A FASII inhibitor prevents staphylococcal evasion of daptomycin by inhibiting phospholipid decoy production

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Abstract

Daptomycin is a treatment of last resort for serious infections caused by drug-resistant Gram-positive pathogens such as methicillin-resistant *Staphylococcus aureus*. We have shown recently that *S. aureus* can evade daptomycin by releasing phospholipid decoys that sequester and inactivate the antibiotic, leading to treatment failure. Since phospholipid release occurs via an active process, we hypothesised that it could be inhibited, thereby increasing daptomycin efficacy. To identify opportunities for therapeutic interventions that block phospholipid release, we first determined how the host environment influenced the release of phospholipids and inactivation of daptomycin by *S. aureus*. The addition of certain host-associated fatty acids to the growth medium enhanced phospholipid release. However, in serum, the sequestration of fatty acids by albumin restricted their availability to *S. aureus* sufficiently to prevent their use in the generation of released phospholipids. This finding implied that in host tissues *S. aureus* may be completely dependent upon endogenous phospholipid biosynthesis to generate lipids for release, providing a target for therapeutic intervention. To test this, we exposed *S. aureus* to AFN-1252, an inhibitor of the staphylococcal FASII fatty acid biosynthetic pathway, together with daptomycin. AFN-1252 efficiently blocked daptomycin-induced phospholipid decoy production, even in the case of isolates resistant to AFN-1252, which prevented the inactivation of daptomycin and resulted in sustained bacterial killing. In turn, daptomycin prevented the fatty acid-dependent emergence of AFN-1252-resistant isolates *in vitro*. In summary, AFN-1252 significantly enhances daptomycin activity against *S. aureus* *in vitro* by blocking the production of phospholipid decoys, whilst daptomycin blocks the emergence of resistance to AFN-1252.
Introduction

Daptomycin is a lipopeptide antibiotic of last resort used to treat infections caused by drug-resistant Gram-positive pathogens such as methicillin-resistant *S. aureus* (MRSA) and vancomycin-resistant enterococci (VRE) [1,2]. The target of daptomycin is the bacterial membrane, where it causes mis-localisation of enzymes required for cell wall biosynthesis, loss of membrane potential and integrity, and rapid bacterial death [1,3,4].

Resistance to daptomycin can arise spontaneously via mutations in genes associated with phospholipid or peptidoglycan biosynthesis [1,5,6]. However, whilst resistance has been reported to arise during treatment, it is a rare occurrence and does not explain why daptomycin treatment failure has been reported in up to 20% of cases of infective endocarditis and up to 30% of cases of complicated skin and soft-tissue infection or osteomyelitis, most commonly caused by *S. aureus* [7,8]. Treatment failure is reduced at higher therapeutic doses of daptomycin, but host toxicity limits the concentration of the drug that can be used [1,7,8]. In a bid to identify additional mechanisms by which *S. aureus* can withstand daptomycin treatment, we discovered that upon exposure to the antibiotic, *S. aureus* releases phospholipids into the extracellular space [9]. These phospholipids act as decoys, sequestering daptomycin and preventing it from inserting into the bacterial membrane.

This decoy-mediated antibiotic inactivation led to treatment failure in a murine model of invasive MRSA infection, suggesting that it could affect daptomycin efficacy in patients [9]. Furthermore, the production of phospholipid decoys also occurs in enterococci and streptococci, suggesting a broadly conserved defence against membrane-acting antimicrobials [10].

The ability of released membrane phospholipids to inactivate daptomycin can be compromised in *S. aureus* by the quorum-sensing-triggered production of small cytolytic peptides known as the alpha phenol soluble modulins (PSMα) [9]. These peptides appear to compete with daptomycin for the phospholipid and thereby prevent inactivation of the antibiotic [9]. Whilst this may appear paradoxical, many invasive infections are caused by *S. aureus* strains defective for PSMα production due to defects in the Accessory gene regulator (Agr) quorum-sensing system that triggers expression of the peptides [11-13]. Furthermore, as serum apolipoproteins inhibit the Agr system and sequester PSMs, wild-type bacteria would be expected to inactivate daptomycin in the bloodstream [14-17].

The mechanism by which daptomycin triggers phospholipid release is currently undefined. However, we have shown that it is an active process that requires energy, as well as protein, cell wall and lipid biosynthesis [9,10]. The requirement for fatty acid biosynthesis for phospholipid release is important
because it raises the prospect of targeting this process to enhance daptomycin efficacy. We have shown previously that inhibition of the FabF component of the fatty acid synthesis (FASII) pathway, using the antibiotic platensimycin, completely blocked phospholipid release [9,10]. Whilst platensimycin is unsuitable as a therapeutic drug due to poor pharmacological properties, the FabI inhibitor AFN-1252 shows more promising characteristics and a pro-drug variant is currently undergoing phase 2 clinical trials [18,19]. However, despite excellent in vitro activity, the therapeutic value of FASII inhibitors as mono-therapeutic agents has attracted much debate [20,21]. Several bacteria, including S. aureus, can utilise fatty acids present in the host to generate phospholipids [21-24]. Although wild-type S. aureus strains cannot fully substitute exogenous fatty acids for endogenous fatty acids synthesised via FASII, there is evidence that some clinical isolates (up to 7%) have acquired mutations that enable them to fully or partially bypass endogenous fatty acid biosynthesis by utilising host-derived fatty acids [22,25,26]. Furthermore, in vitro experimentation suggests that the acquisition of such mutations is dependent upon the presence of host-associated fatty acids, which means that the frequency at which resistance to AFN-1252 emerges in vivo may have been under-estimated [25,26]. As such, the long-term viability of fatty acid synthesis inhibitors, such as AFN-1252, as mono-therapeutic antibacterial drugs is unclear and their ability to block daptomycin-induced phospholipid release in the presence of exogenous fatty acids undetermined [20,21].

Therefore, the aims of this work were to understand how the availability of fatty acids in the host influences the production of phospholipid decoys and determine whether AFN-1252 could be used in combination with daptomycin to provide a viable approach to combatting MRSA infection.

Results

Exogenous fatty acids modulate daptomycin-induced phospholipid release

Since S. aureus can incorporate exogenous fatty acids into membrane phospholipid production, it was hypothesised that host-derived fatty acids would contribute to the production of lipids required for daptomycin-induced phospholipid release [21-24].

To enable accurate measurements of phospholipid release, these experiments were done in tryptic soy broth (TSB) containing, or not, one of several different fatty acids found in normal human serum [27]. To avoid the Agr system compromising daptomycin inactivation, these initial experiments employed the S. aureus USA300 LAC ΔagrA mutant (Table 1), which has the same daptomycin minimum inhibitory concentration (MIC) as the wild-type (Table 2) [9].
Exposure of the *S. aureus* USA300 ΔagrA mutant to daptomycin in the absence of exogenous fatty acids resulted in the release of phospholipids into the extracellular space (Fig. 1A). Supplementation of the TSB growth medium with linoleic acid had no effect on the rate or quantity of phospholipid released, whilst the presence of myristic or palmitic acids resulted in a small increase in the quantity of phospholipids released at the latest time point (Fig. 1A). By contrast, the presence of oleic or lauric acids significantly enhanced both the rate and quantity of phospholipids released, relative to TSB without fatty acids (Fig. 1A).

The increased release of phospholipids from bacteria incubated with oleic or lauric acids resulted in a slightly faster rate of daptomycin inactivation, whilst the presence of linoleic, palmitic or myristic acids reduced the rate of daptomycin inactivation (Fig. 1B). Of note, *S. aureus* failed to fully inactivate daptomycin in the presence of palmitic or linoleic acids, indicating that exogenous fatty acids can retard as well as promote the rate of phospholipid-mediated daptomycin inactivation (Fig. 1B).

In keeping with the effect of individual fatty acids on daptomycin inactivation, the presence of oleic or lauric acid promoted bacterial survival 10-fold above that seen for *S. aureus* incubated without fatty acids by 8 h. By contrast, the presence of palmitic or linoleic acids reduced survival approximately 10-fold, whilst myristic acid had no effect (Fig. 1C).

Next, we determined how the concentration of exogenous fatty acid affected phospholipid release and bacterial survival. As shown previously in Figure 1A, the presence of 20 µM oleic acid promoted phospholipid release in response to daptomycin challenge (Fig. 2A). However, increasing the concentration of oleic acid up to 100 µM (which is similar to that found in serum [27]), did not increase phospholipid release above that seen with 20 µM (Fig. 2A). In keeping with this, the presence of 100 µM oleic acid did not significantly affect the rate of daptomycin inactivation relative to 20 µM, nor did the higher concentration of the fatty acid reduce the initial rate of daptomycin-mediated killing (Fig. 2B,C). However, the highest concentrations of oleic acid did promote the rate of recovery once daptomycin was inactivated, presumably by providing precursors to the energetically-expensive process of membrane biogenesis (Fig. 2B,C).

Serum albumin restricts the utilisation of oleic acid by *S. aureus* for phospholipid release

Having established that fatty acids can modulate phospholipid release in TSB, we wanted to determine whether their presence in the host context had a similar effect. To do this, we firstly supplemented TSB with 50% delipidated human serum, which is deficient for fatty acids. Similarly to
what was seen in TSB alone, exposure of the ΔagrA mutant to daptomycin in TSB containing 50% delipidated human serum resulted in an initial fall in colony forming unit (CFU) counts, followed by a period of recovery (Fig. 3A). However, in contrast to our observations for TSB (Fig. 1C), the addition of oleic acid to TSB containing 50% delipidated serum had no effect on bacterial survival (Fig. 3A). In keeping with these data, the presence of oleic acid had no effect on the rate at which the bacteria inactivated daptomycin (Fig. 3B). This indicated that the ability of \textit{S. aureus} to use oleic acid to promote phospholipid release was restricted by a factor found in serum but not TSB, although this was not quantified directly as serum components interfered with the dye-based assay system.

Fatty acids present in the bloodstream are typically bound to serum albumin, which acts as a carrier protein [28]. To determine whether the presence of this host protein restricted the availability of oleic acid for use in phospholipid release-mediated inactivation of daptomycin, the \textit{S. aureus} ΔagrA mutant was exposed to daptomycin in TSB containing oleic acid and human serum albumin (HSA). By contrast to TSB only, the presence of HSA completely abrogated the increased rate of daptomycin inactivation and bacterial survival observed on supplementation with oleic acid, presumably due to sequestration of the fatty acid by the protein (Fig. 3C,D).

**AFN-1252 blocks daptomycin-induced phospholipid release in the presence of unbound oleic acid**

The finding that HSA prevented the use of exogenous oleic acid by \textit{S. aureus} to promote the rate of daptomycin inactivation indicated that this process is likely to be entirely dependent upon the FASII pathway \textit{in vivo}. Therefore, we hypothesised that the FASII inhibitor AFN-1252 would enhance daptomycin activity against \textit{S. aureus} by blocking the production of phospholipid decoys.

Alone, AFN-1252 (0.15 µg ml\(^{-1}\)) showed bacteriostatic activity (<10-fold drop in CFU counts after 8 h) (Fig. 4A). As described previously, CFU counts of the \textit{S. aureus} ΔagrA mutant exposed to daptomycin fell initially, before recovering due to the release of phospholipids that led to the inactivation of the antibiotic (Fig. 4A,B,C) [9]. However, when the \textit{S. aureus} ΔagrA mutant was exposed to daptomycin in the presence of AFN-1252, there was a >500-fold drop in CFU counts, with no recovery of the bacterial population (Fig. 4A). Further analysis revealed that AFN-1252 almost completely blocked daptomycin-induced phospholipid release and the associated daptomycin inactivation (Fig. 4B,C), providing an explanation for the synergy observed when these antibiotics were used in combination.

Whilst our data indicated that HSA restricts the utilisation of exogenous fatty acids for phospholipid release (Fig. 3C,D), we considered the possibility that some unbound lipids may arise
during infection because of damage to host tissues. Therefore, we repeated the experiments described in Figures 4A,B,C in the presence of oleic acid without HSA, since this lipid had previously been shown to significantly promote phospholipid release (Fig. 1A). The data generated from these experiments were almost identical to those from experiments done in the absence of the fatty acid (Fig. 4D,E,F). AFN-1252 showed clear synergistic activity when used in combination with daptomycin by blocking phospholipid release, even in the presence of unbound oleic acid (Fig. 4E). This resulted in the maintenance of daptomycin activity and a sustained killing effect on S. aureus (Fig. 4D,F).

Together, these data demonstrate that AFN-1252 prevents the production of phospholipid decoys, even in the presence of exogenous fatty acids which would otherwise enhance phospholipid release.

AFN-1252 blocks phospholipid release triggered by a range of daptomycin concentrations

The bactericidal activity of daptomycin is dependent upon the concentration of both the antibiotic and calcium ions [1]. To determine how these factors affected the inhibition of phospholipid release by AFN-1252 and the consequences for bacterial survival, both wild-type (WT) and ΔagrA mutant S. aureus were exposed to various concentrations of daptomycin in broth supplemented with 0.5 mM or 1.25 mM CaCl₂ in the presence or absence of the FASII inhibitor (0.15 µg ml⁻¹).

Daptomycin caused dose-dependent killing of both WT and ΔagrA mutant S. aureus, which was greater at 1.25 mM than 0.5 mM CaCl₂, with >1000-fold reduction in CFU counts at 40 µg ml⁻¹ of the antibiotic (Fig. 5A-D) [1,10]. As expected from our earlier studies, at both CaCl₂ concentrations survival of the ΔagrA mutant was greater than the WT at lower concentrations of daptomycin but killing was similar between strains at the highest concentration of the antibiotic (40 µg ml⁻¹) (Fig. 5A-D) [9]. At lower concentrations of daptomycin, the presence of AFN-1252 reduced bacterial survival by ~10-100-fold but had no effect on bacterial survival in the presence of the highest concentration of the lipopeptide antibiotic (Fig. 5A-D).

As observed previously, phospholipid release was generally greater at lower concentrations of daptomycin [10] and reduced in the presence of the higher concentration of calcium (Fig. 5E-H). However, regardless of the experimental conditions or quantity of phospholipids released, the presence of AFN-1252 significantly reduced phospholipid release from WT or ΔagrA mutant S. aureus to almost undetectable levels (Fig. 5E-H).

In agreement with previous work, the ΔagrA mutant was significantly more efficient than the WT at inactivating daptomycin (Fig. 5I-L) [9]. In the presence of 0.5 mM CaCl₂, WT S. aureus could only partially inactivate 10 µg ml⁻¹ daptomycin whereas the ΔagrA mutant was able to completely...
inactivate the lipopeptide at 20 µg ml⁻¹ (Fig. 5I-J). At 1.25 mM CaCl₂, WT *S. aureus* fully inactivated
daptomycin at 5 µg ml⁻¹ but the ΔagrA mutant inactivated the antibiotic at 10 µg ml⁻¹ (Fig. 5K-L).

However, for both WT and ΔagrA mutant, the presence of AFN-1252 prevented the inactivation of
daptomycin, in keeping with the ability of this antibiotic to prevent phospholipid release (Fig. 5I-L) [9,10].

In summary, at concentrations of daptomycin that are inactivated by released phospholipids, AFN-
1252 promotes bacterial killing. However, at concentrations of daptomycin that cannot be
inactivated by *S. aureus*, AFN-1252 has little or no effect on bacterial survival. This provides
additional evidence that the FASII inhibitor synergises with the lipopeptide antibiotic by blocking the
release of phospholipids that inactivate daptomycin.

**AFN-1252 blocks daptomycin-induced phospholipid release in human serum**

To further explore how the host environment might influence daptomycin-induced phospholipid
release and whether AFN-1252 would be expected to block this, we used TSB containing 50% normal
human serum. In addition to providing fatty acids in their natural state and concentration, this
system also accounts for the effects of antibiotic binding to serum proteins and the suppression of
Agr activity by apolipoproteins.

As reported, the presence of serum resulted in slightly increased MICs of some strains for
both daptomycin and AFN-1252, due to the binding of the antibiotics by serum proteins (Table 2)
[29,30]. Exposure of wild-type USA300 *S. aureus* to daptomycin alone resulted in a brief decline in
CFU counts over the first 2 h, followed by an increase in bacterial numbers (Fig. 6A). Unfortunately,
the high lipid content of serum prevented accurate measurement of phospholipid release. However,
bacterial survival correlated well with the inactivation of daptomycin, which occurred within 4 h (Fig.
6B). A broadly similar survival profile was seen for the ΔagrA mutant, suggesting that the presence
of serum negates previously reported differences in daptomycin inactivation mediated by Agr (Fig.
6C,D) [9].

Despite the increased MIC for AFN-1252 in serum, the presence of the FASII inhibitor
prevented daptomycin inactivation by both wild-type *S. aureus* and the ΔagrA mutant, resulting in
increased bacterial killing over the duration of the assay (Fig. 6A,B,C,D).

**AFN-1252 blocks daptomycin-induced phospholipid release by clinical isolates**
To test whether daptomycin-induced phospholipid release is a common property of clinical MRSA isolates and whether it is blocked in these strains by AFN-1252, we examined a panel of 10 MRSA isolates from bloodstream infections. In keeping with previous reports, some of these isolates were hemolytic, whilst others were not, indicative of a loss of Agr activity (Table 1) [11].

Exposure of each of the 10 isolates to daptomycin in the presence of normal human serum resulted in a wide variation in survival levels, with CFU counts of some strains increasing slightly, whilst others declined >10,000-fold after 8 h challenge, which was independent of their Agr activity (Fig. 7A, Table 1). Measurement of daptomycin activity at the end of the experiment revealed that 6 strains had inactivated daptomycin fully or by at least 80%, whilst the other 4 strains did not significantly reduce the activity of the lipopeptide antibiotic (Fig. 7B). Of note, all 6 of the isolates that fully or partially inactivated daptomycin survived at higher levels (>5% survival) than the 4 isolates that did not reduce the activity of the antibiotic (<0.07% survival). There was no correlation between oxacillin MIC and the ability of an isolate to inactivate daptomycin (table 2).

In keeping with our experiments with the USA300 strain, the presence of AFN-1252 blocked the inactivation of daptomycin, which correlated with a significant reduction in the survival of the daptomycin-inactivating bacterial isolates (Fig. 7A,B). By contrast, AFN-1252 did not significantly affect the survival of bacteria that did not inactivate daptomycin, providing additional evidence that AFN-1252 promotes daptomycin’s bactericidal activity by preventing S. aureus from releasing phospholipid decoys that enable the bacterium to evade the lipopeptide antibiotic (Fig. 7A,B).

Exogenous fatty acids enable emergence of resistance to AFN-1252

The data described above indicated that the FASII inhibitor AFN-1252 in combination with daptomycin may be a promising therapeutic approach. To determine the propensity of S. aureus to acquire spontaneous resistance to AFN-1252, 10 parallel cultures of the USA300 ΔagrA mutant were repeatedly challenged with AFN-1252 (0.15 µg ml⁻¹) in the absence or presence of a physiologically relevant fatty acid cocktail as described previously [26]. Given the impact of HSA on fatty acid sequestration, parallel assays were done with or without the serum protein. After each exposure, bacterial susceptibility to AFN-1252 was determined by broth microdilution assays to establish the MIC.

As expected from a previous report, there was very little change in bacterial growth (Fig. 8A) or MIC (Fig. 8B) when S. aureus was repeatedly exposed to AFN-1252 in the absence of fatty acids [26]. However, in keeping with previous work, by the third round of exposure to AFN-1252 in the presence of fatty acids, with or without HSA, S. aureus was able to replicate in the presence of the
antibiotic (Fig. 8A) [26]. The ability of *S. aureus* to grow in the presence of AFN-1252 after repeated exposure to the antibiotic in the presence of fatty acids, regardless of the presence of HSA, correlated well with data from subsequent MIC assays (Fig. 8C,D). When fatty acids were included in the MIC assays, there was a significant and large increase in the MICs of most cultures from 0.03125 µg ml⁻¹ to more than 16 µg ml⁻¹ (>512-fold) for bacteria that were exposed to AFN-1252 in the presence of exogenous fatty acids (Fig. 8A). Since fatty acid-dependent AFN-1252 resistance has been most commonly linked to mutations in the fabD gene [26], we examined this locus in two randomly selected AFN-1252 resistant isolates from this assay. This revealed a 826G>T substitution, which corresponds to FabD G276 STOP, resulting in a truncated protein in one isolate, whilst the other had a 3G>A substitution, which would be expected to result in failure of the ribosome to recognise the ATG start codon, resulting in a lack of FabD production.

Together, these data confirm previous work showing that repeated exposure of *S. aureus* to AFN-1252 in the presence of exogenous fatty acids facilitated the emergence of fatty acid-dependent resistance to this antibiotic, at least in part via mutations in the *fabD* gene [26].

**Daptomycin prevents fatty acid-dependent emergence of resistance to AFN-1252**

Having confirmed that AFN-1252 resistance can arise in the presence of fatty acids, the next objective was to test whether combination therapy with daptomycin could prevent this. As expected from previous data (Fig. 4), bacterial killing with daptomycin/AFN-1252 combination therapy was highly effective for the first two exposures, where bacterial survival was 1% or less after 8 h. An increase in bacterial survival was observed on the third exposure, but bacterial growth was still inhibited with CFU counts not exceeding that of the original inoculum (Fig. 9A). Furthermore, this increase in survival was independent of the presence of fatty acids (Fig. 9A).

By contrast to experiments with AFN-1252 alone, repeated exposure of *S. aureus* to AFN-1252 in the presence of daptomycin did not lead to an increase in MIC of the FASII inhibitor, even in the presence of fatty acids (Fig. 9B,C,D). Neither was there any increase in the daptomycin MIC (Fig. 9E,F,G). Together, these data demonstrate that daptomycin prevented the emergence of fatty acid-dependent resistance to AFN-1252 when the two antibiotics were used in combination.

Despite the increase in bacterial survival on the third exposure, this did not exceed the original inoculum (Fig. 9A), and the unchanged MIC values (Fig. 9B,C,D,E,F,G) indicated that AFN-1252 and daptomycin still had bacteriostatic activity (i.e. whilst the antibiotics did not cause a drop in CFU counts, they still prevented bacterial replication).

**AFN-1252 blocks daptomycin-induced phospholipid release in AFN-1252-resistant strains**
Having established that the combination of daptomycin and AFN-1252 prevented the emergence of AFN-1252 resistance, we next wanted to understand the underlying mechanism.

As described above (Fig. 4), two independent colony picks of the ΔagrA mutant that had not previously been exposed to antibiotics survived exposure to daptomycin by releasing phospholipids that completely inactivated the antibiotic (Fig. 10A,B,C). However, the presence of AFN-1252 increased the bactericidal activity of daptomycin by preventing phospholipid release and thus preserving the activity of the lipopeptide antibiotic, regardless of the presence of fatty acids (Fig. 10A,B,C).

Next, we assessed the survival of bacteria from 3 independent cultures that had acquired resistance to AFN-1252 during exposure to the antibiotic in the presence of fatty acids but not HSA (AFN-1252 R). Of these 3 isolates, 2 were more susceptible to daptomycin than the ΔagrA mutant, apparently because they released lower levels of phospholipids that failed to fully inactivate the lipopeptide antibiotic (Fig. 10D,E,F). The remaining isolate reduced daptomycin activity by 70%, explaining its enhanced survival in the presence of daptomycin relative to the other 2 isolates. However, the presence of AFN-1252 completely abolished the ability of any of these isolates to inactivate daptomycin, even when exogenous fatty acids were present (Fig. 10D,E,F).

We then examined S. aureus isolates from 3 independent cultures that had acquired resistance to AFN-1252 during exposure to the antibiotic in the presence of fatty acids and HSA (AFN-1252 R HSA). Survival of these three AFN-1252-resistant isolates after exposure to daptomycin alone was not significantly lower than that seen for the AFN-1252-sensitive ΔagrA mutant. This was due to the release of sufficient phospholipid to inactivate all or most of the daptomycin that the bacteria were incubated with (Fig. 10G,H,I). However, despite the ability of these bacteria to grow in the presence of AFN-1252 when exogenous fatty acids were available, the FASII inhibitor almost completely blocked daptomycin-induced phospholipid release from all three isolates, even when the fatty acid cocktail was present (Fig. 10G,H,I).

Together, these data reveal that fatty acid-enabled AFN-1252 resistance results in a reduced ability to release phospholipids in response to daptomycin alone (Fig. 10E, H). Furthermore, although these strains were deemed resistant to AFN-1252, daptomycin-induced phospholipid release was inhibited by the FASII inhibitor, even in the presence of exogenous fatty acids (Fig. 10F,I). This provides additional evidence that daptomycin-induced phospholipid release is dependent upon endogenous, FASII-mediated fatty acid biosynthesis, and that utilisation of exogenous fatty acids to bypass FASII for lipid synthesis does not enable daptomycin-induced phospholipid release. As such, daptomycin-induced phospholipid release is efficiently blocked by AFN-1252, preventing inactivation of the lipopeptide antibiotic.
Discussion

The high rate of daptomycin treatment failure for osteomyelitis and complicated skin infections caused by *S. aureus* warrants efforts to understand the determinants of therapeutic outcomes and identify new approaches to enhance bacterial clearance [8]. In agreement with our previous work [9,10] and that of others [31], the data presented here revealed that *S. aureus* at high density can inactivate daptomycin, which promotes the survival of bacteria exposed to this antibiotic. Subsequent *in vitro* studies revealed that the FASII inhibitor AFN-1252 prevents the inactivation of daptomycin by clinical *S. aureus* isolates, whilst daptomycin reduces the emergence of spontaneous fatty acid-dependent resistance to the FASII inhibitor, at least for the USA300 LAC strain examined here.

It is increasingly clear that the host environment modulates the susceptibility of bacterial pathogens to antibiotics due to the scarcity of nutrients and the induction of stress responses that result in changes in bacterial physiology [32,33]. Serum contains high concentrations of fatty acids, which can be exploited by *S. aureus* to produce phospholipids, reducing the metabolic costs associated with membrane biogenesis [21,23]. In keeping with this, we found that the presence of specific exogenous fatty acids, such as oleic or lauric acids, enhanced phospholipid release in response to daptomycin. However, *S. aureus* has strict requirements for the type of fatty acids that it can incorporate and, at least for wild-type strains, each phospholipid must have at least one fatty acid tail synthesised endogenously via FASII [33]. This requirement for FASII-mediated fatty acid biosynthesis to generate phospholipids was underlined by the ability of AFN-1252 to completely block phospholipid decoy release, regardless of the presence of oleic acid [34]. This provides evidence that daptomycin/AFN-1252 combination therapy may not be compromised by the availability of fatty acids in the host.

Whilst some exogenous fatty acids can be used for phospholipid biosynthesis during staphylococcal growth, it appears that their contribution to daptomycin-induced phospholipid release is severely compromised by the presence of serum albumin, which sequesters the fatty acids [28]. As described above, there is clear evidence that *S. aureus* can partially substitute endogenous fatty acid biosynthesis for exogenous host-derived fatty acids in the generation of phospholipids.

However, our data demonstrate that the presence of serum albumin reduces the efficiency of this process sufficiently to prevent their use in daptomycin-induced phospholipid release, which must occur quickly if the bacteria are to survive exposure to the rapidly bactericidal antibiotic.
In addition to providing nutrients, the host environment can also modulate bacterial signalling systems and virulence factor production. We have shown previously that the inactivation of daptomycin by released phospholipids is inhibited by the concomitant production of PSMα peptides in response to activation of the Agr quorum-sensing system [9]. However, serum blocks Agr signalling and sequesters PSMs [14-17], which explains why both the Agr-competent wild-type and ΔagrA mutant were able to inactivate daptomycin when experiments were conducted in human serum. Similarly, the Agr status of clinical isolates had no impact on their ability to inactivate daptomycin in the presence of serum, by contrast to what we have seen previously in TSB alone [9].

The mechanism by which S. aureus releases phospholipids in response to daptomycin is unknown. However, the finding that a majority, but not all clinical isolates can inactivate daptomycin suggests that it may be possible to identify the genetic determinants of phospholipid release by whole genome sequencing of clinical isolates and subsequent genome-wide association studies. Clearly, the release of phospholipids that can inactivate daptomycin occurs via an active process [9,10]. It appears that this system functions most efficiently at lower concentrations of daptomycin, since presumably higher concentrations of the antibiotic kill the bacteria before they can synthesise and release the phospholipids. This may explain why the efficacy of daptomycin is greater at higher therapeutic dose of the antibiotic, particularly for endocarditis [8]. Unfortunately, the toxicity of the lipopeptide antibiotic limits the concentration that can be used to treat infection [11]. Therefore, the finding that the bactericidal activity of low concentrations of daptomycin can be promoted by AFN-1252, even in the presence of serum, may have clinical value as a route to improving patient outcomes.

The successful clinical development of AFN-1252 would be a welcome addition to the arsenal of anti-staphylococcal antibiotics. However, although wild-type bacteria are dependent upon the endogenous FASII pathway to generate fatty acids for phospholipid biosynthesis, our data provide additional evidence that this is not the case in strains that have acquired mutations within the fabD lipid biosynthetic gene loci [25,26]. These mutants can bypass FASII-mediated fatty acid production, conferring resistance to AFN-1252 in the presence of exogenous fatty acids [25,26]. It has been suggested that FASII bypass could compromise the long-term therapeutic viability of FASII inhibitors such as AFN-1252, a view that is supported by the identification of clinical isolates that are able to resist AFN-1252 in the presence of exogenous fatty acids [25]. Despite this, early clinical studies have shown that AFN-1252 can successfully treat skin and soft tissue infections, albeit in a relatively small number of patients [18]. Furthermore, a study using a murine thigh infection model suggested that AFN-1252 is efficacious for the treatment of deep-seated infection where host-derived fatty acids are likely to be available to S. aureus [35]. Therefore, it remains to be seen...
whether resistance to AFN-1252 becomes a significant clinical problem. However, given the ability of
S. aureus to rapidly acquire resistance to antibiotics, it seems prudent to develop therapeutic
strategies to prevent or overcome the emergence of resistance to AFN-1252. Our data provide
support for the concept of spontaneous AFN-1252 resistance via fatty acid-dependent FASII bypass,
but also demonstrate that the frequency at which resistance emerges can be significantly reduced by
the presence of daptomycin, at least in vitro.

The combination of AFN-1252 and daptomycin could be described as a mutually-beneficial
pairing; whilst AFN-1252 promotes daptomycin activity by blocking phospholipid release,
daptomycin enhances AFN-1252 efficacy by preventing the emergence of resistance. This finding
contributes to our growing appreciation for the potential of combination therapy approaches to
circumvent resistance mechanisms. A well-established example of this is the combination of
daptomycin and β-lactams that target penicillin-binding protein (PBP) 1. The mechanisms
responsible are complex and not fully defined. However, daptomycin increases the expression of
pbpA, which appears to be important to enable the bacterium to survive exposure to the lipopeptide
[36,37]. Blockage of PBP1 function therefore promotes daptomycin activity against S. aureus,
possibly via the increased binding of the lipopeptide antibiotic to the bacterial membrane [36,37]. In
turn, daptomycin reduces the quantity of PBP2a available, which reduces the resistance of S. aureus
to β-lactams [38,39]. This phenomenon, known as the see-saw effect, significantly promotes killing
of S. aureus relative to each of the antibiotics individually and is currently being assessed as a
therapeutic option in a clinical trial [40].

In summary, the presence of AFN-1252 prevented the phospholipid-mediated inactivation of
daptomycin by clinical MRSA isolates, whilst daptomycin inhibited the fatty-acid dependent
emergence of resistance to AFN-1252. Therefore, we propose that the combination of AFN-1252 and
daptomycin may have therapeutic value for the treatment of serious MRSA infections.

Methods

Bacterial strains and growth conditions
Staphylococcus aureus strains USA300 wild-type and ΔagrA mutant [9] or clinical isolates (Table 1)
were grown in tryptic soy broth (TSB) or on tryptic soy agar (TSA). For some assays TSB was
supplemented with fatty acids including oleic acid, linoleic acid, palmitic acid, myristic acid or lauric
acid (all obtained from Sigma). Since the serum concentrations of these fatty acids vary from 2 μM
(lauric acid) to 122 μM (oleic acid) [27], assays were initially done with a single concentration (20
μM) within this range, although some assays with oleic acid used up to 100 μM of the fatty acid. For
some assays, HSA was included (10 mg ml⁻¹) to sequester fatty acids [22]. Some assays used TSB
containing 0.5 mM MgCl\(_2\) and 1.25 mM CaCl\(_2\) supplemented with 50% normal human serum (AB-positive, Sigma) to mimic the host environment. Bacteria inoculated onto TSA plates were incubated statically at 37 °C for 15-17 hours in air unless otherwise stated. Clinical isolates were also plated onto Columbia blood agar (CBA) containing 5% sheep’s blood to enable assessment of haemolysis, which is a useful proxy for Agr activity [9]. Liquid cultures were grown in 3 ml broth in 30 ml universal tubes by suspending a single colony from TSA plates, and incubated at 37 °C, with shaking at 180 RPM to facilitate aeration for 15-17 hours to stationary phase. Staphylococcal colony forming units (CFU) were enumerated by serial dilution in sterile phosphate buffered saline (PBS) and plating of aliquots onto TSA. Bacterial stocks were stored in growth medium containing 20% glycerol at -80 °C.

**Antibiotic killing kinetics**

*S. aureus* was grown to stationary-phase in 3 ml TSB with shaking (180 RPM) at 37 °C in 30 ml universals as described above. Bacteria were subsequently adjusted to a concentration of \(\sim 1 \times 10^8\) bacteria ml\(^{-1}\) in fresh TSB containing 0.5 mM CaCl\(_2\) to maintain consistency with previous work from our group [9] and others [42] in resistance emergence assays, before antibiotics were added at the following concentrations: daptomycin (20 μg ml\(^{-1}\), Tocris), AFN-1252 (0.15 μg ml\(^{-1}\), Medchemexpress). For some experiments, TSB was supplemented with 50% normal human serum (Sigma), human serum albumin or fatty acids as indicated. For assays with 50% normal human serum, the TSB component was supplemented with 0.5 mM MgCl\(_2\) and 1.25 mM CaCl\(_2\) to provide physiological concentrations. Cultures were then incubated at 37 °C with shaking (180 RPM) and bacterial viability determined by CFU counts from samples taken every 2 h for 8 h.

**Daptomycin activity determination**

The activity of daptomycin during incubation with *S. aureus* was quantified as described previously [9,10]. A well of 10 mm was made in TSA plates containing 0.5 mM CaCl\(_2\), followed by the spreading of stationary phase wild-type USA300 LAC (60 μl, \(~10^6\) ml\(^{-1}\) in TSB) across the surface. When AFN-1252 was used in assays, TSA was spread with *Streptococcus agalactiae* COH1 instead of *S. aureus* as this bacterium is naturally resistant to the FASII inhibitor but susceptible to daptomycin. Thereafter the plate was dried before the wells were filled with filter-sterilised culture supernatant. Plates were then incubated for 16 h at 37 °C before the zone of growth inhibition around the well was measured at 4 perpendicular points. To accurately quantify daptomycin activity, a standard plot was generated for the zone of growth inhibition around wells that were filled with TSB supplemented with range of
daptomycin concentrations. This enabled the conversion of the size of the zone of inhibition into percentage daptomycin activity.

**Phospholipid detection and quantification**

*S. aureus* membrane lipid was detected and quantified using FM-4-64 (Life Technologies) as described previously [9,10]. Bacterial culture supernatants (200 µl) were recovered by centrifugation (17,000 x g, 5 min) and then mixed with FM-4-64 to a final concentration of 5 µg ml\(^{-1}\) in the wells of clear flat-bottom microtitre plates with black walls appropriate for fluorescence readings (Greiner Bio-one). Fluorescence was measured using a Tecan microplate reader, with excitation at 565 nm and emission at 660 nm to generate values expressed as relative fluorescence units (RFU). Samples were measured in triplicate for each biological repeat. TSB with or without fatty acids was mixed with the FM-4-64 dye and used as a blank. The readings were analysed by subtracting the values from the blank readings and plotted against time.

**Antibiotic resistance selection assay**

Stationary phase *S. aureus* was inoculated at \(~10^8\) CFU ml\(^{-1}\) into 3 ml TSB with 0.5 mM CaCl\(_2\) containing antibiotics as specified, for 8 h per exposure. Daptomycin (20 µg ml\(^{-1}\)) and/or AFN-1252 (0.15 µg ml\(^{-1}\)) were used singly or in combination. After 8 h, bacterial survival was determined by calculating the fold-change (for assays with the bacteriostatic AFN-1252 only) or percentage-change (for assays with the bactericidal antibiotic daptomycin) in CFU relative to the inoculum. For repeated antibiotic exposure, 1 ml was removed from each culture post-antibiotic exposure, centrifuged (3 min, 17,000 x g) and the resulting pellet washed once in TSB before resuspension in 100 µl TSB. This was used to inoculate 3 ml TSB before incubation for 16 h at 37 °C with shaking (180 RPM) in the absence of antibiotics. Bacterial exposure to antibiotics was then repeated twice for a total of three repeated exposures. In some experiments, the broth was supplemented with a fatty acid cocktail prepared as follows: myristic, palmitic and oleic acid (all from Sigma-Aldrich) were made up to 100 mM in dimethyl sulfoxide (DMSO) as described previously [26]. Where used, the fatty acid cocktail was diluted 1 in 2000 in culture medium to obtain a final concentration of 50 µM to provide a balance between previous work [26] and physiological relevance [27]. In some cases, TSB was also supplemented with human serum albumin (Sigma-Aldrich) at 10 µg ml\(^{-1}\) to improve solubility of the fatty acids without reducing the activity of the antibiotics.

**Determination of antibiotic minimal inhibitory concentrations**
Antibiotic susceptibility was determined using the broth microdilution procedure as described previously [42] to generate minimal inhibitory concentrations for daptomycin and AFN-1252. Antibiotics were diluted serially in 2-fold steps in TSB containing 0.5 mM CaCl$_2$ in a 96-well microtitre plate to obtain a range of concentrations. In some assays, a fatty acid cocktail (50 µM) was added to the broth as described above for the resistance selection assay. Stationary phase bacteria were added to the wells to give a final concentration of 5 × 10$^5$ CFU ml$^{-1}$ and the microtitre plates incubated statically in air at 37 °C for 18 h. The MIC was defined as the minimum concentration of antibiotic needed to inhibit visible growth of the bacteria [38]. For some assays, fold change in MIC was calculated relative to the MIC of the USA300 ΔagrA mutant which had not been exposed to antibiotics.

**PCR amplification and sequencing of fabD**

PCR amplification of fabD was performed using the colony PCR technique. A single colony was suspended into 50 µl nuclease-free H$_2$O and microwaved for 3 min to lyse cells, then centrifuged for 2 min (13,300 RPM, room temperature) to pellet cell debris. Supernatant (5 µl) containing gDNA was used for each PCR reaction using Phusion High-Fidelity DNA Polymerase (New England Biolabs). PCR cycling conditions were as follows: 98 °C for 10 min; 30 cycles of 98 °C for 30 sec, 56.8 °C for 30 sec, 72 °C for 30 sec, with a final step at 72 °C for 5 min. PCR products were purified using the QIAquick PCR Purification Kit (Qiagen) following manufacturer’s instructions. Purified DNA was sequenced by Sanger sequencing (GATC Biotech, Germany) using the same forward primer used for PCR amplification. Primer sequences were obtained from [26] and are as follows: FabDfd 5’-GAAGGTACTGTAGTTAAAGCACACG-3’; FabDrev 5’-GCTTTGATTTCTTCGACTACTGCTT-3’.

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### Table 1. Strains used in this study.

| Strain                  | Relevant characteristics/information                          | Agr activity (haemolytic activity) | Reference or source |
|-------------------------|-----------------------------------------------------------------|-----------------------------------|---------------------|
| USA300 LAC              | Wild-type community-associated MRSA strain isolated            | +++                               | [41]                |
| USA300 LAC ΔagrA        | Agr-defective mutant lacking the agrA gene                      | -                                 | [9]                 |
| CC6                     | MRSA isolated from a bloodstream infection                      | +++                               | CHX                 |
| CC7                     | MRSA isolated from a bloodstream infection                      | +/-                               | CHX                 |
| CC9                     | MRSA isolated from a bloodstream infection                      | +/-                               | CHX                 |
| CD1                     | MRSA isolated from a bloodstream infection                      | -                                 | CHX                 |
| CD2                     | MRSA isolated from a bloodstream infection                      | ++                                | CHX                 |
| CD3                     | MRSA isolated from a bloodstream infection                      | -                                 | CHX                 |
| CD4                     | MRSA isolated from a bloodstream infection                      | +++                               | CHX                 |
| CD5                     | MRSA isolated from a bloodstream infection                      | -                                 | CHX                 |
| CD6                     | MRSA isolated from a bloodstream infection                      | -                                 | CHX                 |
| CD8                     | MRSA isolated from a bloodstream infection                      | +/-                               | CHX                 |

CHX: Charing Cross Hospital clinical Diagnostic Microbiology Laboratory.
Table 2. MICs of daptomycin, AFN-1252 and oxacillin in relevant growth media (μg ml⁻¹).

| Strain          | USA300 WT | USA300 ΔagrA | CC6 | CC7 | CC9 | CD1 | CD2 | CD3 | CD4 | CD5 | CD6 | CD8 |
|-----------------|-----------|---------------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| **Daptomycin**  |           |               |     |     |     |     |     |     |     |     |     |     |
| TSB             | 1         | 1             | 1   | 1   | 1   | 2   | 2   | 1   | 1   | 1   | 1   | 1   |
| TSB 1.25Ca      | 0.5       | 0.5           | 0.25| 0.25| 0.25| 0.5 | 0.5 | 0.25| 0.25| 0.5 | 0.25| 0.25|
| MHB 1.25Ca      | 0.25      | 0.25          | 0.25| 0.25| 0.25| 0.25| 0.25| 0.25| 0.25| 0.25| 0.25| 0.25|
| TSB/serum       | 0.5       | 0.5           | 0.5 | 0.5 | 0.5 | 1   | 1   | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 |
| **AFN-1252**    |           |               |     |     |     |     |     |     |     |     |     |     |
| TSB 0.015       | 0.015     | 0.015         | 0.015| 0.008| 0.015| 0.015| 0.015| 0.015| 0.015| 0.015| 0.015| 0.008|
| TSB 1.25Ca      | 0.015     | 0.015         | 0.015| 0.008| 0.03 | 0.015| 0.03 | 0.015| 0.03 | 0.015| 0.015| 0.008|
| MHB 1.25Ca      | 0.015     | 0.015         | 0.015| 0.008| 0.015| 0.015| 0.015| 0.008| 0.015| 0.008| 0.008| 0.008|
| TSB/serum       | 0.06      | 0.06          | 0.06 | 0.06 | 0.06 | 0.06 | 0.06 | 0.06 | 0.06 | 0.06 | 0.06 | 0.06 |
| **Oxacillin**   |           |               |     |     |     |     |     |     |     |     |     |     |
| TSB 4           | 4         | 4             | 32  | 8   | 4   | >128| 32  | 128 | 32  | 16  | 16  | 16  |
| TSB 1.25Ca      | 4         | 4             | 8   | 8   | 4   | >128| 8   | 128 | 16  | 8   | 8   | 8   |
| MHB 1.25Ca      | 2         | 2             | 8   | 2   | 2   | >128| 4   | 128 | 8   | 4   | 2   | 2   |
| TSB/serum       | 8         | 8             | 64  | 16  | 8   | >128| 32  | 128 | 32  | 32  | 32  | 32  |

TSB 0.5Ca: TSB containing 0.5 mM CaCl₂, TSB 1.25Ca: TSB containing 1.25 mM CaCl₂, MHB 1.25Ca: MHB containing 1.25 mM CaCl₂, TSB/serum: TSB containing 1.25 mM CaCl₂ and 50% normal human serum.
Figure 1. Effect of exogenous fatty acids on daptomycin-induced phospholipid release, daptomycin inactivation and bacterial survival. *S. aureus* ΔagrA was exposed to daptomycin (20 µg ml⁻¹) in the presence of the indicated fatty acid supplements (20 µM) or none (No FA), and the release of phospholipids (A), antibiotic activity (B) and bacterial survival (C) measured over time. Data...
represent the means of 4 independent experiments and error bars show the standard deviation of the mean. Values significantly different (P < 0.05) from bacteria in broth without fatty acid supplements were identified by 2-way repeated measures ANOVA and Dunnett’s post-hoc test (*).

Figure 2. Effect of increasing concentrations of oleic acid on daptomycin-induced phospholipid release, daptomycin inactivation and bacterial survival. The S. aureus ΔagrA mutant was exposed to daptomycin (20 µg ml⁻¹) in the presence of the indicated concentrations of oleic acid and the release of phospholipids (A), antibiotic activity (B) and bacterial survival (C) measured over time. For (B), the values for 20 µM are obscured by the symbols representing 100 µM. Data represent the means of 4 independent experiments and error bars show the standard deviation of the mean. Values significantly different (P < 0.05) from bacteria in broth without fatty acid supplements were identified by 2-way repeated measures ANOVA and Dunnett’s post-hoc test (*).
Figure 3. Human serum albumin prevents the use of exogenous oleic acid in daptomycin-induced phospholipid release. The *S. aureus* ΔagrA mutant was exposed to daptomycin (20 µg ml$^{-1}$) in TSB.
containing 50% delipidated human serum containing oleic acid (20 µM) or not (No FA), and bacterial
survival (A) and antibiotic activity (B) measured over time. In a similar experiment, the S. aureus
ΔagrA mutant was exposed to daptomycin in TSB containing human serum albumin (HSA) and
supplemented with oleic acid (20 µM) or not (No FA), and bacterial survival (C) and antibiotic activity
(D) measured over time. Data represent the means of 4 independent experiments and error bars
show the standard deviation of the mean. There were no significant differences in values obtained
with oleic acid compared to un-supplemented medium (P >0.05) as determined by 2-way repeated
measures ANOVA.

Figure 4. AFN-1252 blocks phospholipid release and therefore preserves daptomycin activity. The S.
aureus ΔagrA mutant was exposed to daptomycin (20 µg ml⁻¹), AFN-1252 (0.15 µg ml⁻¹), or both
antibiotics in the absence (A,B,C) or presence (D,E,F) of oleic acid (20 µM). During incubation, bacterial survival (A,D), quantity of phospholipid released into the supernatant (B,E) and daptomycin activity (C,F) was measured over 8 h. Data represent the means of 4 independent experiments and error bars show the standard deviation of the mean. Values significantly different (P <0.05) from those obtained with bacteria exposed to daptomycin only were identified by 2-way repeated measures ANOVA and Dunnett’s post-hoc test (*).
Figure 5. AFN-1252 blocks phospholipid release at various concentrations of daptomycin. WT *S. aureus* (A,C,E,G,I,K) or ΔagrA mutant (B,D,F,H,J,L) was exposed to daptomycin at the indicated concentrations in the absence (blue bars) or presence (orange bars) of AFN-1252 (0.15 µg ml⁻¹) in the presence of 0.5 mM CaCl₂ (A,B,E,F,I,J) or 1.25 mM CaCl₂ (C,D,G,H,K,L). After 8 h incubation, bacterial survival (A-C), phospholipid release (E-H) and daptomycin activity (I-L) were measured. Values significantly different for experiments done with AFN-1252 (P <0.05) from those obtained with bacteria exposed to daptomycin only were identified by paired student’s t test (*).
Figure 6. AFN-1252 preserves daptomycin activity in serum. Wild-type *S. aureus* USA300 or the ΔagrA mutant was exposed to daptomycin (20 µg ml⁻¹), AFN-1252 (0.15 µg ml⁻¹), both antibiotics or neither in TSB containing 50% normal human serum. During incubation, bacterial survival (A,B) and daptomycin activity (C,D) was measured over 8 h. Data represent the means of 4 independent experiments and error bars show the standard deviation of the mean. Values significantly different (P < 0.05) from those obtained with bacteria exposed to daptomycin only were identified by 2-way repeated measures ANOVA and Dunnett’s post-hoc test (*).
Figure 7. AFN-1252 prevents daptomycin inactivation by clinical MRSA isolates. Clinical MRSA isolates from bloodstream infections were exposed to daptomycin (20 µg ml$^{-1}$), AFN-1252 (0.15 µg ml$^{-1}$) or both antibiotics in TSB containing 50% normal human serum. After 8 h incubation, bacterial survival (A) and daptomycin activity (B) were measured. Data represent the means of 4 independent experiments and error bars show the standard deviation of the mean. Values significantly different (P < 0.05) from those obtained with bacteria exposed to daptomycin only were identified by paired student’s t test (*).
Figure 8. Exogenous fatty acids enable the acquisition of resistance to AFN-1252. Ten parallel cultures of the *S. aureus* ΔagrA mutant were exposed to 3 rounds of AFN-1252 (0.15 µg ml⁻¹) treatment in the absence or presence of 50 µM fatty acids (FA) cocktail and absence or presence of human serum albumin (HSA) for 8 h before bacterial replication (A) and the AFN-1252 MIC were determined in the absence or presence of fatty acid cocktail (B, C, D). Each symbol represents an independent culture (*n* = 10 in each case). Differences in survival between the 1st and 3rd rounds of AFN-1252 exposure under identical conditions were analysed using a one-way ANOVA with Dunn’s multiple comparisons test (*P* < 0.001).
Figure 9. Daptomycin prevents the acquisition of fatty-acid enabled resistance to AFN-1252. Ten parallel cultures of *S. aureus ΔagrA* were exposed to 3 rounds of daptomycin (20 µg ml⁻¹) and AFN-1252 (0.15 µg ml⁻¹) in the absence or presence of fatty acid cocktail and absence or presence of human serum albumin (HSA) before bacterial survival (A) and the AFN-1252 MICs determined in the absence or presence of fatty acids (B,C,D) as well as the daptomycin MICs (E,G,G) after each round of exposure to the antibiotic combination. Each symbol represents an independent culture (*n* = 10 in each case). Differences in survival between rounds of antibiotic exposure under identical conditions were identified using a one-way ANOVA with Dunn’s multiple comparisons test (*P* < 0.001).
Figure 10. AFN-1252 prevents daptomycin-induced phospholipid release, even in the case of AFN-1252 resistant strains. Two independent isolates (each represented by an individual circle) of the S. aureus ΔagrA mutant (USA300 ΔagrA) that had not been exposed to antibiotic (A,B,C), three independent isolates of the S. aureus ΔagrA mutant (each represented by an individual square) that had acquired resistance to AFN-1252 in the presence of the fatty acid cocktail but absence of HSA (AFN-1252 R) (D,E,F) or three independent isolates of the S. aureus ΔagrA mutant (each represented by an individual triangle) that had acquired resistance to AFN-1252 in the presence of the fatty acid cocktail and HSA (AFN-1252 R HSA) (G,H,I) were exposed to daptomycin (Dap) in the presence or absence of various combinations of AFN-1252 and fatty acid cocktail (FA) for 8 h. After this time, bacterial survival (A,D,G), the quantity of released phospholipid (B,E,H) and the activity of daptomycin (C,F,I) was determined. Data represent the mean of 3 independent experiments and error bars represent the standard deviation of the mean. Differences in survival, phospholipid release or daptomycin activity were compared between the AFN-1252 susceptible USA300 ΔagrA and AFN-1252 resistant strains.
isolates and AFN-1252 resistant isolates using a one-way ANOVA with Dunn’s multiple comparisons test (*P < 0.01).
