An efflux-susceptible antibiotic-adjuvant with systemic efficacy against mouse infections

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Scarcity of effective treatments against sepsis is daunting, especially under the contemporary standpoints on antibiotics resistance, entailing the development of alternative treatment strategies. Here, we describe the design and antibiotic adjuvant properties of a new lipopeptide-like pentamer, decanoyl-bis.diaminobutyrate-aminododecanoyl-diaminobutyrate-amide (C₁₀BBc₁₂B), whose sub-maximal tolerated doses combinations with inefficient antibiotics demonstrated systemic efficacies in murine models of peritonitis-sepsis and urinary-tract infections. Attempts to shed light into the mechanism of action using membrane-active fluorescent probes, suggest outer-membrane interactions to dominate the pentamer's adjuvant properties, which were not associated with typical inner-membrane damages or with delayed bacterial growth. Yet, checkerboard titrations with low micromolar concentrations of C₁₀BBc₁₂B exhibited unprecedented capacities in potentiation of hydrophobic antibiotics towards Gram-negative ESKAPE pathogens, with an apparent low propensity for prompting resistance to the antibiotics. Assessment of the pentamer's potentiating activities upon efflux inhibition incites submission of a hitherto unreported, probable action mechanism implicating the pentamer's de-facto capacity to hijack bacterial efflux pumps for boosting its adjuvant activity through repetitive steps including outer-membrane adhesion, translocation and subsequent expulsion.

Sepsis represents one of the most notorious yet ill-treated medical conditions, annually affecting 49 million patients worldwide, with a towering mortality rate of 20%1,2. Effective treatments are urgently needed as current strategies are limited to short-term immunomodulation3–7 and broad-spectrum antibiotics administration8–10, both of which do not adequately meet the challenge, hence allowing sepsis to persist as leading cause of death1,2. Development of effective treatments is complicated, as they must overcome multiple obstacles pertaining to treatment onset, pathogens diversity, as well as variation in host response1–2. This problem is exacerbated by increased prevalence of antibiotic resistance that further limits current treatments efficacies, particularly with regards to Gram-negative bacteria (GNB), against which, no new antibiotic classes have been successfully developed for over 50 years11. Consequently, exploration of antibiotics combinations is frequently pursued by clinicians, sometimes based on trial and error. Alternatively, immune curbing by agents indifferent to pathogens and/or host variability, may offer a more versatile and potentially preferred treatment strategy. Thus, the ability to sequester contributing factors, such as lipopolysaccharides (LPS) that promote sepsis deterioration to septic-shock, would represent a desirable attribute of such agents.

Cationic antimicrobial peptides (AMPs) are sometimes considered as potential candidates for accomplishing this task. AMPs chemo-physical attributes were evolutionarily designed to selectively target bacterial membranes, including through interactions with anionic moieties of LPS, such as lipid A12–14. Nevertheless, their clinical utilization is deemed challenging, namely due to their relatively short half-lives and host-toxicity upon systemic treatments, whereas peptidomimetic approaches are currently believed to minimize such drawbacks, imparting them with robustness and improved chances for successful clinical implementation15. Albeit, those characterized with membranolytic modes of action might in fact achieve the exact opposite outcome, as they too—appear to instigate unregulated LPS release. In this respect, antibiotic adjuvants (namely those using non-specific mechanisms) may provide an attractive treatment alternative16, as they can reinstate an antibiotic's efficacy as well as reduce high-dosage associated toxicity17. Adjuvants can also bestow potency upon Gram-positive-specific hydrophobic antibiotics towards GNB18, specifically when resistance emanates from their low translocation across the outer-membrane (OM)19. Moreover, owing to their mechanistic distinctions from antibiotics, adjuvants can be impervious to the documented resistance mechanisms, emphasizing an inherent advantage for their preferable
One such group of AMP-mimetics is represented by short lipopeptide-like sequences, composed of amide-linked fatty acids and cationic amino acids, which, owing to their simple yet modular structure, have recently shown considerable aptitude to facilitate fine-tuning of the chemophysical properties and subsequent AMP-like biological attributes. Here, we sought out to investigate additional new analogs designed to function as adjuvants, through substitution of the cationic amino-acids and then attempted to elucidate the mechanism of action of the most promising emerging analog.

Results and discussion
C10BBc12B emerges as promising antibiotics adjuvant. Our previous attempts to design antibiotic adjuvants have focused on short lipopeptide-like sequences corresponding to the pentameric formula $A_xC_yC_z$ where “$A_x$” and “$a_y$”, respectively represent the N-terminal acyl of length x (number of methyl groups) and an aminoacyl of length y, whereas “$C_z$” represents a cationic amino acid. Based on earlier findings, we abstained in the current study from incorporating an exceedingly hydrophobic acyl at the N-terminus, that could generate potent but non-selective antimicrobial lipopeptides, which in turn, might complicate their in-vivo systemic implementation due to toxicity phenomena such as hemolysis and unregulated LPS release. Instead, we used decanoic acid, which emerged from an analogous study as potentially embodying the appropriate hydrophobicity for the sequences under present investigation. In parallel, we also attempted to optimize contributions emanating from the cationic residues, by adjusting their side-chain length (Fig. 1a).

We first attempted to detect hydrophobicity differences among the cationic analogs by comparing their elution properties, using a C$_{18}$ reversed-phase HPLC column. However, they all eluted between 45 and 46% hydrophobic solvent (acetonitrile), including upon co-injection of the four analogs, suggesting that the side-chains contribute very little to these molecules chromatographic behavior. This contrasted with previous observations where reducing or increasing the N-terminal acyl length by only 2 methylenes (e.g., $C_{10}K_{12}K$ → $C_{12}K_{12}K$ → $C_{14}K_{12}K$) resulted in substantial hydrophobicity difference (as deduced from the fact that their elution required approximately 5% reduced or increased acetonitrile concentration, respectively). Additionally, assessment of self-assembly tendencies of these lipopeptide analogs (through measuring their light scattering properties) has also demonstrated little dissimilarities in their critical aggregation concentrations, which were all ≥ 100 µM. Conversely, evaluation of the lipopeptides bioactive properties revealed similarities only between K-, O- and B-based analogs (Fig. 1b), whereas the P-based pentamer presented a divergent activity profile. Namely, $C_{10}PPC_{12}P$ exhibited a hemolytic capacity somewhat more pronounced than that observed with its analogs (i.e., LC$_{50} = 26$ versus > 100 µM, respectively). Additionally, when tested against 10 bacterial strains representing the four species of Gram-negative ESKAPE pathogens, the K-, O- and B-based analogs displayed high minimal inhibitory concentrations (MIC ≥ 50 µM), whereas $C_{10}PPC_{12}P$ occasionally presented somewhat lower MIC values. Notably, $C_{10}PPC_{12}P$ presented a lower selectivity profile (i.e., its MIC and LC$_{50}$ values were comparably scaled) which may be attributed to its side-chain’s amine pKa, as it is less likely to be fully protonated at neutral pH (i.e., its β-NH$_3$ group pKa is 6.37 versus at least 10.428, for the three analogs). This could (at least partly) explain its similar potency against RBCs and bacteria, particularly since selectivity towards bacterial membranes is significantly charge-driven. Notably, no cytotoxicity towards cell-lines was observed at least up to 50 µM.

Having established these analogs overall rather poor antibiotic activity against GNB, we next defined their capacities to function as adjuvants that sensitize bacteria to antibiotics. For this purpose, we determined the analogs ability, at a sub-inhibitory concentration (10 µM) to potentiate erythromycin and rifampin (representing two antibiotic families that are normally ineffective against GNB, namely due to their hydrophobicity) using SPR741 as an adjuvant. Here, we sought to fully protonate at neutral pH (i.e., its β-NH$_3$ group pKa is 6.37 versus at least 10.428, for the three analogs). This could (at least partly) explain its similar potency against RBCs and bacteria, particularly since selectivity towards bacterial membranes is significantly charge-driven. Notably, no cytotoxicity towards cell-lines was observed at least up to 50 µM.

Since cyclization of AMPs was found to improve their potency, we similarly produced and tested the corresponding head-to-tail cyclic analogs of the four pentamers. As depicted in Table 1, while maintaining considerable potencies, the cyclic analogs presented substantially lower SF values than their linear counterparts. This finding suggests that structural rigidity contributes to greater antimicrobial potency, whereas flexibility appears to promote stronger adjuvant properties.

Table 1 also compares the potencies of AMPs against E. coli, and rifampin. Furthermore, the cyclic analogs displayed even higher SF values, indicating a superior ability to promote stronger adjuvant properties.

A broader scope of $C_{10}BBc12B$ potentiation capacities is shown in Fig. 2, indicating that the pentamer is endowed with a similarly exquisite capability for sensitizing all four Gram-negative representatives of ESKAPE pathogens tested, as observed with both erythromycin and rifampin. The isobolograms (presented in panels a and b) display geometries indicative of definite synergistic relationships and in most cases, $C_{10}BBc12B$ has potentiated the antibiotics to a point requiring concentrations well-below their respective resistance breakpoints (i.e., 8 and 1 µg/ml respectively for erythromycin and rifampin, as defined against Staphylococcus). For example, the pentamer reduced erythromycin’s inhibitory concentrations against E. coli and K. pneumoniae from 128 and 512 µg/ml to 0.125 and 0.250 µg/ml (i.e., SF values of 1024 and 2048, respectively). Likewise, rifampin’s MIC against these species was reduced to below 1 ng/ml, reflecting an increased sensitization efficiency of 65,000
Figure 1. Basic Characterization of Pentamer Analogs. (a) General molecular structure of the A₁₀Cₐ₁₂C series; where A₁₀ and a₁₂ stand for decanoyl and aminododecanoyl, whereas “C” represents one of the following cationic amino acids: lysine (K), ornithine (O), diaminobutyric acid (B) or diaminopropionic acid (P) where n (methyl groups number on each side chain) equals 4, 3, 2 or 1, respectively. Molecular weights of these analogs are 753, 711, 669 and 627 g/mol, for K, O, B and P, respectively. (b) Cytotoxic activity of pentamer analogs. a, Minimal inhibitory concentrations determined using the broth microdilution method (n, number of tested strains); b, Pentamer concentration causing 50% inhibition of cells respiration, using Alamar blue; c, Pentamer concentration causing 50% hemolysis of murine RBC. Published data appear in bold fonts, shown for comparison purposes.

Table 1. E. coli sensitization to antibiotics by adjuvants. Values specify bacterial sensitization factors (i.e., fold difference between the antibiotic’s MIC in absence of adjuvant versus in its presence) to each antibiotic, as determined against E. coli 25922. SF values obtained for cyclic analogs are shown in parenthesis. Erythromycin and rifampin MIC values in absence of adjuvant were 128 and 8 µg/ml, respectively. Published data appear in bold fonts. NA, not applicable.
and 130,000 folds, respectively. Such unprecedented values strengthen the proposed stature of C_{10}BB_{12}B as an exceptionally potent antibiotic adjuvant.

**Pathogens sensitization is dissociated from emergence of resistance.** To assess selective pressure effects on the observed bacterial sensitization to antibiotics, we compared the rates of antibiotic-resistance development by *A. baumannii* which occupies the top of the urgent category in the latest CDC’s antibiotics resistance threats report. Panels c and d show that in presence of C_{10}BB_{12}B, the MIC of either antibiotic has not increased by more than 2 folds, which corresponds to the inherent variance of the microdilution technique used during the subculture passages and thus can be regarded as practically unaltered. Conversely, in absence of C_{10}BB_{12}B, antibiotics’ MIC values have increased by 128 and 4 folds, respectively for erythromycin and rifampin (i.e., from 64 to 8,192 µg/ml and from 2 to 8 µg/ml, respectively). This dissimilar increase maybe ascribed to the antibiotics differential modes of action (i.e., bacteriostatic versus bactericidal) or to their respective predominant resistance mechanism (i.e., efflux pumps over-expression or rpoB mutations). Regardless, the fact that in presence of C_{10}BB_{12}B the antibiotics’ MIC remained effectively constant, implies that the pentamer has effectively bypassed bacterial aptitudes for developing antibiotic-resistance (at least throughout the assay duration). While the molecular basis is yet to be elucidated, it is worth mentioning that similar assessment was recently reported for SPR741 on *E. coli*. Moreover, as despite the pentamer’s greater synergetic potency and the consequently greater evolutionary pressure exerted on resistance development, resistance has not materialized, thereby highlighting potential advantages of C_{10}BB_{12}B-based therapies.

**C_{10}BB_{12}B performs as adjuvant in-Vivo to protect mice from infections.** Towards determining the potential of C_{10}BB_{12}B to resolve resilient GNB infections, we used a modified peritonitis-sepsis infection model to verify whether bacteria that were exposed to the pentamer prior to administration to mice, would (in principle) be able to affect the disease course, possibly via LPS neutralization, considering that immune activation by LPS is an underlying factor for sepsis deterioration. Thus, bacteria briefly exposed to C_{10}BB_{12}B (10 µM in PBS, a concentration known to affect outer-membrane (OM) permeability but does not alter bacterial proliferation rates, as will be shown in Fig. 4) were administered intraperitoneally to neutropenic mice and mortality was recorded for three days thereafter, as compared to control mice infected by the same culture pre-treated...
with PBS vehicle. As shown in Fig. 3a1, bacteria that were exposed to the pentamer have delayed the onset of mice mortality and reduced the overall lethality rate, thereby reflecting the pentamer’s potential ability to interfere with the sepsis process upon treating infected mice, assuming proper pharmacokinetic behavior would be achieved. Encouraged by this observation, we next prepared to assess the pentamer’s ability to affect sepsis (using the unmodified peritonitis-sepsis model) upon subcutaneous administration to infected mice of a sub-maximal tolerated dose (MTD).

Thus, MTD determination after administration of increasing doses (i.e., 0, 20 and 40 mg/kg C10BBc12B) to normal mice revealed no apparent physiological signs of distress for any of the mice, from the moment of injection throughout a seven-day monitoring period (i.e., MTD was estimated > 40 mg/kg). This represents a significant improvement compared to its analog C10OOc12O22, for which transient signs of distress were observed in a mouse (score of 3 out of 6) and mortality of another mouse was recorded within an hour after administering the 40 mg/kg dose (i.e., estimated MTD was > 20 but < 40 mg/kg). As the B-based pentamer’s MTD is comparable to that reported for the last-resort antibiotic PMB40, this finding suggests that C10BBc12B might offer an improved safety profile given its molecular attributes as small molecule and milder, non-membranolytic putative mechanism of action (as further addressed below).

Next, we evaluated the lipopeptide’s ability to affect survival of infected neutropenic mice. Figure 3a2 depicts the mice survival rates upon monotherapy or/and combination-therapies. Thus, while the control mice response to infection has evolved similarly to the experiment shown in panel a1 (i.e., 80% mortality in control mice was reached at two–three days post-infection, using similar inoculum size) mice treated with C10BBc12B or rifampin displayed roughly comparable (40 and 50%, respectively) improved survival rates, as compared to the vehicle control, whereas the combination treatment reached 90% survival. The fact that C10BBc12B showed some efficacy
even in absence of rifampin evokes our previous findings\textsuperscript{22,29} where using a similar infection model and treatment, C\textsubscript{10}B\textsubscript{12} has also exhibited significant monotherapy efficacy. While the molecular basis for the pentamers’ abilities to prevent mice death in absence of an exogenous antibiotic is yet to be elucidated, we provided various lines of evidence arguing for the possible role played by one or more endogenous antimicrobials that may have substituted for rifampin’s role. If that were to be the case, one would be allowed to predict a more potent antimicrobial performance of C\textsubscript{10}B\textsubscript{12} in treating infections of wild animals (as opposed to laboratory animals such as the mice used herein) since they are normally endowed with a more robust innate immunity.

Figure 3 (panel a3) provides evidence for considerable systemic efficacy even under harsher infection conditions (i.e., when mice were infected with a higher inoculum size) where lethality of control mice has reached 100% already on day one and mice treated with C\textsubscript{10}B\textsubscript{12} or rifampin displayed virtually no efficacy. Under these conditions, the combination treatment managed to increase the survival rates from 0 to 40%, thus implying a synergistic outcome, reminiscent of the synergy observed in terms of SF values (Fig. 2a, b).
Next, we assessed the pentamer’s ability to affect the course of another disease, using the urinary tract infection (UTI) model, where, uro-pathogenic E. coli bacteria were administered by intra-urethral route and the infected mice treated with either C10BBc12B or rifampin or with a combination thereof, and bacterial loads in bladder and kidneys were quantified 24 h post-infection. To determine efficacy, we assessed different treatment regimens, including an initial single dose of 7.5 or 12.5 mg/kg C10BBc12B administered 1 h post infection, but found these regimens to be essentially ineffective, whether administered alone or in combination with rifampin. However, administration of four doses of C10BBc12B within a 24-h period, have generated a significant efficacy upon combination with rifampin. Thus, while no reduction in bacterial load was observed in bladders with any of the tested treatments (Fig. 3b1), the combination treatment has reduced the kidneys bacterial load, as evidenced by complete bacterial eradication (or prevention of colonization altogether) in 80% of the treated mice (Fig. 3b2).

Towards establishing cause and effect relationships, we next attempted to correlate these efficacy outcomes with the circulating pentamer's levels, in the respective tissues of interest. Figure 3c1, c2 present data summarizing the biodistribution analysis showing that C10BBc12B presence overlapped with the efficacies outlined in Fig. 3a, b, respectively. Thus, similarly to its analogs, the subcutaneous administration of C10BBc12B resulted in a steep increase of its circulating concentrations (reaching its maximum after 30–60 min) followed by a shallower decline that ultimately cleared most of the lipopeptide from the bloodstream after ~ 180 min. Importantly, C10BBc12B maintained antibiotics potentiating concentrations (i.e., ≥ 2.5 µg/ml) for >2 h post-adjuvant administration. Likewise, quantification of C10BBc12B in bladder and kidneys, revealed negligible amounts in the bladder, whereas the pentamer was readily quantifiable in kidneys for at least 3 h post-inoculation. Therefore, these findings align well with those observed in Fig. 3, in the sense that treatment efficacies are correlated with the adequate pentamer's presence.

**Antibiotics potentiation is mediated by C10BBc12B interactions with the OM.** Towards better understanding the synergistic mechanism, we compared individual growth kinetics to determine contributions of each antimicrobial and assessed the type and extent of implicated membrane damages, assuming that low OM-permeability underlies the observed antibiotics inefficiencies by impeding their intracellular accumulation. Figure 4a shows that at synergistic concentrations (i.e., 10 µM C10BBc12B and 1 µg/ml erythromycin or 20 ng/ml rifampin) all individual proponents were unable to significantly alter bacterial proliferation (i.e., growth curves obtained for untreated control and individual treatments, were virtually indistinguishable) whereas upon combining treatments, bacterial proliferation was arrested for at least 6 h, followed by bacterial death levels that nearly eradicated the cultures at the 24 h endpoint. Using equivalent conditions, similar growth curves have also characterized K. pneumoniae, P. aeruginosa and A. baumannii (data not shown). Importantly, while sub-inhibitory concentrations of some reported antibiotic potentiators (such as C14(d5)OOC10(O23 or PMBN41) have transiently inhibited bacterial growth in absence of antibiotics, the fact that C10BBc12B is devoid of this attribute cements our proposed classification as adjuvant. Also, C10BBc12B combinations with erythromycin or rifampin exhibited similar behaviors—despite the antibiotics distinct modes of action, hence providing additional support to the notion that their potentiation maybe mechanistically related, where the pentamer plays a facilitator role, in both cases. To test this postulate, we next assessed the extent of OM damage using N-phenyl-1-naphthylamine (NPN) fluorescence measurements (Fig. 4). As shown in panel b1, C10BBc12B, like PMB (deemed gold-standard OM-permeabilizer), increased E. coli’s OM permeability to NPN in a dose-dependent manner, albeit in a milder fashion. Validation of these findings is provided by the observation that high concentrations of MgCl2 have attenuated the NPN fluorescence for both agents, correspondingly. Thus, the resulting fluorescence reduction (i.e., almost complete for C10BBc12B and much less for PMB) enforces the concept that C10BBc12B and PMB differ in their affinities towards LPS. This notion was further corroborated using dansyl-PMB (DPMB) competition experiments (Fig. 4b panel b2) by confirming the inferior ability of C10BBc12B to replace the LPS-bound DPMB (the relevance of this affinity difference will be addressed while discussing Fig. 6).

Another experiment stressing mechanistic difference(s) between these lipopeptides (C10BBc12B and PMB) is provided through their ability to affect cytoplasmic membrane (CM) permeability. Unlike PMB whose bactericidal activity was associated with a rapid CM-depolarization (Fig. 4 panel b3), C10BBc12B was clearly unable to affect the trans-membrane potential (TMP) at the tested concentration range (i.e., up to 10 µM). The fact that a more hydrophilic analog C10BBc12B (data not shown) displayed dose–response depolarization traces that are indistinguishable (i.e., identical to that shown in Fig. 4 panel b3), supports the notion that both derivatives are mechanistically puzzling. Alternatively, while C10BBc12B appears to hold a higher potency of C10BBc12B (eightfold reduction in MIC value) was observed against the mutant biofilm, whereas the pentamer was readily quantifiable in kidneys for at least 3 h post-inoculation. Therefore, these findings align well with those observed in Fig. 3, in the sense that treatment efficacies are correlated with the adequate pentamer’s presence.

**Implication of efflux machinery in antibiotics potentiation.** While C10BBc12B appears to hold mechanistic attributes similar to those of polymyxins, we find that these lipopeptides are distinguishable by various aspects (besides the aforementioned differences) including susceptibility to undergo efflux. When comparatively assessed against the isogenic pair of wild-type E. coli Ag100 and its efflux deficient mutant Ag100a (ΔacrAB) a higher potency of C10BBc12B (eightfold reduction in MIC value) was observed against the mutant
strain (Table 2). Similar relationships were recorded with another isogenic pair of *Salmonella enterica* serovar Typhimurium. These outcomes would sit well with the notion that C10BBc12B is an efflux substrate, very much as previously proposed for its analogs C10KKc12K26 and C10OOc12O29, unlike C14OOc12O23 or C14KKc12K24. Accordingly, the polymyxins (e.g., PMB or colistin), often described as unlikely efflux-substrates43–45 are in fact efficient CM-destabilizers that swiftly dissipate the TMP, thereby obstructing the proper proton-dependent function of resistance-nodulation-division (RND) efflux pumps, including the AcrAB-TolC system commonly expressed in Enterobacteriaceae.

Thus, although both PMBN and C10BBc12B may act as antibiotic adjuvants by increasing OM-permeability, we find that they differ in relation to how they affect the TMP and how they are affected by efflux: the former causes TMP dissipation, possibly inactivating proton-dependent efflux (including of itself)47 whereas C10BBc12B appears relatively reluctant to interact with the CM despite its hydrophobic and cationic attributes, thereby sustaining de-facto, its availability for efflux from the periplasm or from superficial adhesion to the CM (as proposed for C10KKc12K26 but not C14KKc12K24).

Another distinguishing aspect emanated from the lipopeptides divergent behavior in presence of efflux pump inhibitors (EPIs). Since PMBN was reported to synergize with EPIs in potentiating antibiotics activity41, we verified whether C10BBc12B potentiation could be similarly enhanced in presence of EPIs especially since, in the pentamer’s case, efflux function stands presumably unaffected. However, introduction of EPIs appears to rather dampen the pentamer’s capacity for antibiotics potentiation. Thus, towards isolating relationships between C10BBc12B, EPI and efflux machinery, we used rifampin (instead of erythromycin) owing to its non-susceptibility for efflux by RND pumps48 (and in Table 2). Figure 5a shows that C10BBc12B SF values, as determined in absence

| Compound       | MIC (µM) in LB E. coli | MIC (µM) in LB S. Typhimurium |
|----------------|------------------------|-------------------------------|
| Erythromycin   | >50                    | >50                           |
| Rifampin       | 19.4                   | 19.4                          |
| Polymyxin B    | 0.7                    | 0.7                           |
| C10KKc12K      | 6.2                    | 6.2                           |
| C10OOc12O      | >50                    | >50                           |
| C10BBc12B      | 50                     | >50                           |

Table 2. Effect of RND pumps on growth-inhibitory activity. *Minimal inhibitory concentrations determined using the broth microdilution method, as respectively assessed against wild-type (Ag100 and 14028) and efflux deficient (Ag100a and 14028ΔacrAB) isogenic strains of *E. coli* and *S. Typhimurium*. Published data appear in bold fonts.

Figure 5. Efflux Role in C10BBc12B OM-Permeabilization Using *E. coli* 25922. (a) Sensitization factors (SF) determined for rifampin in presence of 10 µM C10BBc12B, solid green bars; 10 µM C10BBc12B + 5 µg/ml PAβN, striped green bars; 5 µg/ml PAβN, white bars. (b) OM permeabilization assessed using a modified NPN assay, following 5 min incubation with 100 µM CCCP. C10BBc12B, solid green bars, C10BBc12B in presence of 100 µM CCCP, striped green bars. Data represent fluorescence after 1 min. Asterisks denote P<0.05; ns, not significant. RFU, relative fluorescence units. Error bars represent standard deviations.
of the EPI phenylalanine-arginine β-naphthylamide (PAβN)\textsuperscript{49,50}, were in fact greater than those obtained in its presence, thereby suggesting an antagonistic relationship between C\textsubscript{10}BBc\textsubscript{12}B and PAβN or even another EPI\textsuperscript{49,51}, unlike PMBN's\textsuperscript{41}. It is ought to be mentioned that while functioning as efflux pumps inhibitors, these EPIs\textsuperscript{52,53} were additionally reported to increase OM permeability (as might also be inferred from our observations, as depicted in Fig. 5a). Unfortunately therefore, as this dual mechanism of action of PAβN (i.e., pump inhibitor and OM permeabilizer), complicates the assignment of either one of its functions to the antagonistic relationship with C\textsubscript{10}BBc\textsubscript{12}B, we attempted to circumvent this challenge by using an alternative approach for limiting efflux function, and depleted the fuel required for proper function of RND pumps (the proton motive force) by introducing the ionophore carbonyl cyanide m-chlorophenyl hydrazone (CCCP)\textsuperscript{54,55}. As shown in Fig. 5b, CCCP has diminished the OM-permeabilization exerted by C\textsubscript{10}BBc\textsubscript{12}B. Importantly, NPN fluorescent signals obtained with the untreated control and with the CCCP-treated control (without lipopeptide) were practically identical, indicating that CCCP affected the proton motive force, without compromising OM integrity. These findings therefore support a scenario in which proper efflux function could boost OM-permeabilization by C\textsubscript{10}BBc\textsubscript{12}B.

**Reiterated Efflux-Propelled OM Interactions Might Boost C\textsubscript{10}BBc\textsubscript{12}B Potency.** The data collected so far (particularly observations detailed in Figs. 4 and 5) insinuate mechanistic aspects that might explain the extraordinary potentiation capacities of C\textsubscript{10}BBc\textsubscript{12}B. In our understanding, they could be rationalized to reflect a cyclic scenario consisting of three main steps, as depicted in Fig. 6.

1. **Adhesion:** As described for PMB by the self-promoted uptake theory\textsuperscript{56}, electrostatic attraction would initially lead to C\textsubscript{10}BBc\textsubscript{12}B adherence to anionic OM components (namely LPS). This interaction effectively displaces LPS-bound bivalent cations (that normally limit lateral motion by “gluing” adjacent lipid-A molecules) thereby expanding the monolayer fluidity. This effect is likely exacerbated by the lipopeptide's larger molecular volume that further increases the intermolecular distances, thereby leading to transient crack formations, through which hydrophobic molecules may silt inwards.

2. **Translocation:** These presumably short bursts of membrane disruption events could nevertheless eventually mount to the translocation of otherwise excluded hydrophobic compounds (exemplified in this work by rifampin and erythromycin) thereby facilitating their interaction with cytoplasmic targets. Concomitantly, this resultant OM-permeabilization also promotes the translocation of additional C\textsubscript{10}BBc\textsubscript{12}B molecules.

3. **Expulsion:** After translocating into the periplasm, the pentamer's fate can theoretically follow several routes, including exit the periplasm autonomously (unlikely, since it would require diffusion opposing the lipopeptide's concentration gradient), interaction with periplasmic constituents including the CM outer leaflet and/or expulsion by efflux. Likely therefore, at this stage, C\textsubscript{10}BBc\textsubscript{12}B would be simultaneously attracted to both...
the CM anionic phospholipids and to the efflux pump hydrophobic pocket\textsuperscript{57–59}. Here too, the lipopeptide's moderate hydrophobicity might serve as a key determinant for the subsequent outcome, as it was shown that hydrophobic analogs (such as C\textsubscript{10}K\textsubscript{2}K\textsubscript{2}) might escape extrusion by efflux pumps via strong interactions and deep insertion within the CM\textsuperscript{60,61}, where they would instigate damages to various extents\textsuperscript{24,29}. In this respect, the pentamer's hydrophobicity appears to be sufficiently low so as to restrict itself to a superficial CM-adherence at most, which would limit its escape from efflux (or at least prolong its unbound state in the periplasm, which increases its chances for expulsion). Notably, the intact TMP sustains the pump's ability to expel the pentamer, which now would be able to re-adhere to the OM and repeat the cycle all over again.

To our knowledge, the finding of an adjuvant whose activity is enhanced—rather than repressed—after efflux, is hitherto unreported. One might ask, at this point: If the K-, O and B-based analogs are efflux substrates (as implied by MIC data, Table 2), how come only C\textsubscript{10}O\textsubscript{2}C\textsubscript{12}B is positively affected by efflux? A plausible reason is suggested by comparing prior studies with current data indicating that C\textsubscript{10}O\textsubscript{2}C\textsubscript{12}B may lack enough binding affinity to induce even partial CM-depolarization (Fig. 4b3) whereas using the very same assay tools, C\textsubscript{10}O\textsubscript{2}C\textsubscript{12}O and C\textsubscript{10}K\textsubscript{2}K\textsubscript{2}K were found to interact with the CM to the point of inducing its depolarization\textsuperscript{52,29}. Based on these observations, one might additionally speculate that transition from K to B has led to a gradual gain in OM-interacting capacity (associated with bacterial sensitization potency) while gradually losing CM-interacting capacity (associated with growth-inhibitory potency).

Moreover, beyond re-introduction to its site of action (i.e., the OM) the pentamer's expulsion might bear another logical consequence, which is to mitigate the pumps ability to extrude other substrates (erythromycin for example). While susceptibility to efflux is \textit{a priori} considered as inherently disadvantageous for a drug's potency, this specific case suggests otherwise. Indeed, many antimicrobial peptides act (solely or partly) by interacting with the CM or the cytoplasm, whereas C\textsubscript{10}O\textsubscript{2}C\textsubscript{12}B seems to act exclusively on the OM—therefore its efflux actually brings the pentamer closer to its target rather than distancing it. This notion, while maybe counterintuitive, suggests that adjuvants acting as OM-permeabilizers may potentially benefit from their own efflux by effectively hijacking this resistance mechanism. In other words, susceptibility to efflux might be henceforth regarded as desirable rather than detrimental for such adjuvants, for instance during drug-screening processes or repurposing schemes.

In conclusion, this study presented several novel intriguing findings supporting the unanticipated notion that antibiotic adjuvants targeting the outer-membrane may benefit from their susceptibility to efflux and may achieve unprecedented capacities for potentiating antibiotics. The data also provide evidence supporting a novel efflux-hijacking scenario that enhances the adjuvant's potency.

Moreover, compared with its close analog C\textsubscript{10}O\textsubscript{2}C\textsubscript{12}O, administration of C\textsubscript{10}O\textsubscript{2}C\textsubscript{12}B to mice was both better tolerated and enabled a higher degree of protection from lethal sepsis. These remarkable feats were realized despite a lower circulating concentration, a drawback overcome thanks to its higher capacity for sensitizing GNB to hydrophobic antibiotics.

**Methods**

**Synthesis.** Lipopeptides were synthesized in-house following standard solid-phase peptide synthesis (SPPS)\textsuperscript{63} procedures, employing 9-fluorenylmethyloxycarbonyl (Fmoc) chemistry\textsuperscript{64} (Applied Biosystems 433A Peptide Synthesizer; Foster City, CA, USA). Linear analogs were synthesized on ring-amide 4-methylbenzhydrylamine (MBHA) resins, followed by cleavage and removal of BOC protecting groups using 95% trifluoroacetic acid:DCM:methanol (5:4:1). Head-to-tail cyclization took place in solution under high dilution conditions, followed by removal of BOC protecting groups as previously described. Lipopeptides were then purified to > 95% homogeneity by reverse-phase high performance liquid chromatography using a C\textsubscript{18} column, employing an increasing linear gradient (1%/min) of acetonitrile in Milli-Q water. For synthesis of cyclic analogs, linear lipopeptides were first synthesized on 2-CT resins, where the N-terminal acyl was substituted with an aminated analog. These lipopeptides were cleaved from the resin using acetic acid/DCM:methanol (3:4:1). Head-to-tail cyclization took place in solution under high dilution conditions, followed by removal of BOC protecting groups as previously described. Lipopeptides were then purified to > 95% homogeneity by reverse-phase high performance liquid chromatography using a C\textsubscript{18} column, employing an increasing linear gradient (1%/min) of acetonitrile in Milli-Q water—both containing 0.1% trifluoroacetic acid. Peak identities were determined by electrospray-ionization mass-spectrometry. Purified lipopeptides were lyophilized and stored at −20 °C until use.

**Bacterial strains and culture conditions.** Bacteria used in this study consisted of standard American Type Culture Collection (ATCC; Biological Industries, Beit Haemek, Israel) strains, clinically isolated (CI; Sourasky Medical Center, Tel Aviv, Israel) strains, as well as isogenic mutant strains. Gram-negative bacteria included Acinetobacter baumannii ATCC 19606; Escherichia coli ATCC 25922, 35218, CFT073, CI 14384, 16327, and the isogenic pair AgI00 (wild-type) and its efflux deficient mutant AgI00a (ΔacrAB); Klebsiella pneumoniae CI 1287, 224; Pseudomonas aeruginosa ATCC 27853, CI 1278, 8634; Salmonella enterica serovar Typhimurium ATCC 14028 (wild-type) and its isogenic efflux deficient mutant ΔacrAB. Bacteria were grown in Luria–Bertani broth (LB; 5 g/L NaCl, 5 g/L yeast extract, 10 g/L tryptone; pH = 7.0) at 37 °C with shaking. Overnight cultures were diluted 10-folds into fresh growth-medium and incubated at 37 °C with shaking until reaching mid-logarithmic phase. Then, bacterial concentrations were adjusted using O.D.\textsubscript{600 nm} measurements, and bacteria were subjected to the experimental procedures listed herein.

**Cell lines and culture conditions.** Cell lines used in this study included murine RAW 264.7 macrophages and human embryonic kidney HEK-293FT cells (ATCC, Biological Industries, Beit Haemek, Israel). Cells were grown in Dulbecco's modified eagle medium (DMEM) containing 4.5 mg/L glucose, L-glutamine, sodium bicarbonate and sodium pyruvate, supplemented with 10% fetal bovine serum and pen-strep, at 37 °C and 5% CO\textsubscript{2}.
Self-assembly. Organization in solution was evaluated using static light-scattering measurements (Horiba Fluorolog-3, Jobin–Yvon; Minami Ward, Kyoto, Japan). Lipopeptides were serially twofold diluted in PBS (10 mM phosphate buffer, 154 mM NaCl; pH = 7.4), and incubated for 2 h at room-temperature. Light-scattering at a 90° angle was then recorded, while holding both excitation and emission wavelengths at 400 nm (slit width of 1 nm). The critical aggregation concentration (CAC) was extracted from the intersection of trend-lines generated for free and aggregated lipopeptide forms, on plots correlating lipopeptide concentrations and scattering intensities65.

Hemolysis. Permeabilization of red blood cells (RBC) was evaluated using absorbance measurements of extracellular hemoglobin. Serial twofold dilutions of lipopeptides were incubated with 1% hematocrit of murine RBC for 3 h at 37 °C with shaking. Samples were then centrifuged at 20,000 RCF for 5 min, and absorbance of the supernatants was measured at 450 nm (BioTek Instruments Synergy HT; Winooski, VT, USA). Percent hemolysis was then calculated by \( (A_S - A_N) / (A_P - A_N) \times 100 \); where \( A_S, A_N, \) and \( A_P \) represent the absorbance of the sample, negative control (PBS) and positive control (Milli-Q water), respectively. LC50 values were extracted from trend-lines generated for linear-regions of plots correlating lipopeptide concentrations and percent hemolysis.

Minimal inhibitory concentration (MIC). MIC was determined according to the broth microdilution method: antimicrobials were subjected to serial twofold dilutions in LB medium, and incubated with \( 5 \times 10^5 \) CFU/ml of bacteria (final volume of 200 µl) for 18–24 h at 37 °C. Then, O.D.620 nm was measured (BioTek Instruments Synergy HT; Winooski, VT, USA), and the MIC was defined as the lowest antimicrobial concentration for which no increase in O.D.620 nm was evident, compared to the untreated control.

Cytotoxicity. Lipopeptides cytotoxicity was determined using fluorescent measurements of reduced resazurin. \( 2 \times 10^4 \) cells were first cultured in 96-well plates over-night (final volume of 100 µl) at 37 °C, 5% CO2. Then, the medium was removed and cells were incubated for an additional 24 h with serial twofold dilutions of the lipopeptides in growth medium. Then, 10 µl of Alamar blue were added and plates were incubated for 3 h, after which fluorescence (excitation: 530 nm; emission: 590 nm) of the reduced resazurin was recorded (BioTek Instruments Synergy HT; Winooski, VT, USA).

Combination with antibiotics. Lipopeptides and antibiotics combinations were assessed using a modified version of the checkerboard titration method: Antibiotics MIC was determined in LB medium containing sub-inhibitory lipopeptide concentrations. The sensitization factor (SF)66 was then calculated by \( SF = MIC_\alpha / MIC_C \), where \( MIC_\alpha \) is the antibiotic MIC in absence of an adjuvant and \( MIC_C \) is the antibiotic MIC in presence of an adjuvant, for a specified adjuvant concentration. Combinations were classified as synergistic when antibiotics MIC were decreased by >4 folds. Alternatively, the fractional inhibitory concentration index (FICI) was calculated by \( FICI = MIC_{C1}/MIC_{A1} + MIC_{C2}/MIC_{A2} \), where \( MIC_{C1} \) and \( MIC_{C2} \) respectively specify the antibiotic and the adjuvant MIC values upon their combination, whereas \( MIC_{A1} \) and \( MIC_{A2} \) respectively specify the antibiotic and the adjuvant MIC values when tested individually. Combinations were classified as synergistic for FICI < 0.5.

Selective pressure. The likelihood of antimicrobial-resistance development was assessed by recording MIC changes upon consecutive rounds of antimicrobials application, as in the MIC assay. Following each round, bacteria from wells corresponding to \( 1/2 \) of the resulting MIC were regrown in fresh media to mid-logarithmic phase and subjected to the same procedure for 15 consecutive days66.

Time-kill kinetics. Antimicrobials mode of action was determined according to the rate of bacterial killing. Varying concentrations of lipopeptides, antibiotics and combinations thereof were mixed in LB medium with \( 5 \times 10^8 \) CFU/ml of bacteria (final volume of 1 ml) and incubated at 37 °C with shaking. At the designated time-points aliquots were serially tenfold diluted in saline, plated on LB agar, and incubated over-night at 37 °C for enumeration.

Outer membrane permeabilization. Lipopeptides ability to permeabilize the OM was evaluated using fluorescent measurements of N-phenyl-1-naphthylamine (NPN)67. 200 µl of an over-night culture were diluted into 10 ml of fresh LB medium and incubated at 37 °C with shaking until reaching O.D.660 nm = 0.5. The culture was then centrifuged at 10,000 RCF for 3 min, the supernatant aspirated, and the pellet resuspended in equal volume of 5 mM HEPES containing 5 mM glucose (pH = 7.2). This suspension was then mixed with NPN (5 mM in acetonitrile) to reach a final NPN concentration of 10 µM. Then, 190 µl of this mixture were dispensed into black microtiter plates, mixed with 10 µl of HEPES containing serial twofold dilutions of lipopeptides and fluorescence (excitation: 360 nm; emission: 460 nm) was recorded immediately for up to 10 min at 37 °C with shaking (BioTek Instruments Synergy HT; Winooski, VT, USA). In a modified version of this assay, in which efflux was inhibited, bacteria (similarly prepared) were incubated with the lipopeptides in presence or absence of 100 µM CCCP for 5 min. Samples were then twice centrifuged at 10,000 RCF for 3 min, the supernatant aspirated, and the pellet resuspended in equal volume of 5 mM HEPES containing 20 mM glucose (pH = 7.2), so as to re-energize the efflux machinery. NPN was then added (final concentration of 10 µM) and fluorescence was recorded immediately thereafter.
Binding to lipopolysaccharide. Affinity to lipopolysaccharide (LPS) was assessed using fluorescent measurements of displaced mono-dansylated polymyxin B (DPMB)\(^{68}\). 180 µl of 5 mM HEPES containing 2 µM DPMB and 3 µg/ml LPS (from *E. coli*) were dispensed into black microtiter plates and mixed with 20 µl of HEPES containing serial twofold dilutions of lipopeptides. Plates were incubated in the dark for 1.5 h at room-temperature and fluorescence (excitation: 340 nm; emission: 485 nm) was recorded immediately thereafter (BioTek Instruments Synergy HT; Winooski, VT, USA).

Cytoplasmic membrane depolarization. Lipopeptides aptitude to depolarize the CM was evaluated using fluorescent measurements of the transmembrane potential sensitive dye 3,3′-Dipropylthiadicarbocyanine iodide (DiSC\(_3\)(5))\(^{69}\). Bacteria (O.D.\(_{600}\) nm = 0.1) were washed by centrifugation at 10,000 RCF for 5 min, supernatant aspiration, and pellet resuspension in 5 mM HEPES containing 20 mM glucose, 50 mM KCl and 0.2 mM EDTA (pH 7.2). This suspension was mixed with DiSC\(_3\)(5) (400 µM in DMSO) to reach a final DiSC\(_3\)(5) concentration of 4 µM and was incubated in the dark for 1 h at 37 °C with shaking. Then, 180 µl of this mixture were dispensed into black microtiter plates and fluorescence was recorded (excitation: 622 nm; emission: 670 nm) at 37 °C with shaking until baseline stabilization (BioTek Instruments Synergy HT; Winooski, VT, USA). Finally, 20 µl of HEPES containing serial twofold dilutions of lipopeptides were added and fluorescence was recorded for up to 30 min at the same conditions\(^{69}\).

In-vivo studies. Animal procedures were reviewed and approved by the Institutional Animal Care and Use Committee (ethics no. IL-0800519, IL-0760711, IL-0550618, IL-0320320, IL-1270821) and performed in accordance with the "Guide for the Care and Use of Laboratory Animals", published by the US National Research Council\(^{70}\). The study is reported in accordance with ARRIVE guidelines. All protocols employed 4–5-week-old male ICR mice, weighing 20–25 g (Envigo; Jerusalem, Israel). Mice were kept on a 12 h light/dark cycle and provided with food and water *ad libitum*. At the designated experimental end-points mice were euthanized by means of CO\(_2\) asphyxiation. Unless otherwise specified, all administered solutions were freshly prepared in sterile PBS immediately before administration.

Acute toxicity. The maximal tolerated dose (MTD) was determined after sub-cutaneous (S.C.) administration of lipopeptides solutions (0.2 ml). Mice (N = 3 per group) were monitored for up to 7 days and their physiological condition was scored on a scale of 1 to 6, in which a score of 1 indicates no apparent signs of distress, and a score of 6 signifies severe distress necessitating the mice immediate euthanasia.

Biodistribution. To assess lipopeptides circulating concentrations, mice (N = 3 per group) were S.C. administered with lipopeptides solutions (0.2 ml), sacrificed at specified times afterwards and their blood was asexptically collected. Blood samples were centrifuged at 6,000 RCF for 2 min, and 0.2 ml of the supernatant plasma were mixed with 0.5 ml of acetonitrile:methanol (1:1). This mixture was shaken for 30 min, and then centrifuged at 10,000 RCF for 10 min. 0.5 ml of the supernatant were diluted 2-folds with Milli-Q water and analyzed by UPLC-MS using a C\(_{18}\) column. Quantification was performed using a calibration-curve generated with similarly processed blood samples, initially spiked with known lipopeptide concentrations. Accumulation in organs (bladder and kidneys) was similarly determined, but was preceded with organs suspension in 5 ml of sterile PBS and homogenization for 2 min using a bench-top homogenizer, after which 0.5 ml of each sample were mixed with 1.5 ml of acetonitrile:methanol (1:1). Quantification was performed using a calibration-curve generated with fresh lipopeptide solutions of known concentrations prepared in Milli-Q water\(^{71}\).

Efficacy. Lipopeptides potential application in monotherapy and/or combination-therapies was explored with two infection models. In the peritonitis-sepsis model\(^{71}\), mice (n = 10 per group) were first rendered neutropenic by two intraperitoneal (I.P.) injections of 0.3 ml containing 150 mg/kg and 100 mg/kg cyclophosphamide, administered 4 days and 1 day before the experiment, respectively. Mice were then infected I.P. with 1.7 × 10\(^6\) or penic by two intraperitoneal (I.P.) injections of 0.3 ml containing 150 mg/kg and 100 mg/kg cyclophosphamide, (BioTek Instruments Synergy HT; Winooski, VT, USA).

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Statistics. Unless otherwise specified, *in-vitro* data were obtained from three independent experiments performed in duplicate. Statistical analysis was performed using one-tailed t-test, assuming equal variance. The UTI assay was analyzed using Kruskal–Wallis analysis followed by the Mann–Whitney U test with Tukey’s fence of k = 3.

The maximal tolerated dose (MTD) was determined after sub-cutaneous (S.C.) administration of lipopeptides solutions (0.2 ml). Mice (N = 3 per group) were monitored for up to 7 days and their physiological condition was scored on a scale of 1 to 6, in which a score of 1 indicates no apparent signs of distress, and a score of 6 signifies severe distress necessitating the mice immediate euthanasia.
Institutional review board statement. The study was conducted according to the guidelines of the Technion Animal Care and Use committee that approved all procedures, care, and handling of animals. Ethics approval codes: IL-0800519, IL-0760711, IL-0550618, IL-0320320, IL-1270821. The study is reported in accordance with ARRIVE guidelines. Mice were obtained from Envigo; Jerusalem, Israel.

Data availability
All data generated or analyzed during this study are included in this published article.

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Author contributions
O.M. designed experiments, synthesized reagents, performed research, analyzed data, and wrote the manuscript; E.Z. synthesized reagents (DPMB), participated in in-vivo studies, analyzed data, and wrote the manuscript; A.M. conceived and supervised the study, analyzed data, and wrote the manuscript. All authors read and approved the final version of the manuscript.

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Competing interests
The authors declare no competing interests.

Additional information
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