Mutual inhibition through hybrid oligomer formation of daptomycin and the semisynthetic lipopeptide antibiotic CB-182,462

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Abstract

Daptomycin is a clinically important lipopeptide antibiotic that kills Gram-positive bacteria through membrane depolarization. Its activity requires calcium and the presence of phosphatidylglycerol in the target membrane. Calcium and phosphatidylglycerol also promote the formation of daptomycin oligomers, which have been assumed but not proven to be required for the bactericidal effect. Daptomycin shares substantial structural similarity with another lipopeptide antibiotic, A54145; the two have identical amino acid residues in 5 out of 13 positions and similar ones in 4 more positions. We here examined whether these conserved residues are sufficient for oligomer formation. To this end, we used fluorescence energy transfer and excimer fluorescence to detect hybrid oligomers of daptomycin and CB-182,462, a semisynthetic derivative of A54145. Mixtures of the two compounds indeed produced hybrid oligomers, but at the same time displayed a significantly less than additive antibacterial activity against *Bacillus subtilis*. The existence of functionally impaired oligomers indicates that oligomer formation is indeed important for antibacterial function. However, it also shows that oligomerization is not sufficient; once formed, the oligomers must take another step in order to acquire antibacterial activity. Thus, the amino acid residues shared between daptomycin and CB-182,462 suffice for formation of the oligomer, but not for its subsequent activation.

Introduction

The lipopeptide antibiotic daptomycin is used clinically against infections by Gram-positive bacteria, including strains of staphylococci and enterococci that are resistant to other antibiotics [1-3]. It binds to and causes depolarization of the bacterial cytoplasmic membrane, which is considered to be the mechanism of its rapid bactericidal action [4, 5]. Electron microscopy of daptomycin-exposed cells does not reveal any discontinuity of or morphological change to the lipid bilayer [6]. Both this observation and the selective nature of the membrane permeability defect [7] support the notion that daptomycin forms small, discrete membrane lesions. It was proposed earlier that these discrete lesions are formed by oligomeric assemblies of daptomycin molecules [5]. However, experimental evidence of oligomer formation has been obtained only recently [8, 9], and direct proof of their involvement in membrane permeabilization is still lacking.

Daptomycin consists of 13 amino acids, including several nonstandard ones [10]. The ten C-terminal residues form a ring that is closed by an ester bond (Figure 1). The exocyclic N-terminal tryptophan carries a fatty acyl residue, which in the clinical compound is decanoic acid, although the length of this acyl tail is subject to variation in the natural compound. The same architecture occurs in the related antibiotic A54145 [11], and the two molecules also share significant sequence homology, with five identical residues and four more similar ones. We reasoned that testing the two molecules for their ability to form hybrid oligomers should provide information on the contributions of the conserved and the non-conserved residues, respectively, to the oligomerization process.

The experiments were performed with various fluorescent derivatives of daptomycin and of CB-182,462, a semisynthetic derivative of A54145 [12]. Our results readily demonstrate the formation of such hybrid oligomers, both on model membranes and on bacterial cells; therefore, the amino acid residues conserved between daptomycin and CB-182,462 are sufficient for oligomerization. Remarkably, however, the hybrid oligomers exhibit reduced antibacterial activity. The observation of oligomers with impaired antibacterial activity shows, on the one hand, that the oligomer is indeed involved in the antibacterial effect. On the other hand, it indicates that oligomer formation as such is not sufficient for activity. The oligomer must undergo some additional event, such as for example cooperative membrane insertion, in order to acquire bactericidal activity.

Materials and Methods

Synthesis and purification of NBD-CB-182,462 and of perylene-daptomycin. Unlabeled CB-182,462 was kindly provided by Jared Silverman (Cubist Pharmaceuticals Inc., Lexington, MA, USA). Reaction of CB-182,462 with NBD-Cl (4-Chloro,7-nitro-2,1,3-benzoxadiazole; Fluka) and HPLC purification were performed as described before for NBD-daptomycin [8]. Molecular weight and homogeneity were confirmed by mass spectrometry on a Micromass Q-TOF Ultima GLOBAL mass spectrometer. The synthesis of perylene-daptomycin has been described as well [9].

Preparation of PC/PG large unilamellar vesicles (LUV). 1,2-Dimyristoyl-sn-glycero-3-phosphocholine (DMPC) and 1,2-dimyristoyl-sn-glycero-3-[phospho-rac-(1-glycerol)] (DMPG) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Large unilamellar vesicles were prepared by polycarbonate membrane extrusion as described [8]. HEPES buffer (20 mM, pH 7.4) containing 150 mM sodium chloride was used throughout.

Steady-state fluorescence measurements on PC/PG liposomes and on bacterial cells. Fluorescence emission spectra were acquired using a PTI QuantaMaster spectrofluorometer. Excitation wavelengths were 282 nm for tryptophan, 430 nm for perylene, and 465 nm for directly excited NBD fluorescence. Excitation and emission band passes were typically 2 nm but were occasionally adjusted in order to increase or reduce sensitivity. Unlabeled daptomycin and CB-182,462 or NBD-labeled CB-182,462 and perylene-labeled daptomycin were applied in the quantities indicated in the Results section to the liposomes (200 or 250 μM total lipid) in HEPES/NaCl with calcium (5 mM). Samples were incubated for 5 or 10 minutes before acquisition of emission spectra, and in some instances measured repeatedly after longer time intervals as indicated.

For fluorescence measurements on bacteria, cells from a fresh overnight culture of *Bacillus subtilis* ATCC 1046 were harvested and repeatedly washed with HEPES/NaCl buffer by centrifugation in a table-top centrifuge. The pelleted cells were resuspended in approximately 20 volumes of buffer. Of the resulting cell suspension, 150 μl were incubated in the presence of $CaCl_2$ (5 mM) for 10 minutes with the amounts of labeled or unlabeled daptomycin and CB-182,462 stated in the Results section. The cells were again washed repeatedly by centrifugation with HEPES/NaCl/$CaCl_2$, resuspended in the same buffer and measured as described above for liposome samples.

Antibacterial activity of daptomycin and CB-182,462. Overnight cultures of *Bacillus subtilis* ATCC 1046 were grown at 37 °C in LB broth. Daptomycin and CB-182,462, alone or combined in various

ratios, were serially diluted in LB broth supplemented with 5 mM $CaCl_2$. Each serial dilution was inoculated with 1% by volume of the *Bacillus subtilis* overnight culture, and the culture tubes were incubated with shaking at 37°C overnight. Growth was evaluated visually by turbidity. Growth and sterility controls were included in each experiment.

Results

Oligomerization of CB-182,462 on liposome membranes. We have previously shown that daptomycin forms oligomers on model membranes and bacterial cells [8, 9, 13]. While the similarity between the two compounds suggests that CB-182,462 should do the same, this has not been directly demonstrated. Therefore, a fluorescently labeled derivative was prepared with nitrobenzoxadiazole (NBD) attached to its unique free amino group (Figure 1). While the absorption of NBD is highest around 470 nm, there is a smaller absorption peak around 340 nm [14] that overlaps the fluorescence emission spectrum of tryptophan. This allows the detection of FRET between the tryptophan of unlabeled CB-182,462 and the NBD-labeled derivative. In the experiment shown in Figure 2A, the two species were mixed before application to PC/PG liposomes in the presence of calcium. The tryptophan fluorescence of the unlabeled molecules is very strongly reduced by FRET, which indicates a close association of the two species. FRET is still observed, but to a lesser extent, when the two species are applied sequentially. Under these conditions, the labeled molecules and the unlabeled ones should undergo oligomerization separately, and therefore FRET will only occur between, but not within oligomers, which accounts for the reduced extent of FRET.

NBD exhibits concentration-dependent self-quenching [15]. The local concentration of NBD is higher, and therefore quenching is more pronounced, in pure NBD-CB-182,462 oligomers than in hybrid oligomers formed from a mixture of the NBD-labeled compound and an excess of the unlabeled one (Figure 2B). The results from both FRET and self-quenching experiments are completely analogous to our previous observations with daptomycin [8].

Formation of daptomycin/CB-182,462 hybrid oligomers on liposomes. The intrinsic fluorescence of tryptophan also overlaps the absorption spectrum of kynurenine, which in daptomycin causes virtually complete FRET from tryptophan to kynurenine [16]. FRET between tryptophan and kynurenine can also be used to detect formation of hybrid CB-182,462/daptomycin oligomers. When a premixed sample of the two is applied to PC/PG liposomes, the tryptophan fluorescence of CB-182,462 is largely

suppressed by FRET (Figure 3A). Again, sequential application reduces FRET, which is consistent with formation of separate oligomers.

While daptomycin oligomers are largely stable on a time scale of one or a few hours [8], it seems possible that hybrid oligomers of CB-182,462 might be less stable. In the experiment shown in Figure 3B, a sample prepared by sequential application of the two antibiotics was incubated, and the fluorescence emission measured repeatedly after various time intervals. While the extent of FRET increases slightly with time, it remains much lower than that observed with a premixed sample after 60 minutes. This suggests that the rate of subunit exchange between oligomers is low, and oligomers are largely stable on the time scale of the experiment.

Formation of hybrid oligomers on bacterial cells. While PC/PG membranes are a useful model to observe the activity of daptomycin and CB-182,462, the lipid composition of bacterial membranes is different, and it is therefore pertinent to examine the formation of hybrid oligomers on bacterial cells as well. Due to the abundance of tryptophan in bacterial proteins, the intrinsic tryptophan fluorescence of CB-182,462 could not be used in these experiments; however, two alternative approaches allowed for the detection of hybrid oligomers on *Bacillus subtilis* cells. Firstly, the concentration-dependent self-quenching of NBD in NBD-labeled CB-182,462 can be inhibited not only by unlabeled CB-182,462 but also using unlabeled daptomycin (Figure 4A). Secondly, the formation of perylene excimers in oligomers of perylene-labeled daptomycin [9] is suppressed by both unlabeled daptomycin and unlabeled CB-182,462 (Figure 4B). Between the two experiments, it is evident that both the labeled and the unlabeled forms of daptomycin and CB-182,462 are capable of hybrid oligomer formation on bacterial cell membranes.

Antibacterial activity of daptomycin/CB-182,462 mixtures. If two different drugs act independently but share the same target and mode of action, their mixtures will display additive effectiveness. This can be detected in an isobologram [17]. In such a graph, the concentrations of the two drugs in question are varied independently, and lines are drawn to connect equieffective dosages of various combinations. If the two drugs in question indeed behave additively, the equieffective dosages of all mixtures will fall on a straight line that connects the equieffective dosages of the two pure drugs.

Figure 5 shows an isobologram for the minimum inhibitory concentrations (MICs) of daptomycin and CB-182,462. It is clear that the MICs deviate from such an ideal straight line. With the mixtures, greater than additive dosages are required to reach the MIC, indicating that the two drugs inhibit one

another. The apex of mutual inhibition occurs at equimolar ratio. While any binary mixture should produce a distribution of oligomers that vary with respect to both the fractions of the two antibiotics incorporated and the positions within the oligomer occupied by each, an equimolar ratio should maximize the extent of scrambling and minimize the residual fractions of homogeneous oligomers. The observation that mutual inhibition is strongest at this ratio therefore supports the notion that hybrid oligomers have impaired antibacterial activity.

Discussion

In previous studies, it was shown that daptomycin forms oligomers on PG-containing membranes [8, 9], and that the oligomers have a stoichiometry of approximately 6–7 subunits [13]. A role of membrane-associated oligomers in the antibacterial activity of daptomycin has widely been assumed and accepted as plausible [5]; however, direct experimental evidence has been lacking. The mutual inhibition between daptomycin and the semisynthetic homologous lipopeptide CB-182,462 reported in this study constitutes the first such evidence; for if each individual monomer contributed independently and proportionally to the antibacterial action, combinations of the two antibiotics should display additive activity. In addition, however, the reduced antibacterial activity of hybrid oligomers also shows that oligomer formation as such is not sufficient for the antibacterial effect. One must therefore assume that the oligomer, once formed, has to undergo an additional step to acquire bactericidal activity. A similar functional sequence exists with many pore-forming protein toxins, such as for example *Staphylococcus aureus* α-toxin [18, 19], anthrax toxin protective antigen [20] and the cholesterol-dependent cytolysins of Gram-positive bacteria [21], all of which first assemble into oligomers atop the target membrane before cooperatively inserting into and permeabilizing it. By analogy, one may hypothesize that the oligomers of daptomycin and related antibiotics also undergo such a cooperative membrane insertion (Figure 6); however, the structural correlate of oligomer activation clearly needs further experimental study. The same applies to the functional roles of individual amino acid residues in the daptomycin molecule. While our study makes an initial distinction between two functional groups of residues – namely, the ones shared between daptomycin and CB-182,462, which suffice for oligomerization, and those not shared between the two molecules, at least some of which are required for oligomer activation – this level of resolution is clearly inadequate for construction of a detailed structural and functional model of the formation and activation of the oligomer. Studies with additional

synthetic or genetically engineered [22, 23] molecular variants of daptomycin may help to address this question.

The high degree of specificity in the mutual interaction of the subunits within the daptomycin and CB-182,462 oligomers agrees with the considerable stability of the oligomers; even the hybrids, which one might expect to be less stable than the homogeneous oligomers, appear mostly stable on a time scale longer than required for exercising the antibacterial effect. Daptomycin has been likened to the large and structurally diverse functional family of antibacterial peptides [24, 25]. However, with most antibacterial peptides, oligomer formation seems to be rather fleeting and transient, or at least more readily reversible [26-29] than with daptomycin and CB-182,462. It appears possible, therefore, that pore-forming protein toxins provide a more useful and valid paradigm than typical antimicrobial peptides to understand daptomycin's mode of pore formation.

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References

[1] Bell, J.M., Turnidge, J.D., Sader, H.S. and Jones, R.N. Antimicrobial activity and spectrum of daptomycin: results from the surveillance program in Australia and New Zealand (2008), Pathology *42* (2010) 470-473.

[2] Cantón, R., Ruiz-Garbajosa, P., Chaves, R.L. and Johnson, A.P. A potential role for daptomycin in enterococcal infections: what is the evidence?, J Antimicrob Chemother *65* (2010) 1126-1136.

[3] Tally, F.P., Zeckel, M., Wasilewski, M.M., Carini, C., Berman, C.L., Drusano, G.L. and Oleson, F.B. Daptomycin: a novel agent for Gram-positive infections, Expert Opin Investig Drugs *8* (1999) 1223-1238.

[4] Alborn, W.E.J., Allen, N.E. and Preston, D.A. Daptomycin disrupts membrane potential in growing Staphylococcus aureus, Antimicrob Agents Chemother *35* (1991) 2282-2287.

[5] Silverman, J.A., Perlmutter, N.G. and Shapiro, H.M. Correlation of daptomycin bactericidal activity and membrane depolarization in Staphylococcus aureus, Antimicrob Agents Chemother *47* (2003) 2538-2544.

[6] Cotroneo, N., Harris, R., Perlmutter, N., Beveridge, T. and Silverman, J.A. Daptomycin exerts bactericidal activity without lysis of Staphylococcus aureus, Antimicrob Agents Chemother *52* (2008) 2223-2225.

[7] Allen, N.E., Alborn, W.E.J. and Hobbs, J.N.J. Inhibition of membrane potential-dependent amino acid transport by daptomycin, Antimicrob Agents Chemother *35* (1991) 2639-2642.

[8] Muraih, J.K., Pearson, A., Silverman, J. and Palmer, M. Oligomerization of daptomycin on membranes, Biochim Biophys Acta *1808* (2011) 1154-1160.

[9] Muraih, J.K., Harris, J., Taylor, S. and Palmer, M. Characterization of daptomycin oligomerization with perylene excimer fluorescence: Stoichiometric binding of phosphatidylglycerol triggers oligomer formation, Biochim Biophys Acta *1818* (2012) 673-678.

[10] Debono, M., Barnhart, M., Carrell, C.B., Hoffmann, J.A., Occolowitz, J.L., Abbott, B.J., Fukuda, D.S., Hamill, R.L., Biemann, K. and Herlihy, W.C. A21978C, a complex of new acidic peptide antibiotics: isolation, chemistry, and mass spectral structure elucidation, J Antibiot (Tokyo) *40* (1987) 761-777.

[11] Boeck, L.D., Papiska, H.R., Wetzel, R.W., Mynderse, J.S., Fukuda, D.S., Mertz, F.P. and Berry, D.M. A54145, a new lipopeptide antibiotic complex: discovery, taxonomy, fermentation and HPLC, J Antibiot (Tokyo) *43* (1990) 587-593.

[12] Mascolo, C., Townsend, K., Cotroneo, N. and Silverman, J. Microbiological Characterization of a Novel Lipopeptide Antibiotic with Activity in Pulmonary Surfactant (2009). Conference presentation, ICAAC.

[13] Muraih, J.K. and Palmer, M. Estimation of the subunit stoichiometry of the membrane-associated daptomycin oligomer by FRET, Biochim Biophys Acta *1818* (2012) 1642-1647.

[14] Lancet, D. and Pecht, I. Spectroscopic and immunochemical studies with nitrobenzoxadiazolealanine, a fluorescent dinitrophenyl analogue, Biochemistry *16* (1977) 5150-5157.

[15] Brown, R.S., Brennan, J.D. and Krull, U.J. Self-quenching of nitrobenzoxadiazole labeled phospholipids in lipid membranes, The Journal of Chemical Physics *100* (1994) 6019-6027.

[16] Lakey, J.H. and Ptak, M. Fluorescence indicates a calcium-dependent interaction between the lipopeptide antibiotic LY146032 and phospholipid membranes, Biochemistry *27* (1988) 4639-4645.

[17] Gessner, P.K. Isobolographic analysis of interactions: an update on applications and utility, Toxicology *105* (1995) 161-179.

[18] Jursch, R., Hildebrand, A., Hobom, G., Tranum-Jensen, J., Ward, R., Kehoe, M. and Bhakdi, S. Histidine residues near the N terminus of staphylococcal alpha-toxin as reporters of regions that are critical for oligomerization and pore formation, Infect Immun *62* (1994) 2249-2256.

[19] Walker, B., Braha, O., Cheley, S. and Bayley, H. An intermediate in the assembly of a pore-forming protein trapped with a genetically-engineered switch, Chem Biol *2* (1995) 99-105.

[20] Miller, C.J., Elliott, J.L. and Collier, R.J. Anthrax protective antigen: prepore-to-pore conversion, Biochemistry *38* (1999) 10432-10441.

[21] Hotze, E., Wilson-Kubalek, E., Rossjohn, J., Parker, M., Johnson, A. and Tweten, R. Arresting pore formation of a cholesterol-dependent cytolysin by disulphide trapping synchonizes the insertion of the transmembrane beta-sheet from a pre-pore intermediate., Journal of Biological Chemistry *276* (2001) 8261-8268.

[22] Nguyen, K.T., He, X., Alexander, D.C., Li, C., Gu, J., Mascio, C., Praagh, A.V., Mortin, L., Chu, M., Silverman, J.A., Brian, P. and Baltz, R.H. Genetically Engineered Lipopeptide Antibiotics Related to A54145 and Daptomycin with Improved Properties, Antimicrob Agents Chemother (2010) .

[23] Strieker, M. and Marahiel, M.A. The structural diversity of acidic lipopeptide antibiotics, Chembiochem *10* (2009) 607-616.

[24] Mishra, N.N., McKinnell, J., Yeaman, M.R., Rubio, A., Nast, C.C., Chen, L., Kreiswirth, B.N. and Bayer, A.S. In vitro cross-resistance to daptomycin and host defense cationic antimicrobial peptides in clinical methicillin-resistant Staphylococcus aureus isolates, Antimicrob Agents Chemother *55* (2011) 4012-4018.

[25] Straus, S.K. and Hancock, R.E.W. Mode of action of the new antibiotic for Gram-positive pathogens daptomycin: comparison with cationic antimicrobial peptides and lipopeptides, Biochim Biophys Acta *1758* (2006) 1215-1223.

[26] Cafiso, D.S. Alamethicin: a peptide model for voltage gating and protein-membrane interactions, Annu Rev Biophys Biomol Struct *23* (1994) 141-165.

[27] Shai, Y. Mechanism of the binding, insertion and destabilization of phospholipid bilayer membranes by alpha-helical antimicrobial and cell non-selective membrane-lytic peptides, Biochim Biophys Acta *1462* (1999) 55-70.

[28] Shai, Y. Mode of action of membrane active antimicrobial peptides, Biopolymers *66* (2002) 236-248.

[29] Brogden, K.A. Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria?, Nat Rev Microbiol *3* (2005) 238-250.

Legends to Figures

Figure 1: Structures of daptomycin and of CB-182,462 and of their labeled derivatives used in this study. Arrows indicate amide or ester bonds, in C to N or C to O direction. In perylene-daptomycin, a perylene-butanoyl residue replaces the N-terminally attached decanoyl residue found in daptomycin. CB-182,462 is a semisynthetic derivative of the natural compound A54145, in which the naturally occurring N-terminally linked fatty acyl residue is replaced by a substituted carbamyl residue. In NBD-CB-182,462, a nitrobenzoxadiazole group is attached to the free amine in the side chain of D-lysine. Abbreviated names for non-standard amino acids: Orn, ornithine; MeOGlu, γ-methoxy-glutamate; kyn, kynurenine; HO-Asn, β-hydroxy-asparagine; MeOAsp, β-methoxy-aspartate. The two residues whose intrinsic fluorescence was used in some of the experiments, tryptophan in CB-182,462 and kynurenine in daptomycin, are set in boldface. Note that, in addition to the sequence similarity, the same positions are occupied by D- and L-amino acids in both molecules.

Figure 2: Formation of CB-182,462 oligomers on PC/PG liposomes. NBD-labeled CB-182,462 (0.96 μM) and unlabeled CB-182,462 (4.8 μM), alone or combination, were incubated with PC/PG (1:1, 200 μM total lipid) liposomes in the presence of calcium (5 mM). **A**: Tryptophan fluorescence upon excitation at 282 nm. NBD-CB-182,462 has virtually none, due to FRET from tryptophan to NBD. When the two compounds are mixed before application to liposomes, the tryptophan fluorescence is more strongly quenched than when they are applied separately with a time interval of 5 minutes between both applications. This is consistent with the formation of hybrid oligomers in the first case but mostly segregated oligomers in the second. The emission of NBD was not scanned in this experiment because it overlaps the secondary maximum of the excitation wavelength. **B**: Self-quenching of NBD-CB-182,462. Addition of unlabeled CB-182,462 before application to the PC/PG liposomes increases the fluorescence intensity of NBD-CB-182,462 upon direct excitation of NBD at 465 nm, indicating that the latter is subject to self-quenching in homogeneous oligomers. If the unlabeled CB-182,462 is added 5 minutes after the NBD-labeled sample, it has little effect on the extent of quenching.

Figure 3: Formation of hybrid oligomers of native CB-182,462 and daptomycin on liposomes. **A**: When both compounds (2 μM each) are mixed before addition to PC/PG liposomes (250 μM total lipid) and calcium (5 mM), the tryptophan fluorescence of CB-182,462 is strongly reduced by FRET, and the kynurenine emission of daptomycin (around 445 nm) is increased. The extent of FRET is

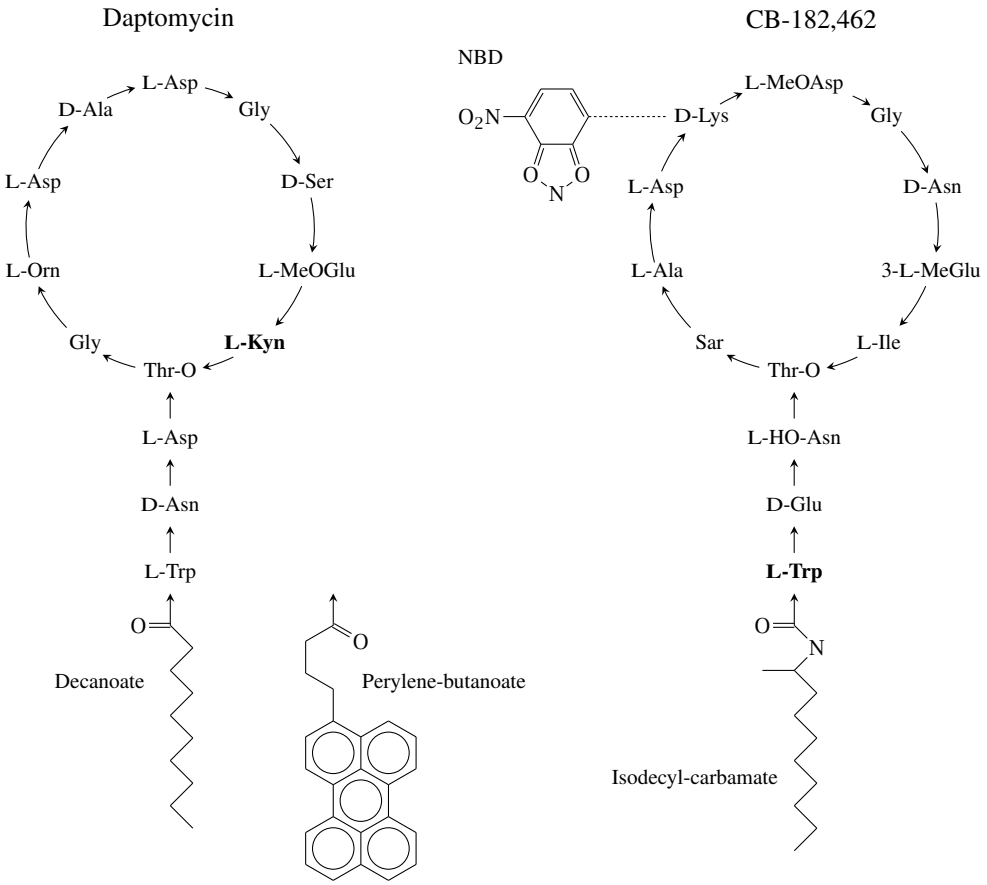
smaller when one compound is applied to the liposomes 5 minutes after the first one. **B**: Extended incubation of a sequentially prepared sample. The extent of FRET increases slightly with time, but after 60 minutes still does not approach that of a premixed sample, indicating that daptomycin and CB-182,462 mostly do not reassemble into hybrid oligomers within this time period. Thin dotted lines represent several time points between 5 and 60 minutes.

Figure 4: Formation of hybrid CB-182,462 oligomers on *Bacillus subtilis* cell membranes. **A**: Alleviation of NBD-CB-182,462 self-quenching by unlabeled CB-182,462 or daptomycin. NBD-CB-182,462 (4 μM), alone or premixed with unlabeled CB-182,462 or daptomycin (20 μM), was incubated with *Bacillus subtilis* ATCC 1046 cells in the presence of calcium (5 mM). After incubation for 10 minutes, the cells were washed repeatedly by centrifugation, re-suspended in buffer, and the NBD fluorescence measured upon excitation at 465 nm. **B**: Inhibition of excimer formation by perylene-daptomycin by unlabeled daptomycin or CB-182,462. Perylene-daptomycin monomers emit maximally at 455 nm, whereas the excimers emit maximally at about 525 nm [9]. Concentrations of labeled and unlabeled compounds, and other conditions as in A.

Figure 5: Mutual inhibition of bactericidal action between daptomycin and CB-182,462. *Bacillus subtilis* ATCC 1046 was pre-grown in LB broth and inoculated 1:100 into LB broth supplemented with calcium (5 mM) and either or both antibiotics as indicated. The data points represent the MIC values determined in two independent representative experiments. The dotted line (partly covered) represents the expected relationship for additive drug action.

Figure 6: Hypothetical model for daptomycin action. Membrane binding (1) precedes oligomerization (2), which in turn must be followed by membrane insertion (3) in order to produce functional membrane lesions (top). In mixtures of daptomycin and CB-182,462 (bottom), oligomerization is preserved, but membrane insertion is disrupted.

Figure 1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CB-182,462
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Figure 2

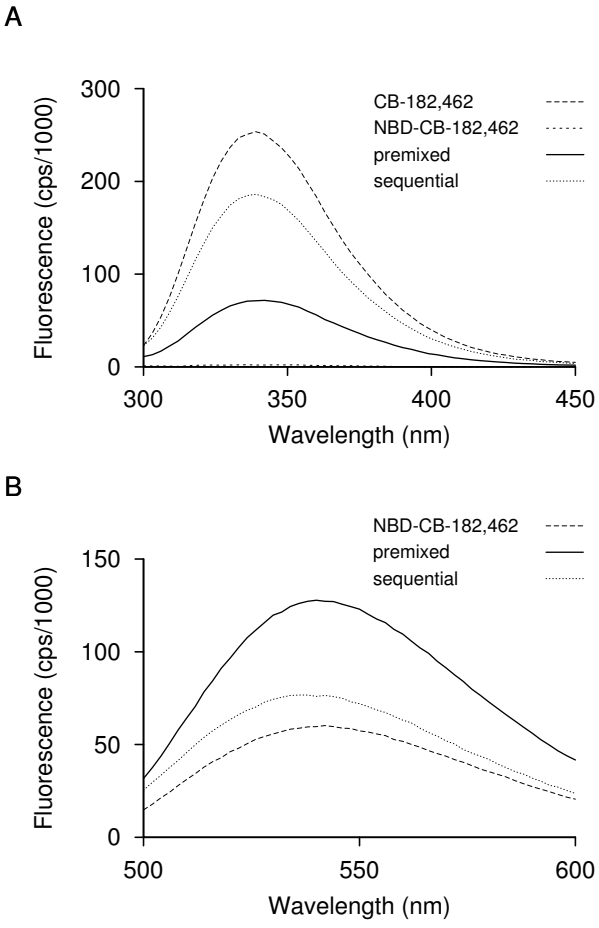
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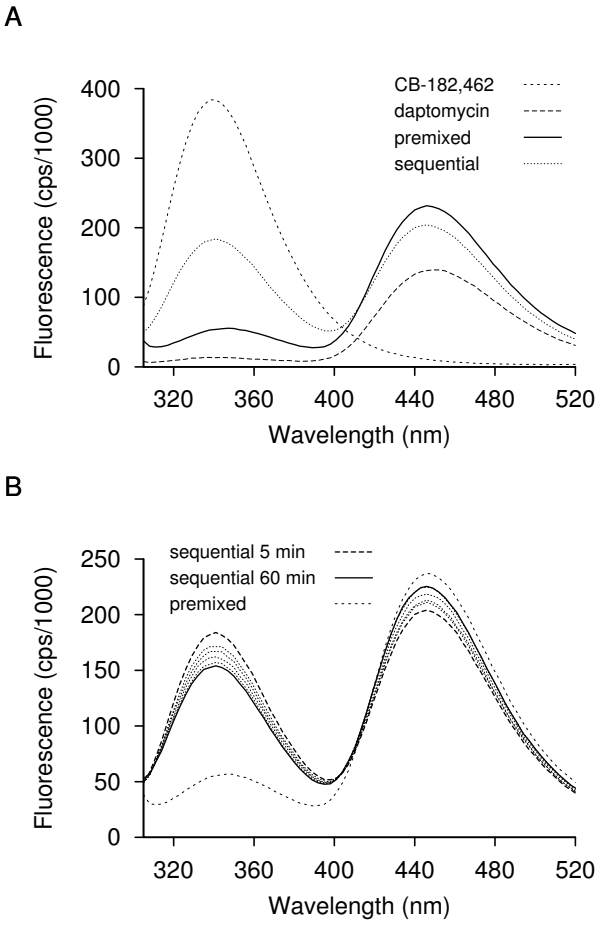
Figure 3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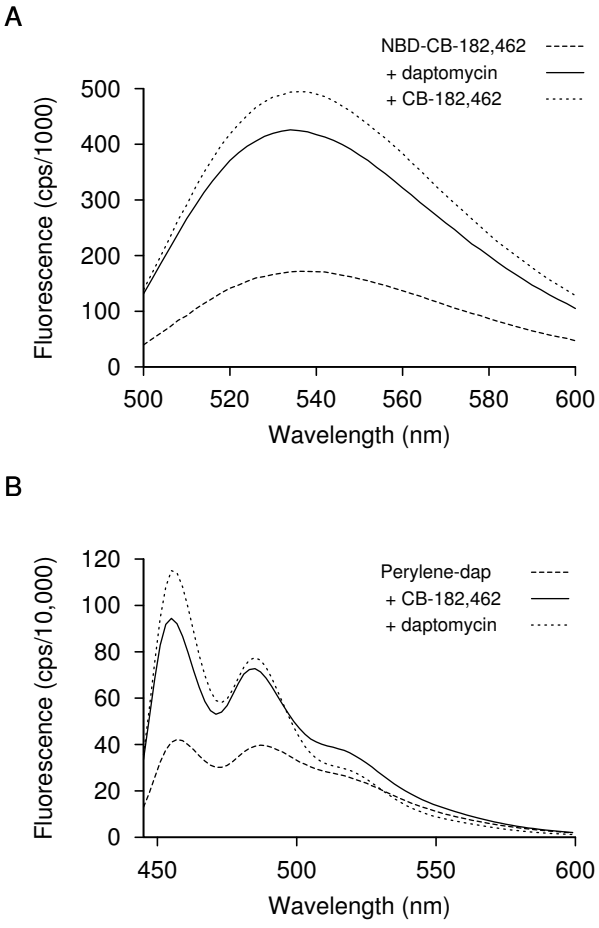
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Figure 5

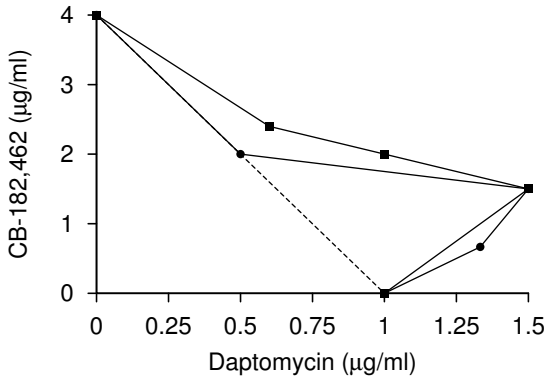

Figure 6