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Desferrioxamine: gallium-pluronic micelles increase outer membrane permeability and potentiate antibiotic activity against *Pseudomonas aeruginosa*[†]

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Abstract

The outer membrane of *Pseudomonas aeruginosa* functions primarily as a permeability barrier and imparts a broad spectrum of intrinsic antibiotic resistance. Herein, we describe the synthesis, characterization, and antimicrobial evaluation of a targeted polymeric micelle that specifically permeabilizes the outer membrane and potentiates antibiotic activity against *P. aeruginosa*.

The rapid spread of antibiotic resistance has become a major threat to public health, with the World Health Organization currently designating carbapenem-resistant *Pseudomonas aeruginosa* as a critical priority for the development of new antibiotic strategies.^{1–3} As the frequency of multi-drug resistant (MDR) infections rises, the number of effective treatments in our antibiotic armamentarium continues to shrink, highlighting the urgent need for new therapeutics for this challenging pathogen.^{4–7}

For Gram-negative species, attaining a sufficient antibiotic concentration at the bacterial target site is particularly challenging due to limited outer membrane (OM) permeability, especially in the case of *P. aeruginosa*, which has approximately 12-fold lower OM permeability relative to *E. coli*.^{8,9} Many compounds that exhibit potent activity against Gram-positive organisms are notably less effective against *P. aeruginosa* due to restricted OM permeability.¹⁰ Cationic peptides and small molecule permeabilizers have been shown to increase OM permeability and improve the antipseudomonal activity of high molecular weight (HMW) antibiotics (larger than approximately 700 Da) such as erythromycin and rifampicin against Gram-negative pathogens, but these tend to be less effective against *P. aeruginosa* in particular and often have non-specific activity resulting in toxicity to mammalian cells.^{11–13}

Poloxamers, amphiphilic copolymers comprised of a poly-propylene oxide (PPO) block flanked by two polyethylene oxide (PEO) blocks, are an alternative strategy for increasing

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Conflicts of interest

There are no conflicts to declare.

OM permeability. These polymers have been studied extensively for their ability to enhance drug delivery to mammalian cells, typically with regards to antitumor agents, by forming transient pores in the lipid bilayer and inhibiting efflux pumps.^{14,15} However, poloxamers are unable to accumulate in sufficiently high concentrations on Gram-negative bacterial cell surfaces to produce the permeabilization effects seen in mammalian cells.

Herein, we present a novel OM permeabilizer consisting of Pluronic (poloxamer) F127 micelles conjugated to the siderophore desferrioxamine B (DFO) complexed to Ga, DFO:Ga^{III} (DG). DG was chosen as a targeting ligand because during infection, *P. aeruginosa* upregulates the expression of OM receptors for iron uptake by its native siderophores pyoverdine and pyochelin, as well as receptors for xenosiderophores such as DFO:Fe^{III} (ferrioxamine) and ferrichrome.^{16,17} Similar to the ferrioxamine complex, DG is readily recognized by *P. aeruginosa* but avoids inadvertent delivery of nutritional iron to bacterial cells, and has furthermore demonstrated encouraging antibiotic activity.¹⁸ By increasing the local concentration of Pluronic micelles on the surface of Gram-negative bacteria through this targeting strategy, the surfactant was surprisingly effective at disrupting the OM permeability of *P. aeruginosa* (Scheme 1). The potentiation properties of F127-DG₂ micelles in the presence of several antibiotics with poor OM permeability were evaluated against two reference strains of *P. aeruginosa* (ATCC 27853 and PAO1) and three clinically-isolated carbapenem-resistant strains (Table S1, ESI[†]).¹⁹

Starting from F127, the terminal hydroxy groups were oxidized to carboxylic acids and then conjugated to the free amine of DFO *via* an amide bond by established literature procedures (Fig. S1–S5, ESI[†]).²⁰ The modified polymer was chelated to Ga^{III} based on ¹H NMR and MS (Fig. S6 and S7, ESI[†]); *ca.* 90% of terminal –OH groups converted to DG based on AAS and UV-Vis to generate F127-DG₂ constructs. F127-DG₂ retained solution structure similar to unmodified F127 micelles (21.6 nm) and readily formed 24.4 nm micelles in aqueous solution at 37 °C as verified by DLS and TEM (Fig S8A, ESI[†]).

To investigate the interaction of F127-DG₂ on the surface of *P. aeruginosa* by confocal laser scanning microscopy (CLSM), the aggregation-induced fluorophore tetraphenylethylene (TPE) was loaded into F127 and F127-DG₂ micelles.²⁰ Micelles retained similar size structures after TPE loading (F127/TPE = 23.2 nm, F127-DG₂/TPE = 24.8 nm) (Fig. S8B, ESI[†]). Based on TPE fluorescence intensity, CLSM visually confirmed that F127-DG₂/TPE micelles accumulated to a greater extent on the cell surface of *P. aeruginosa* compared to untargeted F127/TPE micelles (Fig. 1A and Fig. S9, ESI[†]). Interestingly, *E. coli* had similar degrees of nonspecific labeling by both micelle formulations, which may be due to a lack of OM receptors for the DG ligand.

OM permeabilization of *P. aeruginosa* was visualized by CLSM using hexidium iodide (HI), a fluorescent stain for Gram-positive organisms with limited ability to diffuse across the OM of Gram-negative species.²¹ Increased OM permeability should therefore result in a greater accumulation of HI within affected cells. Incubation of *P. aeruginosa* with F127-DG₂ resulted in noticeably more HI fluorescence compared to untreated cells or cells treated with

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unmodified F127 plus DG (Fig. 1B and Fig. S10, ESI[†]). *E. coli* did not show any increases in HI labeling, further supporting that the lack of DG receptors in this species prevents OM targeting by F127-DG₂.

Increased OM permeability was also evaluated using nitrocefin (NCF), a chromogenic β -lactam compound which undergoes a color change from yellow (A390) to red (A485) upon hydrolysis. In whole cell Gram-negative bacteria, β -lactamases are primarily located in the periplasm, therefore the NCF hydrolysis rate is primarily limited by its rate of diffusion across the OM.²² Bacterial cells incubated with NCF in the presence of F127-DG₂ micelles resulted in significantly faster hydrolysis by *P. aeruginosa* compared to unmodified F127 micelles plus free DG (Fig. 1C), indicating that chemical conjugation of F127 micelles to DG is necessary for the observed increase in OM permeability. *E. coli* OM permeability was unaffected, indicating that the targeting effect of F127-DG₂ is specific to *P. aeruginosa* due to its expression of the necessary OM receptors.

The antimicrobial activity of F127-DG₂ was evaluated alone and in combination with three HMW antibiotics characterized by a limited ability to diffuse across the OM: erythromycin (ERY), rifampicin (RIF), and vancomycin (VAN) were selected as model drugs with diverse target sites and mechanisms of action. Antimicrobial efficacy was evaluated using a checkerboard broth microdilution assay performed per CLSI guidelines in cation-adjusted Mueller-Hinton broth.²³ Synergy was evaluated by calculating the fractional inhibitory concentration (FICI).

F127-DG₂ alone did not completely inhibit growth of any of the tested pathogens, even at concentrations as high as 1024 μ M. F127-DG₂ was moderately synergistic with ERY or RIF against all tested strains of *P. aeruginosa*, and highly synergistic with VAN (Table 1). Improved antibiotic activity was observed in MDR strains of *P. aeruginosa* as well, indicating that the action of F127-DG₂ on the OM is not affected by clinically observed mechanisms of evolved antibiotic resistance. No synergy was observed between F127 plus DG combined with antibiotics against *P. aeruginosa*, indicating that only the targeted Pluronic micelles were able to permeabilize the OM and allow antibiotics to diffuse across. Neither F127-DG₂ nor F127 plus DG potentiated antibiotic activity against *E. coli* due to lack of the necessary OM receptors for DG.

Differences in synergistic activity observed for each antibiotic are attributed to their different target sites as well as secondary mechanisms of resistance. ERY and RIF bind to the 50S ribosomal subunit and RNA polymerase, respectively, both of which are cytoplasmic targets.^{24,25} This requires ERY and RIF to diffuse across the inner membrane in order to have activity, so increased OM permeability alone does not allow direct access to the target site. Additionally, efflux transporters in the OM can reduce periplasmic antibiotic accumulation, thus competition between greater influx of antibiotics and existing efflux systems results in only a modest increase in antimicrobial activity.^{26,27} VAN targets the cross-linking enzymes responsible for synthesis of the bacterial cell wall, which occurs in the periplasm of Gram-negative organisms.²⁸ OM permeabilization allows VAN to directly reach its target site in *P. aeruginosa*, therefore resulting in highly synergistic activity with F127-DG₂. Partial growth

curves using F127-DG₂ or F127 plus DG combined with the selected antibiotics for each strain show a dose-dependent relationship (Fig. S11–S16, ESI†).

To confirm that the antimicrobial activity observed was due to the action of the selected antibiotics themselves, a survival assay was used to differentiate between bacteriostatic or bactericidal activity. Single plate serial dilution spotting was used to track the growth of *P. aeruginosa* cultures incubated with either F127-DG₂ or F127 plus DG combined with selected antibiotics.²⁹ F127-DG₂ combined with ERY was bacteriostatic, while RIF and VAN both demonstrated bactericidal activity, which is consistent with their known mechanisms of action against Gram-positive organisms (Fig. 2A).^{24,25,28} When combined with F127-DG₂, CFU counts revealed no growth for ERY and an approximately 2.6 log reduction in viable colonies for RIF and VAN after 4 hour incubation (Fig. 2B). Antibiotics combined with F127 plus DG did result in a slight decrease in bacterial growth relative to the untreated control, but no inhibitory activity was observed. *E. coli* was essentially unaffected at the tested potentiator and antibiotic concentrations.

The toxicity of F127-DG₂ to mammalian cells was assessed with HeLa cells as a model human cell line using a metabolism-based resazurin assay.³⁰ Free DG, F127, and F127-DG₂ were considerably less toxic than free DFO even at the highest tested concentrations, suggesting that the system would be well tolerated *in vivo* (Fig. 3A). While F127 is relatively non-toxic, membrane permeabilization leading to mammalian toxicity has been observed *in vivo* at high doses (1.0–1.3 g kg⁻¹ IV in rats, or *ca.* 1200–1600 μM).³¹ Cell membrane damage was evaluated by a hemolysis assay using sheep red blood cells and measuring the released hemoglobin after two hours relative to a total lysis control.³² F127-DG₂ resulted in <5% hemolysis at 1024 μM, comparable to unmodified F127 (Fig. 3B), indicating that F127-DG₂ does not induce significant mammalian cell membrane toxicity. The ideal F127-DG₂ concentrations for antibiotic potentiation was 128 μM (equivalent to approximately 0.13 g kg⁻¹ IV) in most cases, which is considerably lower than the observed and previously reported thresholds for mammalian cell toxicity, indicating strong pathogen-specific activity.

The results obtained in this study provide a new strategy for OM permeabilization and antibiotic potentiation in *P. aeruginosa*. F127-DG₂ is non-toxic to mammalian cells and selectively targets the OM receptors for DG in *P. aeruginosa*, leading to increased accumulation of the amphiphilic Pluronic surfactant on the bacterial cell surface, and although the mechanism by which this occurs is currently under investigation, the effect leads to enhanced OM permeability. In doing so, F127-DG₂ potentiates the activity of several HMW antibiotics, resulting in moderate synergy with ERY or RIF and strong synergy with VAN. Encouragingly, F127-DG₂ was effective against reference strains of *P. aeruginosa* as well as strains expressing clinically-relevant MDR phenotypes, suggesting a potential therapeutic impact in cases where current antibiotic regimens are insufficient.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

F127-DG₂ increases the OM permeability of *P. aeruginosa*. (A) Targeted F127-DG₂/TPE micelles exhibit greater binding to the OM of *P. aeruginosa* than untargeted F127/TPE micelles based on increased TPE fluorescence (blue). F127-DG₂/TPE does not target *E. coli* due to lack of the necessary OM receptors. Positive control staining performed with FM 4-64FX (red). (B) OM permeabilization with F127-DG₂ (64 μ M) results in greater HI accumulation (red) in *P. aeruginosa* than unmodified F127 (64 μ M) + DG (128 μ M), while *E. coli* OM permeability is unchanged. Positive control staining performed with SYTO13

(green). (C) NCF hydrolysis occurs more rapidly in *P. aeruginosa* treated with F127-DG₂ (128 μ M) than unmodified F127 (128 μ M) + DG (256 μ M); *E. coli* OM permeability is unaffected by either polymer. Scale bars represent 2 μ m.

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Fig. 2.

Survival of *P. aeruginosa* cells treated for 4 h shows bacteriostatic activity for ERY when combined with F127-DG₂, while RIF and VAN combinations were bactericidal. (A) MHA plates at 0 and 4 hour for cultures of *P. aeruginosa* treated with F127-DG₂ combined with ERY, RIF, or VAN. (A) F127-DG₂ combined with ERY is bacteriostatic against *P. aeruginosa* whereas RIF or VAN are bactericidal. Unmodified F127 + DG combined with tested antibiotics did not result in inhibitory activity against *P. aeruginosa* and *E. coli* was also relatively unaffected by either formulation. Note: 128 μ M F127-DG₂ (or 128 μ M F127+ 256 μ M DG) and 96 μ g mL⁻¹ ERY, 12 μ g mL⁻¹ RIF, or 48 μ g mL⁻¹ were used against *P. aeruginosa*. One-way ANOVA performed for *P. aeruginosa* with F127-DG₂ plus antibiotics relative to *t* = 0 h positive control, ****p* < 0.001.

Fig. 3.

Mammalian cell toxicity of F127-DG₂. (A) No cytotoxicity observed at the highest concentration of F127-DG₂ (1024 μM), which is considerably higher than the concentration at which OM permeabilization of *P. aeruginosa* is observed (128 μM), indicating pathogen-specific activity. (B) <5% hemolysis occurred in RBCs treated with free DG, hemolysis decreased further when the DG complex is conjugated to F127.

Scheme 1.

F127-DG₂ micelles target OM ferrioxamine receptors expressed by *P. aeruginosa*, leading to cell surface accumulation. High local concentrations of the amphiphilic polymer perturbs OM integrity and increases permeability.

Antimicrobial activity of F127-DG₂ or F127 + DG combined with selected antibiotics against *P. aeruginosa* and *E. coli*. The MIC of F127-DG₂ alone or free DG was greater than 1024 µM for all strains. FICI o 0.25 considered high synergistic activity, 0.25 o FICI o 0.75 considered moderate synergistic activity, and FICI > 0.75 considered no synergistic activity

Table 1

Potentiator	Antibiotic	<i>P. aeruginosa</i>				<i>E. coli</i>	
		ATCC 27853 ^a	PAOI ^a	MDR 2638 ^a	MDR 3072 ^a	MDR 24530 ^a	ATCC 25922 ^a
None	ERY	256	256	512	256	512	32
	RIF	32	16	8	16	16	4
	VAN	>1024	>1024	512	>1024	1024	128
F127-DG ₂	ERY	64(0.38)	128(0.53)	64(0.25)	128(0.63)	256(0.53)	16(0.75)
	RIF	8(0.31)	8(0.56)	4(0.53)	8(0.56)	8(0.53)	4(1.25)
	VAN	32(0.16)	64(0.19)	64(0.19)	64(0.19)	128(0.25)	128(1.25)

^aInhibitory concentrations for antibiotics are given in µg mL⁻¹, followed by FICIs given in parentheses.