Sensitization of Gram-negative bacteria to rifampin and OAK combinations

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The spread of multidrug resistance (MDR) among pathogenic bacteria continues to challenge modern medicine. In particular, shortage in new antibiotics for treating Gram-negative bacteria (GNB) infections is disquieting, stressing a growing urgency for alternative solutions1-4. Oligo-acyl-lysyls (OAKs) represent a potentially useful approach for developing safe, efficient and economically viable antimicrobial small molecules to meet the global and ever increasing MDR-associated threats5-7. Previously, the prototypical OAK sequence acyl-lysyl-lysyl-aminocacyl-lysyl proved to generate OAK derivatives targeting Gram-positive bacteria (GPB)8,9 and more recently, prompted inefficient antibiotics to improve activity against GNB10. Superficial OAK interactions with both the cytoplasmic and outer membrane (CM and OM, respectively, Fig. 1a) were implicated in this chemo-sensitization property, causing naturally resistant bacteria to become sensitive to formerly inactive antibiotics10 or to overcome acquired resistance mechanisms11.

Here, we challenged these putative OAK-membrane interactions by testing their capacity to mediate uptake of otherwise excluded antibiotics, such as rifampin12. Various similar studies were conducted using a panoply of chemicals entities13-16 ranging from cation-chelators to host defense peptides, highlighting the potential usefulness of these combinations that may provide future therapeutic alternatives to GNB infections, albeit not always without dispute17. Rifampin and penicillin are hydrophobic antibiotics, respectively targeting cytoplasmic and periplasmic bacterial components. As inactivity of penicillin on GNB is often due to β-lactam processing enzymes, its periplasmic accumulation is not expected to benefit from OAK's action, unlike rifampin whose inefficacy over GNB usually results from its natural low OM-permeability12,18, although other mechanisms were reported to confer additional resistance to rifampin, including mutations in RNA polymerase gene, rpoB19,20. Note that rifampin (member of the rifamycin family) is a highly effective anti-mycobacterial drug, even though the mycobacterial outer membrane hinders, to some extent, its entry. Only in several mycobacteria species this reduced permeability results in variable degrees of resistance21,22.

**Results**

Minimal inhibitory concentration (MIC) assays demonstrated that OAKs and antibiotics were individually unable to efficiently inhibit growth of GNB, whereas rifampin became extremely potent in presence of sub-MIC of the reference OAK C[12(0)](v7)X (Structure shown in Fig. 1b). Figure 2a illustrates the case of *E. coli* while Supplementary Fig. S1 (Supplementary Results online) summarizes data obtained with three additional species representing medically relevant GNB, collectively revealing the extent to which rifampin's MIC was reduced in presence of sub-MIC OAK. Similar data obtained with erythromycin19 is included for comparison. Remarkably, sub-MIC levels of rifampin or erythromycin have also potentiated the OAK's antibacterial activity against *E. coli*, reducing the MIC from >50 down to <1 μg/ml (Fig. 2b). Supplementary Fig. S2 shows that similar outcome is obtained with additional bacterial strains and species, thereby confirming the occurrence of a mutually synergistic process in GNB. Figure 2cd suggests that sub-MIC OAK and rifampin might induce membrane depolarization at micromolar concentrations, while underlining significant differences in terms of dose and kinetics (mechanistic relevance is discussed below).
Table 1 summarizes the biophysical attributes of OAK derivatives, including published data concerning two derivatives whose N-terminal dodecenoyl was replaced with a saturated version (C12\(\text{v}_7\)) or deleted\(^6,9,23\), indicating that the hydrophobic analog undergoes self-assembly at lower concentrations and is more efficient in hemolysis and antibacterial activities. As evident from the middle part of Table 1, this analog was as potent as C12\(\text{v}_7\)X in sensitizing E. coli to rifampin. These properties were diminished in absence of the N-terminal acyl, as expected for excessively hydrophilic OAKs\(^5,6\), raising the question of how the OAK would behave if the N-terminal acyl was replaced with analogs having intermediate hydrophobicity values. We therefore produced two new derivatives, C10X and C8X, which revealed rather intriguing biological profiles: they were less active in hemolytic and antibacterial tests but they sensitized bacteria to rifampin, nonetheless. As shown in Supplementary Table S1, sensitization persisted against different species, thereby establishing C10X as a potent sensitizer of GNB to rifampin since it reduced active concentrations from high micromolar- to low nanomolar-range. Note that C10X also displayed reciprocal synergism with rifampin (Supplementary Fig. S3) as observed for the reference OAK, whereas these OAKs were unable to sensitize the tested bacteria to penicillin.

Table 1 | Structure-activity study highlighting biophysical attributes of N-terminal OAK derivatives

| Sequence | %H^+Q^+ | CAC\(^d\) (\(\mu\)M) | LC50\(^c\) (\(\mu\)M) | MIC of rifampin in presence of OAK (\(\mu\)g/ml) | SF | MIC of penicillin G in presence of OAK (\(\mu\)g/ml) | SF |
|---------|----------|----------------|----------------|-----------------|---|----------------|---|
| C12\(\text{v}_7\)X | 49 | 40 | 100 | 8 | 50 | 8–16 | 8–16 | ≤0.06 | 0.004 | 2000–4000 | >512 | >512 | >512 | >512 | 1 |
| C12X | 51 | 12.5 | 29 | 9 | 16 | 8–16 | 8–16 | ≤0.06 | 0.002 | 4000–8000 | >512 | >512 | >512 | >512 | 1 |
| C10\(\text{v}_7\) | 46 | 3 | ≤100 | >100 | >50 | 8–16 | 4 | 0.02 | 0.004 | 2000–4000 | >512 | >512 | >512 | >512 | 1 |
| C10X | 41 | 3 | ≥100 | >100 | >50 | 8–16 | 2 | 0.5 | 0.06 | 133–267 | >512 | >512 | >512 | >512 | 1 |
| C8X | 27 | 4 | ≥100 | >100 | >50 | 8–16 | 8–16 | 8 | 1–2 |

Where: X = lysyl-lysyl-aminododecanoyl-lysyl-amide;\n\(^a\)Hydrophobicity measure, defined as % acetonitrile eluent in C18 HPLC column;\n\(^b\)Molecular charge in physiological conditions;\n\(^c\)Critical aggregation concentration in PBS;\n\(^d\)OAK concentration that induced 50% hemolysis after 3 h incubation in PBS 37°C;\n\(^e\)Treated against E. coli strain ML-35p. SF, sensitization factor defined as the ratio of the MIC in absence of OAK to that in presence of 5 \(\mu\)g/ml of OAK. Bold lines indicate published data.
allow detectable amounts to reach the blood compartment, unlike rifampin that rapidly accumulated up to 13.26 ± 2.25 µg/ml (nearly as reported)\textsuperscript{24,25}. In contrast, subcutaneous OAKs administrations (MTD > 20 mg/Kg) were both well tolerated and enabled plasma levels of around 5 µg/ml when dosed at 12.5 mg/Kg. Consequently, to promote optimal/simultaneous blood concentrations during efficacy tests, we opted for subcutaneous OAKs administration- one hour after rifampin’s oral administration. To test the drugs ability to affect disease course systemically, we used neutropenic mice to nullify neutrophil-mediated immune contributions in resolving bacterial infections\textsuperscript{26}. Inoculation with nullify neutrophil-mediated immune contributions in resolving bac-to affect disease course systemically, we used neutropenic mice to

Figure 2 | Evidence for sensitization of E. coli to antibiotics and OAKs combinations. Panels (a,b) respectively show the MIC evolution when bacteria were treated with rifampin (circles) or erythromycin (triangles) in presence of the specified sub-MIC OAK and vice versa. OAK MIC against this strain is >50 µg/ml (panel a). For Panel b, the standalone antibiotic MIC is 16 and 512 µg/ml respectively for rifampin and erythromycin. Panels (c,d) show the time-dependence of membrane depolarization and the bacterial viability during the experiment, respectively. Symbols: squares, untreated control; circles, rifampin (4 µg/ml); open triangles, OAK (1.3 µg/ml); solid triangles, OAK + rifampin. Panels (e,f) show a pharmacokinetic study using LC/MS analysis to monitor the mean plasma concentrations of C12(0/7)X (triangles) and rifampin (inverted triangles) after oral administration of 20 mg/Kg each, calculated based on their respective calibration curves (e) and the same analysis for subcutaneous administrations of C12(0/7)X (triangles) and C10X (circles) at 12.5 mg/Kg each (f). Error bars = s.d. Panels (g,h) show systemic efficacy in neutropenic mice after monotherapy (g) and combination therapy (h). Symbols: open squares, vehicle treated control; triangles, C12(0/7)X alone; circles, C10X alone; inverted triangles, rifampin alone; solid triangles, combined treatment of C12(0/7)X and rifampin; solid circles, combined treatment of C10X and rifampin. The data represent averages from two independent experiments performed with 10 mice/group (standard deviations were <10%).

Discussion
Rifampin is often used in combination therapy for treating Mycobacterium infections, including tuberculosis and leprosy\textsuperscript{32,33}. At the concentrations used (which were higher than the MIC for poly- myxin), the compounds did not premeabilize the CM, except for C12X that incidentally, was also more potent than its unsaturated analog (Fig. 3b).

To verify whether the peptides OM permeabilization ability involved similar binding affinities to lipopolysaccharides (LPS), we used the dansyl-polymyxin assay\textsuperscript{31}. Supplementary Fig. S4 summarizes the dose-dependent kinetics obtained with LPS from two GNB (E. coli and P. aeruginosa). Figure 3c shows that OAKs affinity increased with increasing hydrophobicity (Table 1) but the OAKs exhibited a significantly lower affinity than polymyxin B. For instance, about 10-fold difference was observed with C10X (P < 0.004). Finally, time-kill kinetics obtained at synergistic concentra-
tions (Fig. 3d,e) with OAK alone and in presence of the biocidal rifampin or the biostatic erythromycin, indicated that after a brief delay, normal bacterial growth has resumed upon exposure to sub-MIC C12(0/7)X. This delay was not observable with C10X (Supplementary Fig. S5), indicating that this more hydrophilic analog, just like erythromycin or rifampin, did not affect the normal growth rates, only their combination with an OAK did. Moreover, synergism vanished if rifampin and OAKs were not added simultaneously: For example, bacterial survival was nearly normal if rifam- pin addition was delayed by 15 min after the OAKs (Fig. 3f) and vice versa (C10X is shown in Supplementary Fig. S5).

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Rifampin is also active on Gram-positive cocci but not on enterobacteriae - a large family of GNB that includes common pathogens, such as E. coli, Klebsiella, Salmonella or Pseudomonas. Clearly, the opportunity to expand rifampin’s activity spectrum and/or to reduce its adverse effects (e.g., hepatotoxicity), would be welcomed34,35. The current work revealed that OAKs have the ability to reduce rifampin’s active concentrations by several orders of magnitude. Moreover, this study represents the first report (to our knowledge) of a reciprocal chemo-sensitization (mutual synergism) process of such a magnitude, imparting potency upon two molecular species acting by distinct mechanisms, hence the efforts invested towards understanding the underlying molecular basis.

We first focused on obtaining evidence susceptible to clarify the individual roles in the synergistic pair. The activities exhibited by C12(X) alone, might partly explain the enhanced potency observed upon combination. However, being virtually devoid of antibacterial activity on its own, chemo-sensitization activity associated with C10(X) is a priori unexpected; thus, synergism is achievable with little regard to individual antibacterial capacity. In that sense, the new analogs represent valuable tools for deciphering the mechanism underlying synergy by assigning individual responsibilities of each reactant. The fact that C10(X) was more potent than C12(X) alone and in combinations with rifampin (d) and erythromycin (e). Symbols: open squares, untreated control; open triangles, C12(X); open circles, rifampin or erythromycin (0.004 or 0.03 μg/ml, respectively); solid triangles, C12(X) + antibiotic. (f) Effect of delayed exposure to rifampin or OAK; Bacteria (K. pneumoniae CI 1287) were exposed to both C12(X) (5 μg/ml) and rifampin (0.008 μg/ml) without delay (t = 0) and after delayed exposure to rifampin (white bars) or OAK (striped bars) by 15 or 30 min in LB culture medium. UC; untreated control. Dashed line indicates the inoculum. Error bars = s.d.

The mechanism enabling antibiotics to potentiate the OAKs is less understood. It seems to result from precursor damages exerted by micromolar sub-MIC antibiotics that somehow produce OAK-hypersusceptible bacteria. This may occur with rifampin by competing with OAKs for membrane interactions due to its hydrophobic/cationic characters, as suggested by the sigmoidal shape of the depolarization curve which was observed only at micromolar combinations of OAK-rifampin. The sigmoidal shape disappeared at nanomolar levels of rifampin (data not shown) or upon replacing rifampin with micromolar levels of the porin-gated erythromycin10. Notwithstanding, reciprocal synergism might also occur between erythromycin and OAKs, should these efflux substrates16,18 hamper each other’s extrusion by populating the binding pockets of resistance-nodulation-division (RND) pumps16. Future studies might clarify this issue.

Of interest is the comparison between OAKs and polymyxins, since these cationic lipopeptides permeabilize the OM with similar potencies (Fig. 3a) despite major differences in chemo-physical attributes (Fig. 1c). OAK derivatives reduced rifampin’s MIC by 4000 folds at 5 μg/ml (8000-fold at 10 μg/ml, data not shown). Under similar conditions, polymyxin B nonapeptide derivatives have reportedly reduced rifampin’s MIC by 85–750 and 250–500 folds, respect-
ively against *E. coli* and *K. pneumoniae* strains. The large difference in sensitization factors suggests that it is not solely due to OM permeabilization, perhaps it is also related to additional factors, including the way each lipopeptide affects the CM structure and function. In this regard, polymyxins maybe rather handicapped because of their higher affinity for LPS.

**Membrane depolarization.** Measurements were performed with 3,3′-dipropylthiadicarbocyanine iodide (DiSC_3(5)), a lipophilic potentiometric dye. Cells were treated with 2 mM EDTA prior to addition of DiSC_3(5) stock solution (final concentration 0.4 μM) and quantifying at RT for 20–30 min. KCi was then added (final concentration 100 mM), the suspension incubated overnight (4 C), 180 μl aliquots were placed in black 96-well plate for 30 min to allow stabilization of the dye signal, then 20 μl of stock solutions of OAK, rifampin, or their combination, were added to obtain the desired final concentrations. Membrane depolarization was monitored by measuring excitation/emission at 620/680 nm, respectively, under shaking at 37°C (BioTek Synergy HT Microplate Reader). Data were obtained from at least two independent experiments performed in duplicate.

**Outer and inner membrane permeabilization.** The mutant *E. coli* ML-35p was used to monitor the ability of the OAK to perforate/perturb the inner and outer membranes. The assay was performed in sterile 96-well plates in a final volume of 200 μl. Bacteria were grown overnight in TSB, washed 3 times in sodium phosphate buffer (50 mM, pH 7.4) and diluted to 10^7 CFU/ml in SPB containing 3% TSB. Aliquots of this suspension (100 μl) were added to 100 μl of SPB containing a test compound and either ortho-nitrophenyl-β-galactoside (ONPG, 2.5 μM) or nitrocefin (25 μM). Hydrolysis of ONPG and nitrocefin was monitored by measuring absorbance at 420 or 486 nm, respectively, at various time intervals, with shaking at 37°C (BioTek Synergy HT Microplate Reader). Data were obtained from at least two independent experiments performed in duplicate.

**Dansyl-polymyxin binding assay.** The affinity of OAKs to LPS from *E. coli* or from *P. aeruginosa* was studied by displacement of bound dansyl-polymyxin, as described. Briefly, Polymyxin B sulphate was dansylated using dansyl chloride followed by monoreduction using DNTB and purification by HPLC. The mixture was incubated in black 96-well plates containing 180 μl of HEPES (5 mM, pH = 7.2), 3 μg/ml LPS, 2 μM mono-DPMB and 20 μl of OAK or polymyxin solution in the desired concentrations (0.6–10 μM). The mixtures were incubated for 1.5 h (RT). The displacement of DPMB was measured as the corresponding decrease in fluorescence (excitation/emission at 340/485 nm) using a BioTek Synergy HT Microplate Reader. Data were obtained from at least two independent experiments performed in duplicate.

**Hemolytic assay.** Hemolytic activity was assessed using fresh Human blood collected into sodium citrate containing test tubes, rinsed 3 times in PBS (centrifuged at 200 × g for 2 min). Packed cells were re-suspended in PBS resulting in 1% hematocrit. 50 μl of this suspension were added to Eppendorf test tubes containing 200 μl of test compound solutions (in serial twofold dilutions), PBS alone (for base-line value), or PBS alone containing 100 μl of LPS, 20 μl of OAK or polymyxin in the desired concentrations. Hemolytic activity was assessed as function of hemoglobin leakage by measuring absorbance of 200 μl of supernatant at 450 nm.

**In vivo studies.** Animal studies were performed using male ICR mice (weight range, 23 ± 2 g) obtained from Harlan Laboratories (Rehovot, Israel). Procedures, care and handling of animals were reviewed and approved by Technion Animal Care and Use Committee.

**Toxicity.** Maximal tolerated dose (MTD) was determined after single-dose subcutaneous (SC) or oral (gavage) administration of OAKs at specified doses using 2 or 10 mice/compound. Animals were inspected for adverse effects during 6 h by recording motor activity, piloreaction, redness in ear lobes, cyanosis, protruding eyeballs, slow or labored breathing, loss of response in the rear leg and convulsions. Mortality was monitored during 7 days thereafter.

**Pharmacokinetic study.** Drugs blood concentrations were determined by LC-MS using calibrated curves essentially as described. Briefly, OAK and/or rifampin were given by SC or oral administration. At specified time intervals mice were euthanized (CO2 asphyxiation) and blood samples collected from venous pouch (8 of mice/time point). For analysis, samples were centrifuged using 5 min, 6000 × g. 200 μl plasma were mixed with 0.5 ml extraction buffer (50% acetonitrile (ACN): 50% methanol) incubated (30 min on plate shaker at 200 rpm, RT), centrifuged (2 min, 14000 × g) and 300 μl supernatant diluted twofold in DDW and analyzed by LC-MS (5 μl injected to Waters Xevo G2 ToF/AQUITY UPLC H-Class system). Flow rate, 0.5 μl/min. Run time, 5 min. Mobile phase, ACN/DDW combination containing 0.1% formic acid using an ACQUITY UPLC BEH column (C8, 1.7 μm) and eluted with a 0–90% ACN gradient. Quantification was by MS detection in positive ionization mode using an identical procedure that was performed in mouse whole blood in order to establish standard calibration curves.

**Efficacy.** Mice were rendered neutropenic by intraperitoneal (IP) injection of cyclophosphamide (150 and 100 mg/kg on days 0 and 3, respectively). The procedure was confirmed to result in severe neutropenia by day 4, at which time infection was induced. The peritonitis-sepsis model was used whereby infection was obtained after IP injection of a logarithmic-phase culture of *E. coli* (*K. pneumoniae* × 10^7 CFU/mice in 0.3 ml PBS). Immediately thereafter, mice were treated orally with rifampin (0.25 mg DDW containing 0.45 mg/mouse), whereas the OAKs were administered subcutaneously, an hour post-inoculation (0.3 ml PBS containing 0.3 mg/mouse). Mice survival was monitored for up to 7 days post-treatment.
Survival data were obtained from 2 independent experiments (n = 10 mice/group/experiment). Statistical analysis was performed using a paired t test were 2 = 0.05.

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Author contributions
J.J. synthesized reagents, performed research, analyzed data, wrote the paper; F.Z. performed research (in-vivo blood concentrations), analyzed data; G.K. performed research (in-vivo toxicity and efficacy experiments), analyzed data; K.G. performed research (part of the antibacterial assay); A.M. designed the experiments, analyzed data, wrote the paper. All authors discussed the results and commented on the manuscript.

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