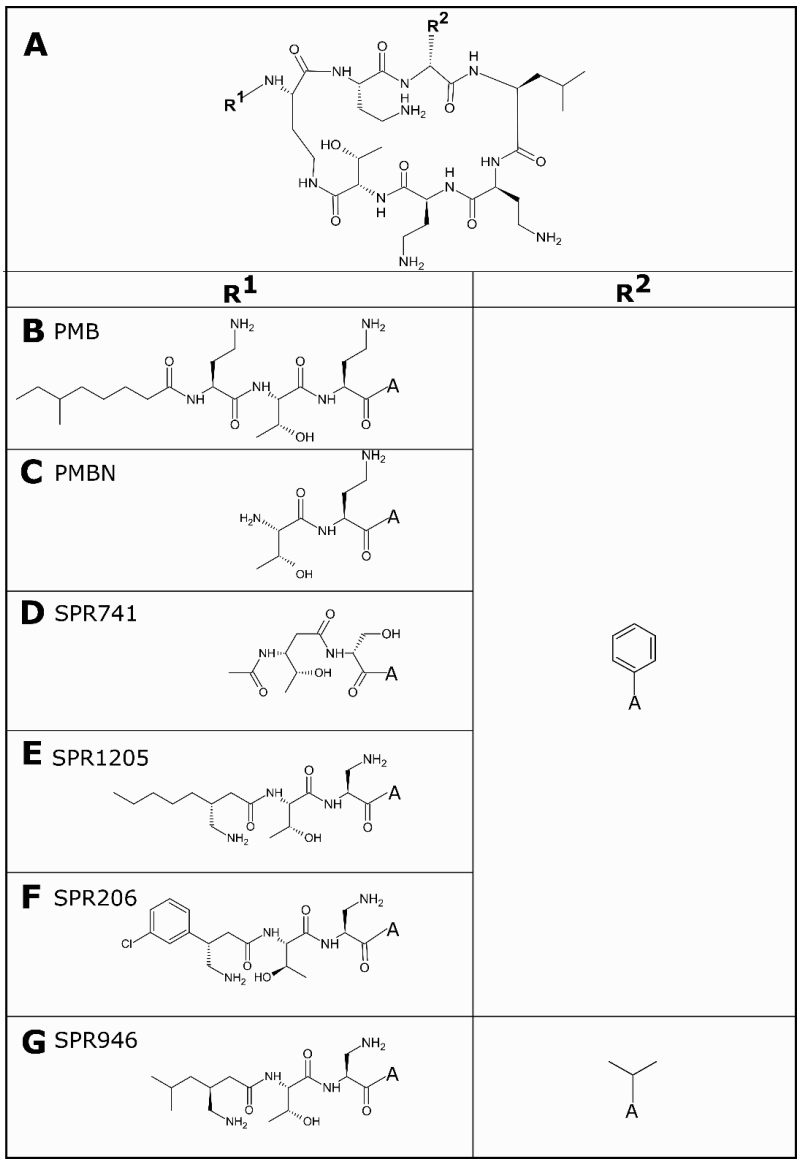
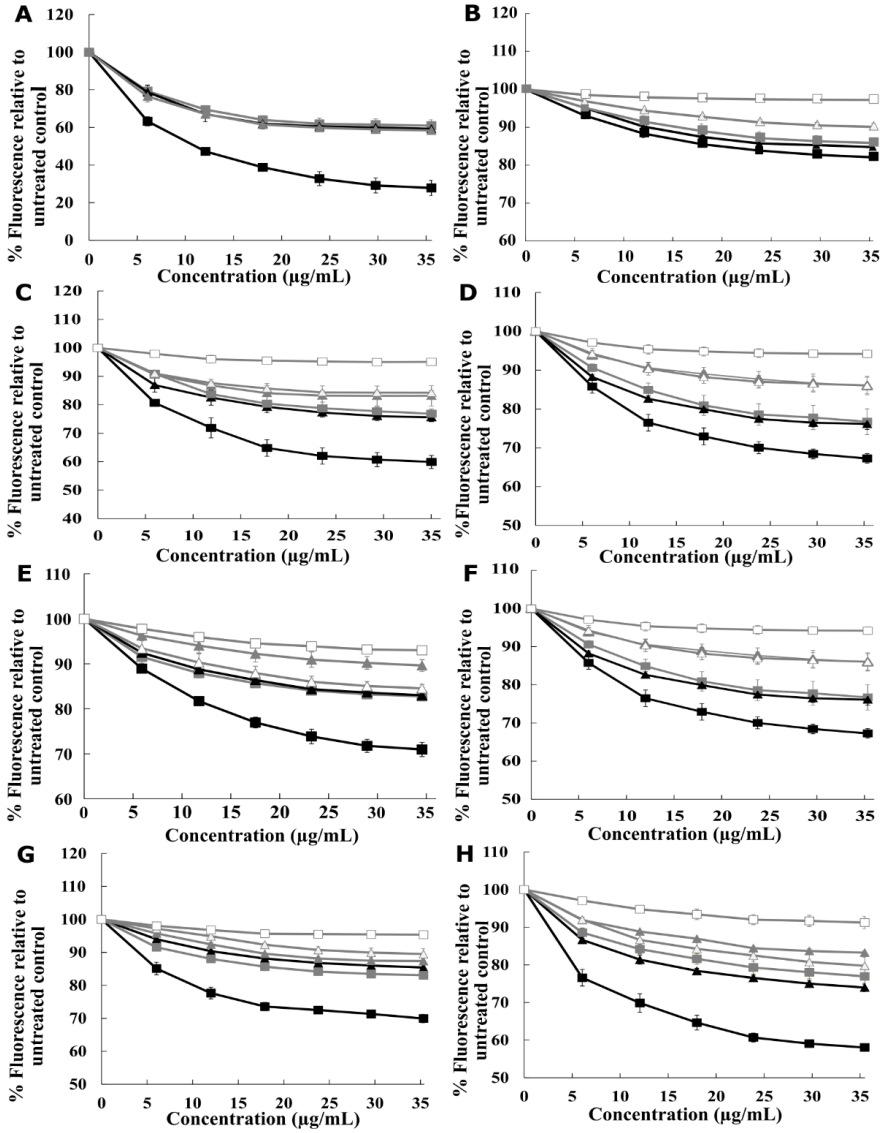
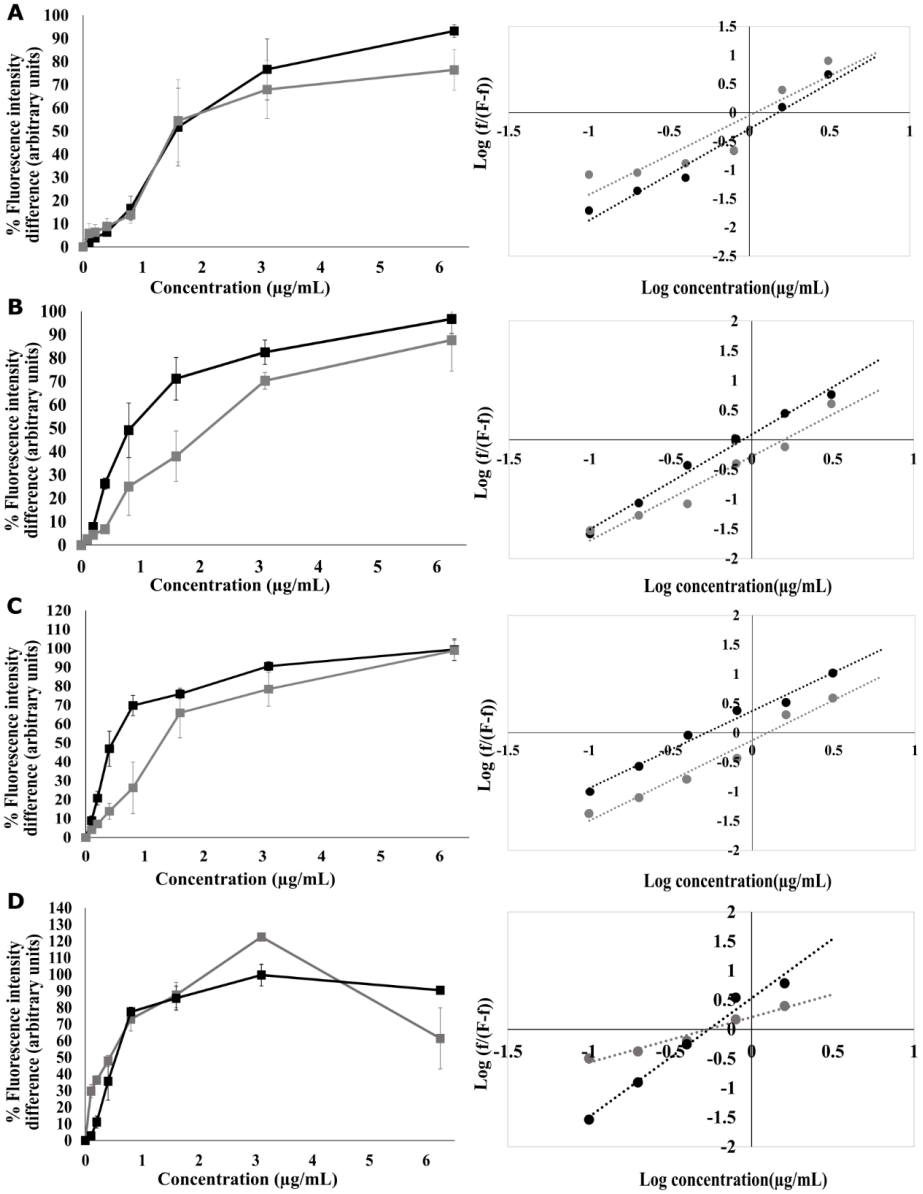


Figure 2. Whole cell DPX displacement by PMB (■) and SPR-compounds. SPR206 (■), SPR1205 (▲), SPR946 (▲), PMBN (△), and SPR741 (□) were used to displace DPX from various intact bacterial cells leading to a reduction in fluorescence. Maximal displacement was observed at ~35 µg/mL of each compound except for PMBN and SPR741 that required up to 200 µg/mL. (A) *E. coli* SC9251 and (B) *E. coli* NCTC 13846 whole cells. (C) *P. aeruginosa* PAO1 and (D) *P. aeruginosa* 9BR whole cells. (E) *Acinetobacter baumannii* ATCC 17978 and (F) *A. baumannii* C4 whole cells. (G) *K. pneumoniae* VA360 and (H) *Klebsiella pneumoniae* 13883-PXR whole cells. Left hand panels show WT or polymyxin susceptible cells; right hand panels show polymyxin or MDR derivatives.



588 **Figure 3. Influence of SPR206 on uptake of NPN across the outer membrane.** This was
589 performed with WT (■) and MDR (■) strains and the corresponding Hill plots for (A) *E. coli*
590 SC9251 and *E. coli* NCTC 13846 , (B) *P. aeruginosa* PAO1 and *P. aeruginosa* 9BR, (C) *A.*
591 *baumannii* ATCC 17978 and *A. baumannii* C4, and (D) *K. pneumoniae* VA360 (■) and *K.*
592 *pneumoniae* 13883-PXR (■). % fluorescence intensity is normalized to maximum fluorescence
593 of PMB for each strain.



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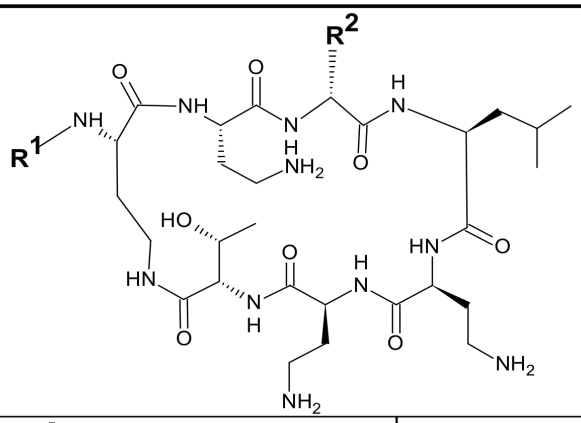

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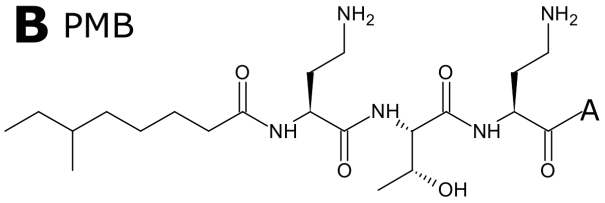
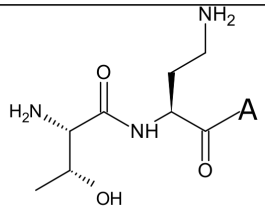
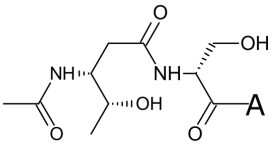
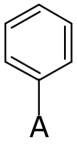
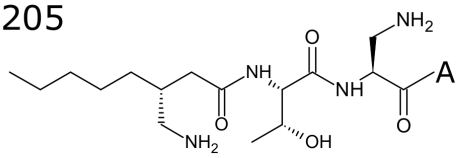
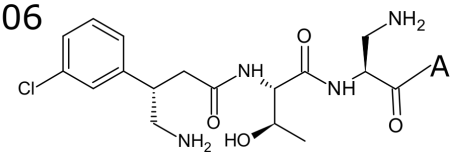
B PMB 	
C PMBN 	
D SPR741 	
E SPR1205 	
F SPR206 	
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