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Abstract

Berberine-INF55 hybrids are a promising class of antibacterials that combine berberine and the NorA multidrug resistance pump inhibitor INF55 (5-nitro-2-phenylindole) together in one molecule via a chemically stable linkage. Previous studies demonstrated the potential of these compounds for countering efflux-mediated antibacterial drug resistance but they didn't establish whether the compounds function as originally intended, i.e. with the berberine moiety providing antibacterial activity and the attached INF55 component independently blocking multidrug resistance pumps, thereby enhancing the activity of berberine by reducing its efflux. We hypothesised that if the proposed mechanism is correct, then hybrids carrying more potent INF55 pump inhibitor structures should show enhanced antibacterial effects relative to those bearing weaker inhibitors. Two INF55 analogues showing graded reductions in NorA inhibitory activity compared with INF55 were identified and their corresponding berberine-INF55 hybrids carrying equivalent INF55 inhibitor structures synthesised. Multiple assays comparing the antibacterial effects of the hybrids and their corresponding berberine-INF55 analogue combinations showed that the three hybrids all show very similar activities, leading us to conclude that the antibacterial mechanism(s) of berberine-INF55 hybrids is different from berberine-INF55 combinations.

Disciplines

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On the Mechanism of Berberine-INF55 (5-Nitro-2-Phenylindole) Hybrid Antibacterials

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Abstract

Berberine-INF55 hybrids are a promising class of antibacterials that combine berberine and the NorA multidrug resistance (MDR) pump inhibitor INF55 (5-nitro-2-phenyindole) together in one molecule via a chemically stable linkage. Previous studies demonstrated the potential of these compounds for countering efflux-mediated antibacterial drug resistance but they didn't establish whether the compounds function as originally intended; i.e. with the berberine moiety providing antibacterial activity and the attached INF55 component independently blocking MDR pumps, thereby enhancing the activity of berberine by reducing its efflux. We hypothesized that if the proposed mechanism is correct then hybrids carrying more potent INF55 pump inhibitor structures should show enhanced antibacterial effects relative to those bearing weaker inhibitors. Two INF55 analogues showing graded reductions in NorA inhibitory activity compared to INF55 were identified and their corresponding berberine-INF55 hybrids carrying equivalent INF55 inhibitor structures synthesised. Multiple assays comparing the antibacterial effects of the hybrids and their corresponding berberine/INF55 analogue combinations showed that the three hybrids all show very similar activities, leading us to conclude that the antibacterial mechanism(s) of berberine-INF55 hybrids is different from berberine/INF55 combinations.

Introduction

A promising strategy for countering efflux-mediated antibiotic resistance in bacteria is to co-administer a small molecule multi-drug resistance (MDR) efflux pump inhibitor (EPI) in combination with an antibacterial.[1] In this strategy, the MDR inhibitor serves to limit efflux of the antibacterial and raise its intracellular concentrations above sub-lethal levels to enhance antibacterial potency. Potential clinical disadvantages of the approach, however, include the requirement for matching pharmacokinetic and physicochemical properties of two structurally unrelated molecules, along with other co-dosing challenges. One possible solution is to covalently link the MDR inhibitor and antibacterial components together into a single (non-cleavable) hybrid molecule.[2-4] Such hybrids carry the potential advantage of delivering equimolar quantities of the two agents to infection sites whilst avoiding the complications of multi-agent co-dosing.[5]

In 2006 Bremner *et al.* reported the first such hybrid, termed SS14-O **1** (Fig. 1),[2] comprising the antibacterial alkaloid berberine substituted at its 13-position *via* a stable 2'-CH_2 linkage to 5-nitro-2-phenyindole **5** (INF55), a well-known inhibitor of the NorA MDR pump in *Staphylococcus aureus*.[6] In designing SS14-O **1** it was reasoned that the berberine moiety (a known substrate for NorA)[7] could show enhanced antibacterial effects (membrane activity and interactions with DNA)[8] as part of a hybrid due to higher intracellular concentrations arising through inhibition of NorA-mediated efflux by the appended INF55 **5** component. SS14-O **1** was shown to accumulate in wild-type, *norA*-knockout and NorA overexpressing strains of *S. aureus* and showed higher antibacterial potency than berberine alone or berberine in combination with INF55 **5**.[2] A follow-up study explored the effects of varying the relative orientations of the berberine and INF55 components in hybrids by comparing the activities of isomers SS14-O **1**, SS14-M **2** and SS14-P **3** (Fig. 1).[9] The three isomers showed remarkably similar minimum inhibitory concentrations (MICs) given their structural differences, which remained essentially unchanged across wild-type, *norA*-knockout, and NorA overexpressing *S. aureus* cells. The three isomers accumulated in *S. aureus* cells and showed identical abilities to block *Enterococcus*

faecalis-mediated killing of the nematode *Caenorhabditis elegans* in a gastrointestinal infection model. A key conclusion from these studies was that berberine-INF55 hybrids are not substrates for NorA, although ethidium bromide uptake/efflux experiments suggested that these hybrids might block the NorA pump.[9] Another study exploring an SS14-O **1** analogue with an extended methylene ether linkage (**4**, Fig. 1) showed that this compound displays similar antibacterial activity to the other hybrids and that its activity remains consistent across *S. aureus* strains expressing varying levels of NorA.[10]

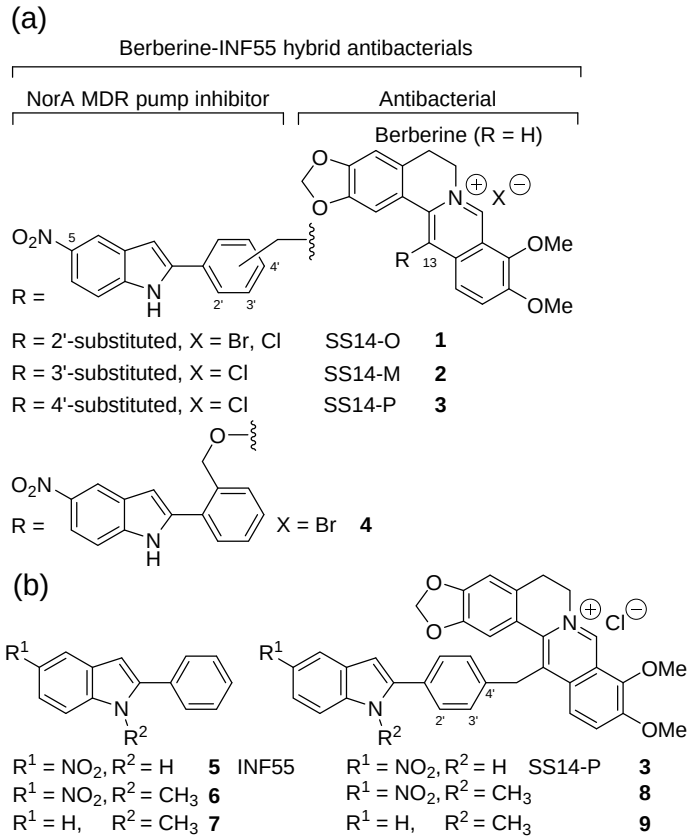


Fig. 1. (a) Berberine-INF55 hybrid antibacterials **1**-**4**.[2,9,10] (b) INF55 (5-nitro-2-phenylindole) **5**, *N*-Methyl-INF55 **6** and *N*-Methyl-2-phenylindole **7** and their corresponding berberine-INF55 hybrids **3**, **8** and **9**.

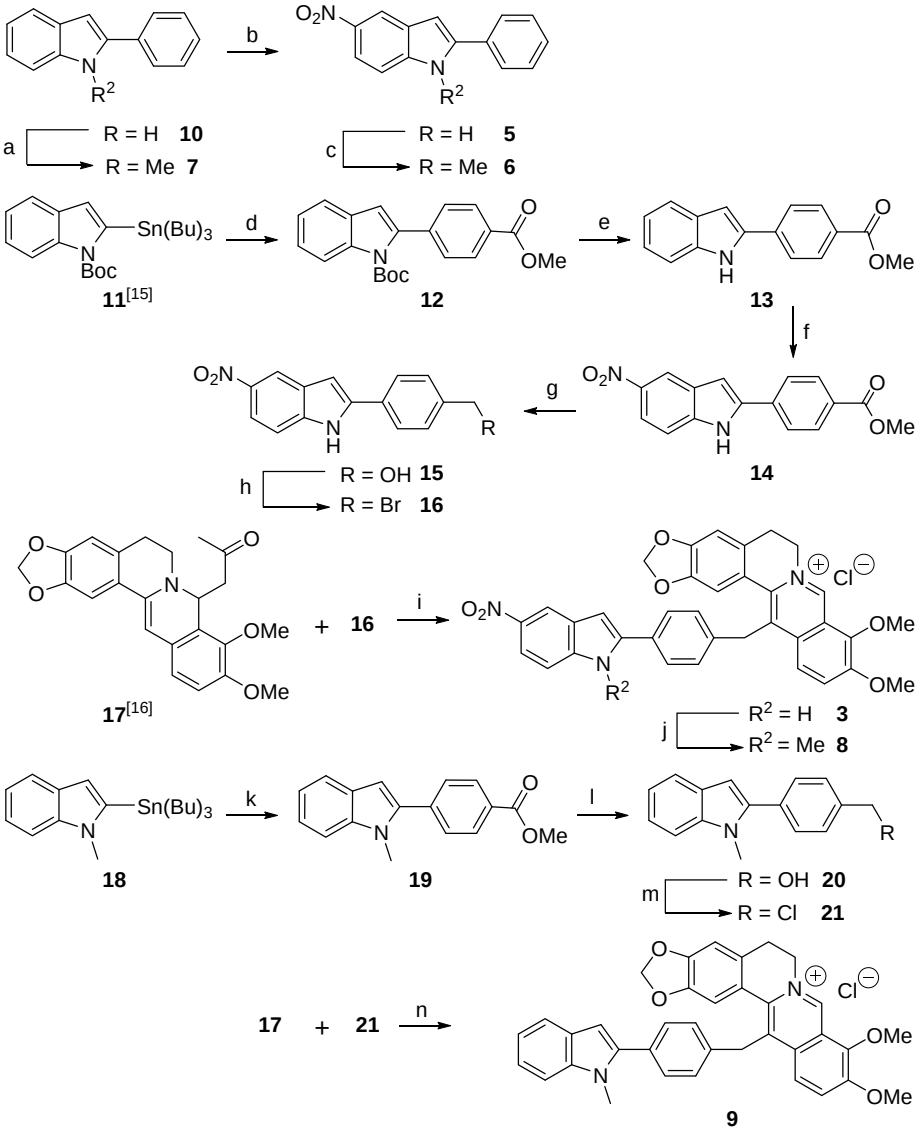
While the above studies demonstrated the promising antibacterial properties of berberine-INF55 hybrids, the observation that the hybrids do not appear to be substrates for NorA cast doubt on whether the hybrids function as originally intended; i.e. with the INF55 moiety serving to block NorA MDR pumps (and thus efflux) and the attached berberine moiety providing (enhanced) antibacterial action. In this current study we further explored whether the proposed mechanism of the hybrids was indeed underpinning their activity. Central to the study was the hypothesis that if the proposed mechanisms were at play then a direct correlation should exist between the ability of structurally distinct INF55-type MDR pump inhibitors to potentiate the antibacterial activity of berberine when co-administered and the activity of the corresponding berberine/INF55 hybrids. In other words, INF55-type MDR pump inhibitors that more strongly potentiate the antibacterial activity of berberine when co-administered should confer a higher level of antibacterial potency to the corresponding berberine-INF55 hybrids.

Exploring this hypothesis required analogues of INF55 **5** that: (1) showed a range of antibacterial potentiation effects when co-administered with berberine and (2) could be attached at the berberine 13-position to create hybrids differing only in the structure of the appended INF55 moiety. Checkerboard assays (see below) identified *N*-Methyl-INF55 **6** and *N*-methyl-2-phenylindole **7** as suitable INF55 **5** analogues (Fig. 1). This paper reports the synthesis and parallel evaluation of berberine/INF55 hybrids **3**, **8** and **9**, which incorporate INF55 **5** and analogues **6** and **7**, respectively (Fig. 1), and their corresponding berberine/**5**,**6**,**7** combinations, in multiple assays aimed at testing the above hypothesis.

Chemistry

The synthesis of INF55 **5**, analogues **6** and **7** and hybrids **3**, **8** and **9** is outlined in Scheme 1. INF55 **5** was obtained in 91% yield via regioselective nitration of 2-phenyl indole **10** using the literature method.[11] *N*-Methyl-INF55 **6** was prepared in 80% yield by stirring INF55 **5** for two hours at room temperature with K_2CO_3 and CH_3I in anhydrous DMF. *N*-Methyl-2-phenylindole **7** was

prepared in 80% yield by stirring 2-phenylindole **10** for 2 hours in anhydrous THF at room temperature with NaH and CH_3I.



Scheme 1. Reagents and conditions: (a) NaH, CH_3I, THF, 80%; (b) $NaNO_3$, H_2SO_4, -10 °C, 70%;[11] (c) K_2CO_3, CH_3I, DMF, 80%; (d) $PdCl_2$, 1,4-dioxane, methyl 4-iodobenzoate, 100 °C, 84%; (e) TFA:CH_2Cl_2 (1:1), 79%; (f) $NaNO_3$, H_2SO_4, -10 °C;[12] (g) $LiBH_4$, THF, 40 °C;[12] (h) CBr_4, PPh_3, THF:Et_2O (1:1);[12] (i) NaI (10 mol %), CH_3CN, 70 ^oC, anion exchange 40%; (j) CH_3I, K_2CO_3, DMF, anion exchange, 75%; (k) $PdCl_2$, methyl 4-iodobenzoate, 1,4-dioxane, 100 °C, 75%; (l) $LiAlH_4$, THF, 40 °C, 85%; (m) CCl_4:CH_2Cl_2 (1:1), PPh_3, ~65% (crude); (n) NaI (10 mol %), CH_3CN, 70 ^oC, anion exchange, 40%.

Our previously reported synthesis of SS14-P **3**[12] involved reacting 8-allyldihydroberberine[13] in the final step with the key benzylic bromide intermediate **16**; prepared *via* functional group manipulations with the precursor methyl ester **13**. In the prior work, **13** was synthesised directly from indole and methyl-4-iodobenzoate in a single step using the rhodium (III)-catalysed indole C2-arylation method reported by Sames et al.[14] While this reaction invariably provides some **13**, the yields are always low (max 28%) and the reaction outcomes unpredictable. A more robust Stille coupling-based approach was therefore developed to install the functionalised 4-carboxymethyl aryl substituent at the indole C2-position. Stille coupling of *N*-Boc-2-tributylstannylindole **11** (prepared in two steps via the literature method)[15] with methyl-4-iodobenzoate using $PdCl_2$ in refluxing 1,4-dioxane gave **12** in 84% yield. Boc-deprotection of **12** using CH_2Cl_2:TFA (1:1) subsequently afforded the NH-indole **13** in 79% yield. While the new route to **13** is longer, it is simple to carry out and reproducible on a multi-gram scale. The key intermediate **16** was then obtained from **13** using our reported 3-step nitration, reduction, bromination sequence.[12]

A new reaction for producing SS14-P **3** was developed wherein bromide **16** was coupled to 8-acetonyldihydroberberine **17**[16] instead of 8-allyldihydroberberine, as had been performed previously.[12] Catalytic Finkelstein conversion of bromide **16** *in situ* to the iodide with 10 mol% NaI in CH_3CN at 70 ^oC in the presence of 8-acetonyldihydroberberine **17** gave SS14-P **3** in 40% yield. Preparative RP-HPLC purification in the presence of 0.1% HCl initially provided mixed Cl^-/Br^-/I^- salts that were subsequently converted to pure Cl^- salts of **3** by anion exchange (Scheme 1). Although the new procedure didn't provide higher yields of SS14-P **3** it was more reproducible than the 8-allyldihydroberberine method,[12] which for unknown reasons sometimes failed to yield any **3**.

SS14-P **3** was converted to the *N*-methylated hybrid **8** in 75% yield by reaction with excess CH_3I and K_2CO_3 in DMF at room temperature. The mixture of Cl^-/I^- salts initially obtained after silica gel column chromatography (CH_3CN:EtOAc:MeOH, 1:1:0.5) was converted to the pure Cl^- salt **8** by anion exchange. Hybrid **9** was prepared by reacting 8-acetonyldihydroberberine **17** with

the benzylic chloride intermediate **21**, which was synthesised in 3 steps from (*N*-methylindol-2-yl)tributylstannane **18** (prepared by the literature method).[15] In the first step, $PdCl_2$ catalysed Stille coupling of **18** with methyl-4-iodobenzoate provided **19** in 75% yield.

Reduction of the methyl ester **19** with $LiAlH_4$ (added portion wise) in anhydrous THF with gentle heating at 40 ^oC gave the benzylic alcohol **20** in 85% yield. Chloride **21** was prepared by stirring **20** in CCl_4:CH_2Cl_2 (1:1) for 10 min before adding PPh_3 (4 eq). TLC analysis (EtOAc:hexane, 8.5:1.5) was used to monitor the reaction and upon completion the mixture was quickly filtered through a plug of neutral alumina and washed with CH_2Cl_2. The filtrates were concentrated *in vacuo* and the residue triturated with pentane. The crude **21** was used immediately due to its instability and was unable to be fully characterised. Reaction of crude **21** with 8-acetonyldihydroberberine **17** in the presence of 10 mol% NaI in CH_3CN at 70 ^oC provided hybrid **9** in 40% yield after preparative RP-HPLC and anion exchange.

Checkerboard assays in *S. aureus* strains with varying NorA expression levels

Preliminary antibacterial checkerboard assays[2] were performed using 8325-4 wild-type, K1758 *norA*-knockout and K2378 NorA overexpressing *S. aureus* cells with berberine/**5**-**7** combinations to confirm their suitability as INF55-based NorA EPIs for use in this study (Fig. 2). Complete growth inhibition was observed in all three *S. aureus* strains with INF55 **5** at 1.25 μg/mL and berberine present at concentrations below 20 μg/mL. Analogues **6** and **7** at 1.25 μg/mL did not inhibit growth of 8325-4 and K1758 cells in the presence of berberine at the highest concentrations tested (125 μg/mL or 30 μg/mL). Growth inhibition of K2378 cells was observed with **6** and **7** at 1.25 μg/mL, but only with berberine present at 125 μg/mL. N-Me-INF55 **6** did not inhibit 8325-4 growth at the highest concentration tested (80 μg/mL) with 125 μg/mL berberine present. The results are consistent with the following rank order of berberine antibacterial potentiation effects for the compounds: **5** > **7** > **6**. The graded reductions in activity confirmed **5**-**7** were a suitable series of INF55-based NorA EPIs for testing the above-stated hypothesis.

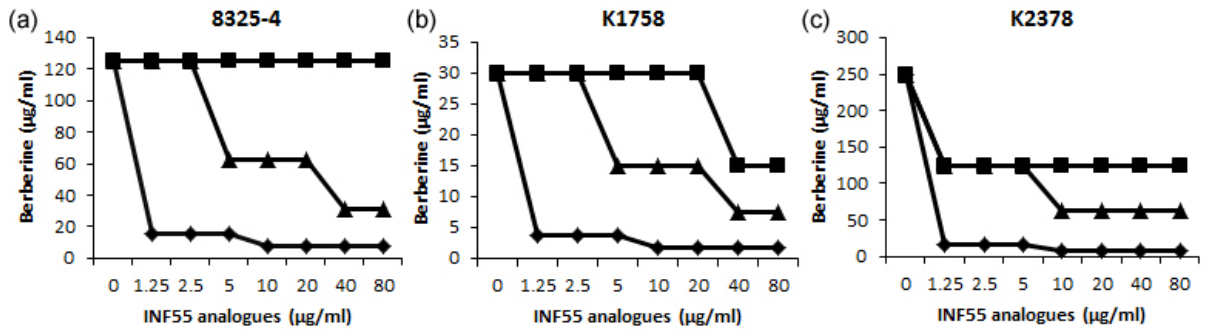


Fig. 2. Checkerboard assays comparing potentiation of berberine's antibacterial effects by INF55 **5** (♦), **6** (■) and **7** (▲) against (a) 8325-4 wild-type, (b) K1758 *norA*-knockout and (c) K2378 NorA overexpressing *S. aureus* cells. Compounds **5-7** showed no antibacterial effects against these strains when administered alone at concentrations below or equal to 80 μg/mL. MICs for berberine alone against 8325-4, K1758 and 2378 were 125 μg/mL, 30 μg/mL and 250 μg/mL, respectively.[2]

Antibacterial activities against *S. aureus* strains

The checkerboard experiments indicated that potentiation of berberine's activity by the three INF55-based NorA EPIs **5-7** decreases in the order **5** > **7** > **6** against 8325-4 wild-type, K1758 *norA*-knockout and K2378 NorA overexpressing *S. aureus* cells. Accordingly, if the above-stated hypothesis were correct then their respective hybrids **3**, **8** and **9** should show antibacterial potencies in the order **3** > **9** > **8** against these cells, assuming no synergistic or antagonistic action between the two components when joined. The MICs for complete inhibition of bacterial growth was measured for hybrids **3**, **8** and **9** against the *S. aureus* panel with vancomycin included as a control (Table 1). All three hybrids showed identical MICs (0.78 μg/mL) against 8325-4 and K2378 and 2-fold higher potencies (0.39 μg/mL) against K1758. The MIC of vancomycin was 1 μg/mL against the three strains. Consistent MICs (< 2-fold difference) for **3**, **8** and **9** confirmed that they are all poor substrates for NorA. Lack of variation in MICs was a feature observed previously with hybrids **1-4** and indicates that the molecular target(s) of berberine-INF55 hybrids is(are) tolerant of structural variations within the INF55 portion. However, unvarying MICs for hybrids **3**, **8** and **9** is

not consistent with the stated hypothesis, since if correct, the MICs would have increased in the order **3** < **9** < **8**.

S. aureus **strains**	**Compound**	**MIC (μg/ml)**
8325-4	**3**	0.78
	8	0.78
	9	0.78
	vancomycin	1
K1758	**3**	0.39
	8	0.39
	9	0.39
	vancomycin	1
K2378	**3**	0.78
	8	0.78
	9	0.78
	vancomycin	1

Table 1. MICs of hybrids **3**, **8**, and **9** and vancomycin (control) against wild-type (8325-4), *norA*-knockout (K1758) and NorA overexpressing (K2378) *S. aureus* strains.

Uptake into *S.aureus* cells

Uptake of hybrids **3**, **8** and **9**, berberine and berberine in the presence of INF55 **5** and analogues **6**, **7** into 8325-4, K1758 and K2378 *S. aureus* cells was compared using our previously reported fluorescence-based method.[9] The interaction of berberine or hybrids with DNA upon entering cells causes an increase in fluorescence at 517 nm (excitation at 355 nm), while expulsion of these compounds from cells *via* efflux leads to lower fluorescence intensities, thus providing a

qualitative measure of compound efflux susceptibility. Fig. 3 indicates that berberine at 3 μM alone does not accumulate significantly in any of the *S. aureus* strains, consistent with its high efflux susceptibility. Lack of berberine uptake into *norA*-knockout strain K1758 suggests that pumps other than NorA must also contribute to its efflux. Uptake of berberine did not increase in any of the strains in the presence of equimolar **7** and only small increases were observed withcompounds **5** and **6** present. These results suggest that NorA inhibition by INF55 **5** and analogues **6**, **7** has only a minor effect on berberine accumulation, possibly due to the countering effects of other pumps not affected by these inhibitors. Nevertheless, the ability of INF55 analogues (**5** in particular) to potentiate the antibacterial activity of berberine (Fig. 2) against the same *S. aureus* strains suggests that this seemingly slight effect on berberine uptake is sufficient to enhance antibacterial effects.

Significantly larger increases in fluorescence were observed with hybrids **3**, **8** and **9** at the same concentrations (3 μM), indicating that they are taken up to a greater extent in these cells than berberine or berberine in the presence **5-7** (Fig.3). For these experiments it was necessary to demonstrate that the high fluorescence observed with hybrids was due to increased cellular uptake and not increased fluorescence intensity of hybrid/DNA complex(es) relative to berberine/DNA complex(es). Cell-free control experiments comparing fluorescence upon binding to calf thymus DNA (CT-DNA)[17] of hybrids **3**, **8**, **9**, berberine and berberine in the presence of **5**, **6** and **7** showed that complexes formed between the three hybrids and CT-DNA exhibited significantly less fluorescence than berberine/DNA complexes (data not shown), supporting the conclusion that higher intracellular uptake of **3**, **8** and **9** had occurred relative to berberine alone or berberine in mixtures with **5-7**.

Uptake of each hybrid was unchanged across the three strains, consistent with the compounds not being substrates for NorA (in agreement with the MIC data, Table 1). While the uptake data confirmed that **3**, **8** and **9** accumulate in these cells, there was no evidence to support that incorporation of higher potency INF55-based EPIs leads to increased uptake. If this were true, uptake of the hybrids should have increased in the order **8** < **9** < **3** (based on the checkerboard data

in Fig. 2). Hybrids **8** and **9** appeared to show identical uptake in all strains that was greater than that of **3** in spite of **3** containing the most potent NorA EPI **5**.

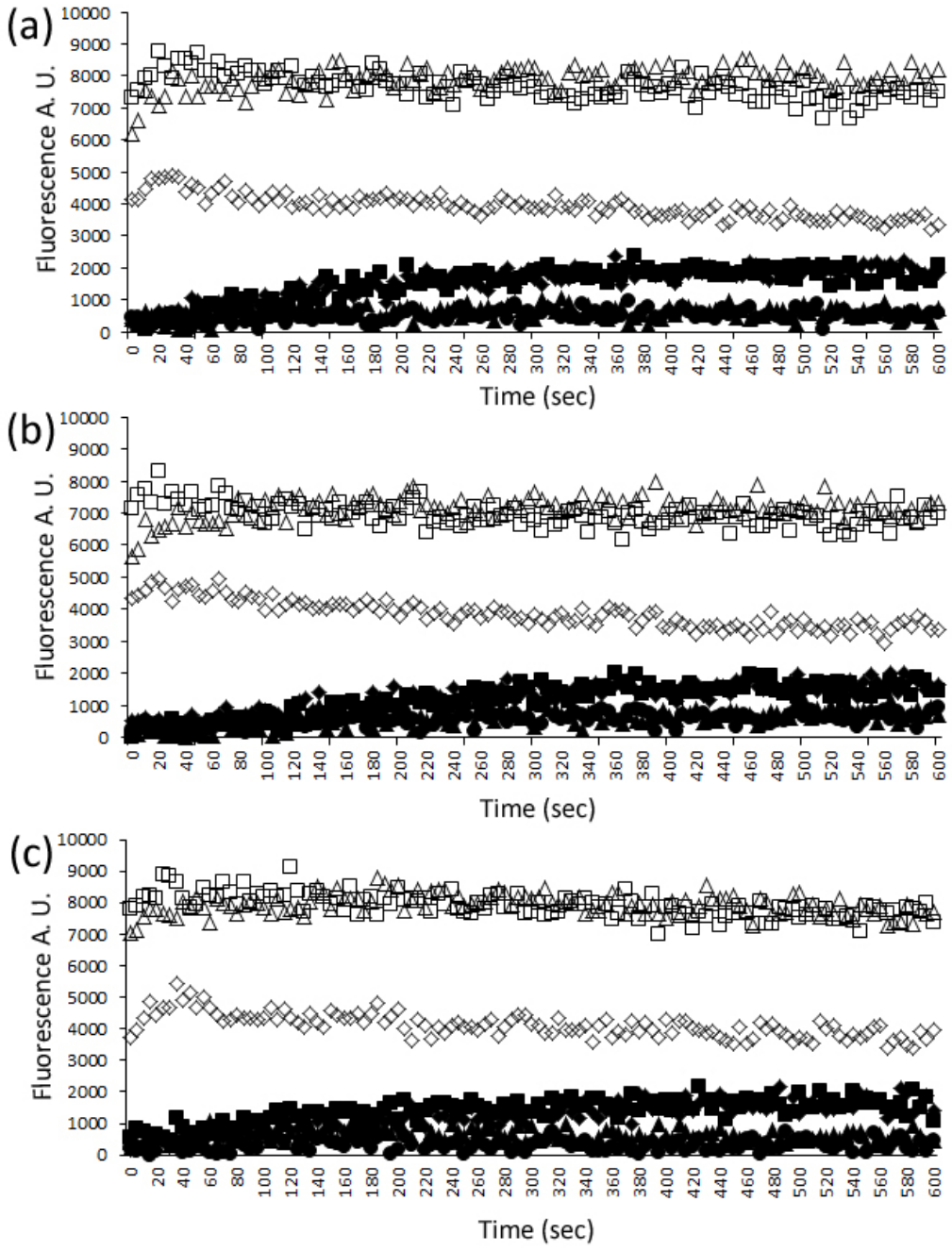


Fig. 3. Uptake of hybrids **3** (◊), **8** (□), **9** (Δ), berberine (●) and berberine in the presence of **5** (♦), **6** (■) and **7** (▲) into (a) 8325-4 wild-type, (b) K1758 *norA*-knockout and (c) K2378 NorA overexpressing *S. aureus* cells. Uptake was measured by monitoring fluorescence at 517 nm (excitation at 355 nm) and is expressed in arbitrary fluorescence units A.U. All compounds were present at 3 μM in 1% DMSO solutions.

Antibacterial activity and checkerboard assays with methicillin-resistant *S. aureus* (MRSA) and *E. faecalis*

Bacterial strains	Compound	MIC (μg/ml)
MRSA MW2	berberine	50
	5	> 100
	6	> 100
	7	> 100
	3	3.13
	8	3.13
	9	< 1.56
	vancomycin	< 1.56
E. faecalis MMH594	berberine	> 100
	5	> 100
	6	> 100
	7	> 100
	3	3.13
	8	3.13
	9	3.13
	vancomycin	< 1.56

Table 2. MICs of INF55 **5**, analogues **6** and **7**, hybrids **3**, **8** and **9** and vancomycin (control) in liquid cultures of MRSA MW2 and *E. faecalis* MMH594.

The above-stated hypothesis was further tested by comparing the curative effects of the hybrids in two *C. elegans* live infection models. In these models, *C. elegans* is infected with methicillin-resistant *S. aureus* (strain MW2) or *E. faecalis* (MMH594) and worm survival is

measured relative to controls. Before performing these experiments it was necessary to measure the MICs of each compound against *S. aureus* MW2 and *E. faecalis* MMH594 cells in liquid cultures (Table 2). Berberine showed an MIC of 50 μg/mL against MRSA MW2 and lower potency (MIC 100 μg/mL) against *E. faecalis* MMH594. All three EPIs **5-7** showed no activity against either strain when administered alone at < 100 μg/mL. Hybrids **3**, **8** and **9** on the other hand, all showed robust but virtually identical activities (MICs 1.56 – 3.13 μg/mL). These data once again conflicted with the hypothesis since MICs for **3**, **8** and **9** should have increased in the order **3** < **9** < **8** if it were supported.

Checkerboard assays were used to compare the berberine potentiation effects of **5-7** in MW2 and MMH594 cells (Fig. 4). As expected, INF55 **5** was the most potent EPI with 25 μg/mL and concentrations of berberine below 10 μg/mL strongly inhibiting MW2. EPIs **6** and **7** required > 50 μg/mL to show any effect. The three EPIs were notably less effective against MMH594, where concentrations of **5** greater than 50 μg/mL and higher concentrations of berberine were required to produce an effect. Compound **7** showed only weak activity at 100 μg/mL and required higher concentrations of berberine. Compound **6** showed no potentiation at the highest concentration tested. The berberine potentiation activity of the EPIs thus decreased in the order **5** > **7** > **6** against MW2 and MMH594, in agreement with the order observed with *S. aureus* strains 8325-4, K1758 and K2378 (Fig. 2). These data supported **5-7** (and their respective hybrids **3**, **8** and **9**) being suitable compounds for testing our berberine/INF55 hybrid mechanism hypothesis in the *C. elegans* live infection models.

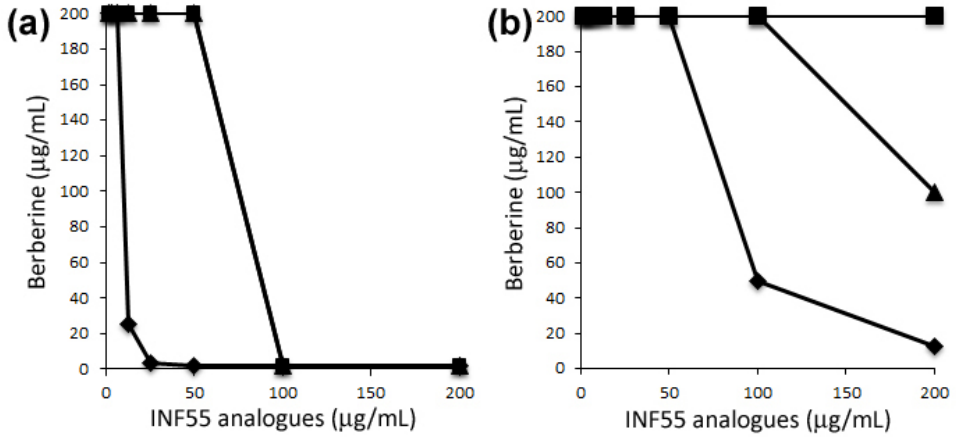


Fig. 4. Checkerboard assays comparing the potentiation of berberine's antibacterial effects by INF55 **5** (♦) and analogues **6** (■) and **7** (▲) against (a) MRSA MW2 and (b) *E. faecalis* MMH594.

C. elegans/MRSA and *C. elegans*/*E. faecalis* live infection models

The *C. elegans*/MRSA live infection model measures worm survival after infection with MRSA MW2.[18] In the absence of antimicrobials the worms die but they can be rescued by treatment with vancomycin (Fig. 5(a), Vanc 20 µg/mL). Increasing concentrations (0-200 µg/mL) of berberine, EPIs **5**-**7** and hybrids **3**, **8** and **9** were used to investigate their effects on MRSA MW2-infected worm survival. Berberine was found to increase survival relative to the 1% DMSO control at concentrations above 50 µg/mL, consistent with its MIC (50 µg/mL) against this species (Table 2). None of the EPIs showed any curative effects when tested alone. Hybrids **3**, **8** and **9**, however, all showed robust curative effects, but there were no clear differences between their potencies. Checkerboard survival assays where berberine was co-administered with EPIs **5**-**7** to worms infected with MRSA were attempted but reproducible data were unable to be obtained, possibly due to the toxicity of INF55-based EPIs to worms.[2]

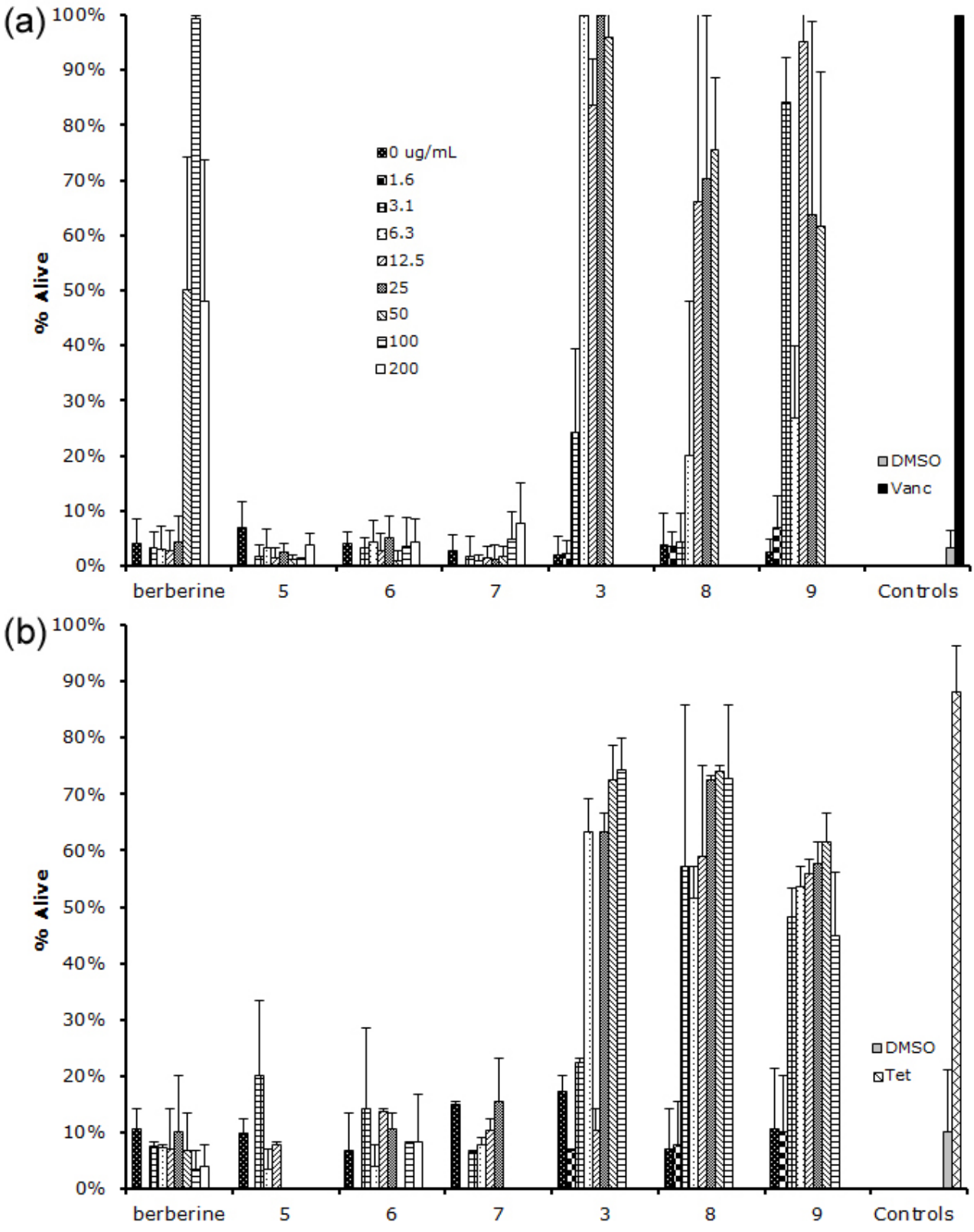


Fig. 5. Survival (%) of *C. elegans* worms infected with (a) MRSA MW2 and (b) *E. faecalis* MMH594 following treatment with increasing concentrations of berberine, INF55 **5** and analogues **6**, **7** and hybrids **3**, **8**, and **9**. Vancomycin (Vanc, 20 μg/mL) and tetracycline (Tet, 20 μg/mL) were included as positive controls in the MRSA and *E. faecalis* experiments, respectively, and 1%

DMSO as a negative control. Compound concentrations are in μg/mL. All assay solutions contained 1% DMSO.

In the *C. elegans*/*E. faecalis* live infection model[9] worms are infected with *E. faecalis* MMH594 and their survival monitored over several days. In the absence of antimicrobials the worms die but they can be rescued with tetracycline (Fig. 5(b), Tet 20 μg/mL). Berberine and EPIs **5**-**7** alone showed no effect on survival over the concentration range tested (0-200 μg/mL). The lack of activity of berberine against *E. faecalis* MMH594 was consistent with its low *in vitro* potency against this organism (MIC > 100 μg/mL, Table 2). The three hybrids **3**, **8** and **9** all showed strong rescuing effects at concentrations above 6.25μg/mL, but once again there were no clear differences between their potencies. As with the MRSA MW2 model, checkerboard experiments were attempted with berberine/ **5**-**7** combinations but reproducible data were unable to be obtained.

Concluding Remarks

This study aimed to test the hypothesis that berberine-INF55 hybrids elicit their antibacterial effects through the combined activities of their two functionally distinct components, with the berberine moiety acting to kill bacterial cells and the INF55 portion serving to block NorA-mediated efflux. Checkerboard assays with three *S. aureus* strains varying in NorA expression levels, along with MRSA MW2 and *E. faecalis* MMH594 strains, established that INF55 analogues **6** and **7** show graded reductions in their berberine potentiation potencies relative to **5**, thus making them suitable compounds for testing the hypothesis. The three EPIs and their corresponding hybrids **3**, **8** and **9** were synthesised using a mix of literature and newly developed chemistry.

The three hybrids showed strong antibacterial activity (measured as MICs) against all strains tested but the activity did not vary between the compounds, a result inconsistent with the hypothesis. Uptake of the three hybrids into *S. aureus* cells was confirmed using fluorescence-based cell assays but again the lack of variation in uptake did not support the hypothesis. The *C. elegans* MRSA and *E. faecalis* live infection experiments clearly demonstrated the robust curative effects of

the hybrids, with worm survival also establishing that the compounds show low toxicity. However, failure to observe significant differences between their activities was further evidence that the hypothesis was not supported. We conclude from this work that the mechanism(s) of antibacterial action of berberine/INF55 hybrids must be different from the mechanisms at play when berberine is co-administered with INF55-based EPIs. Further studies aimed at unravelling the true mechanism of action of this interesting class of antibacterials are therefore warranted.

Experimental

Chemistry

THF and diethyl ether were dried over sodium and DMF and CH_3CN was dried over 4 Å molecular sieves prior to use. 2-Phenylindole **10**, CH_3I, $Sn(Bu)_3Cl$, *n*-butyllithium, $PdCl_2$, CBr_4, PPh_3,$LiAlH_4$, $LiBH_4$ and IRA-904 quaternary ammonium Cl^- anion exchange resin were purchased from Sigma Aldrich. 4-Iodomethylbenzoate was synthesised by $MeOH/H_2SO_4$(cat) esterification of 4-iodobenzoic acid, which was purchased from Matrix scientific. Analytical thin layer chromatography (TLC) was carried out using Merck 0.2 mm silica gel 60 F_{254} coated aluminium plates. Compounds were visualised by UV absorption (λ 254 nm) and/or staining with cerium ammonium molybdate. Column chromatography was performed using Merck silica gel 60 (230-400 mesh). Low resolution electrospray ionisation mass spectra (ESI-MS) were obtained on a micromass Z-path (LCZ) spectrometer. Electron impact high resolution mass spectra (HRMS) were obtained on a Fisions/VG Autospec spectrometer using perfluorokerosene as internal standard. ESI HRMS were obtained on a Waters QT *Ultima* spectrometer using polyethylene glycol or polypropylene glycol as internal standard. ^1H, ^{13}C NMR experiments were performed using a Varian Mercury 300 MHz, Varian Inova 500 MHz or Varian Premium Shielded 500 MHz spectrometer at 25 ^oC. Chemical shifts are reported as δ (ppm) relative to internal TMS (or solvent where indicated). The abbreviations s = singlet, d = doublet, appt = apparent triplet, t = triplet, q = quadruplet, m = multiplet and bs = broad singlet are used throughout. RP-HPLC purifcations of

hybrids **3** and **9** were performed using gradient elutions with solvents A (100% H_2O, 0.1% HCl) and B (90% CH_3CN, 10% H_2O, 0.1% HCl) on a Sunfire ^{TM} Prep C18 OBD^{TM} (5μM) steel jacketed column run at 30 mL.min^{-1} and detection at 254 nm. Analytical HPLC analyses were performed using a Shimadzu CLASS-LC10 *VP* system using gradient elutions with solvent A and B on a Phenomenex Luna μM C18 column run at 1mL.min^{-1} with detection at 254 nm.

9,10-dimethoxy-13-(4-(5-nitro-1H-indol-2-yl)benzyl)-5,6-dihydro-[1,3]diolo[4,5-]isoquinolino[3,2-a]isoquinolin-7-ium chloride **3**

To a stirred solution of key bromide **16** (100 mg, 0.30 mmol) in anhydrous CH_3CN (10 mL) was added sodium iodide (49 mg, 0.33mmol) and the mixture stirred for 1 h at 70 ^oC. 8-Acetonyl dehydroberberine **17** (118.8 mg, 0.30 mmol) was then added and stirring continued at 70 ^oC for another 3-4 h. The reaction was monitored by TLC (MeOH:$CHCl_3$, 1:9 or EtOAc:hexane, 1:4) and ESI-MS. After completion, the reaction was diluted with CH_3CN (5 mL) and the product adsorbed onto silica gel via evaporation of the solvent. Purification was performed using column chromatography (EtOAc:CH_3CN:hexane, 1:1:1) initially followed by (EtOAc:CH_3CN:MeOH, 1:1:0.3) to afford semi pure **3**. Further purification was carried out using RP-HPLC (gradient from A 0% to 100% B over 30 min, R_t = 18.8 min). Fractions containing the product were combined and concentrated by freeze-drying. The salt mixture obtained was stirred with IRA-904 quaternary ammonium Cl^- anion exchange resin in MeOH (5 mL) at rt for 1 h. Filtration and concentration yielded pure **3** as a yellow amorphous solid. (75 mg, 40%). mp 218-220 °C (decomp);^{1}HNMR (500 MHz, DMF-*d6*): δ 3.26 (s, 2H), 4.03 (s, 3H), 4.11 (s, 3H), 4.84 (s, 2H), 5.15 (bs, 2H), 6.06 (s, 2H), 7.04 (s, 1H), 7.11 (s, 1H), 7.17 (s, 1H), 7.32 (d, *J* = 7.5 Hz, 2H), 7.62 (d, *J* = 9.0 Hz, 1H), 7.81-7.93 (m, 3H), 8.07 (m, 2H), 8.46 (s, 1H), 10.1 (s, 1H), 13.05 (s, 1H); ^{13}C NMR (125 MHz, DMF-*d6*): δ 28.3, 36.3, 57.4, 58.1, 62.5, 101.3, 103.1, 109.1, 112.4, 117.4, 121.2, 122.4, 122.5, 126.8, 127.0, 128.9, 129.5, 130.8, 131.0, 133.9, 135.0, 138.4, 140.3, 141.6, 142.0, 142.1, 145.4, 146.2, 147.5, 150.4, 151.2, ESI-MS *m/z* Calc for $C_{35}H_{28}N_3O_6^+$ 586.1973; observed 586.1997.

*1-Methyl-5-nitro-2-phenyl-1H-indole **6***

To a stirred solution of INF55 **5** (1g, 4.2 mmol) in anhydrous DMF (10 mL) was added oven dried K_2CO_3 (1.74 g, 12.6 mmol) and stirred for 15-20 min at rt. Methyl iodide (0.8 mL, 12.6 mmol) in DMF (5 mL) was then added dropwise at rt and stirred for 1-2 h. Progress of the reaction was monitored by TLC (EtOAc:Hexane, 1:4). The reaction mixture was diluted with water and extracted with EtOAc (3 x 10 mL), the organic layer was washed with water and brine and dried over anhydrous Na_2SO_4 before concentrating *in vacuo* to afford **6** as a yellow solid (847 mg, 80%). mp 175-178 ^oC; ^1H NMR (300 MHz, $CDCl_3$): δ 3.80 (s, 3H), 6.71 (s, 1H), 7.37 (d, *J* = 6.0 Hz, 1H), 7.48-7.51 (m, 5H), 8.14 (d, *J* = 6.0 Hz, 1H), 8.58 (s, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 31.9, 103.7, 109.4, 117.2, 117.5, 127.0, 128.7, 129.3, 131.4, 140.9, 141.8, 144.7. ESI-MS *m/z* Calc. for $C_{15}H_{13}N_2O_2$ $[M+H^+]$ 253.0977; observed 253.0970.

1-Methyl-2-phenyl-1H-indole **7**

To a stirred solution of 2-phenylindole **5** (1.0 g, 5.2 mmol) in anhydrous THF (10 mL) was added sodium hydride (372 mg, 15.5 mmol) and the reaction was stirred for 15 min at rt. Methyl iodide (2.20 g, 15.5 mmol) in anhydrous THF (10 mL) was added dropwise to the reaction mixture and stirred for 2 h. The reaction was monitored by TLC (EtOAc:Hexane, 1:9). The reaction mixture was quenched with saturated sodium sulphate and extracted with EtOAc (3 x 10 mL). The organic layer was separated, dried over anhydrous $MgSO_4$ and concentrated *in vacuo* to afford **7** as a white solid (857 mg, 80%). mp 110-112 ^oC; ^1H NMR (500 MHz, $CDCl_3$): δ 3.86 (s, 3H), 6.54 (s, 1H), 7.12 (t, *J* = 8.0 Hz, 1H), 7.22 (t, *J* = 7.0 Hz, 1H), 7.30-7.36 (m, 2H), 7.40-7.48(m, 4H), 7.61 (d, *J* = 7.5 Hz, 1H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 31.1, 101.7, 109.6, 119.2, 120.5, 121.7, 127.8, 128.0, 128.5, 129.4, 132.8, 138.4, 141.6; ESI-MS *m/z* Calc for $C_{15}H_{14}N$ $[M+H^+]$ 208.1126; observed 208.1126.

9,10-dimethoxy-13-(4-(1-methyl-5-nitro-1H-indol-2-yl)benzyl)-5,6-dihydro-[1,3]dioxolo[4,5-g]isoquinolino[3,2-a]isoquinolin-7-ium chloride **8**

To a stirred solution of hybrid **3** (70 mg, 1.1 mmol) in anhydrous DMF (5 mL) under Ar was added oven-dried K_2CO_3 (464 mg, 3.4 mmol) and the mixture was stirred for 15 min. Methyl iodide (0.21 mL, 3.4 mmol) in DMF (2 mL) was added dropwise to the reaction and stirring continued for a further 1-2 h. Upon complete consumption of the starting material (ESI-MS) the mixture was concentrated *in vacuo* and the residue purified by silica gel flash column chromatography (CH_3CN:EtOAc:MeOH, 1:1:0.5) to yield mixed Cl^-/I^- salts. The salt mixture was stirred with IRA-904 quaternary ammonium Cl^- anion exchange resin in MeOH (5 mL) at rt for 1 h. After filtration and concentration the title compound **8** was obtained as a yellow amorphous solid (53.3 mg, 75%). mp 208-212 °C (decomp); ^{1}H NMR (500 MHz, DMF-*d6*): δ 3.34 (s, 2H), 3.91 (s, 3H), 4.15 (s, 3H), 4.21 (s, 3H), 5.00 (s, 2H), 5.18 (bs, 2H), 6.17 (s, 2H), 6.92 (s, 1H), 7.16 (s, 1H), 7.29 (s, 1H), 7.48 (d, *J* = 6.5 Hz, 2H), 7.71 (d, *J* = 7.5 Hz, 2H), 7.75 (d, *J* = 8.5 Hz, 1H), 8.01 (m, 1H), 8.11 (d, *J* = 8.5 Hz,1H), 8.18 (d, *J* = 9.0 Hz,1H), 8.59 (s, 1H), 10.40 (s, 1H); ^{13}C NMR (125 MHz, DMF-*d6*): δ 28.3, 32.2, 36.2, 57.4, 58.0, 62.6, 103.1, 104.4, 109.1, 109.2, 111.2, 117.3, 117.6, 121.1, 122.5, 122.5, 126.8, 127.6, 129.4, 130.6, 130.9, 133.9, 135.2, 138.5, 140.9, 141.9, 142.2, 145.1, 145.6, 146.8, 147.6, 150.4, 151.3; ESI-MS *m/z* Calc for $C_{36}H_{30}N_3O_6$ [M-Cl^-] 600.2129; observed 600.2122.

9,10-dimethoxy-13-(4-(1-methyl-1H-indol-2-yl)benzyl)-5,6-dihydro-[1,3]dioxolo[4,5-g]isoquinolino[3,2-a]isoquinolin-7-ium chloride **9**

To a solution of the crude chloride **21** (128 mg, 0.5 mmol) in anhydrous CH_3CN (10 mL) was added sodium iodide (12.7 mg, 0.53 mmol) and the reaction was stirred for 1 h at 70 ^{o}C. 8-acetonyldihydroberberine **17** (196.8 mg, 0.501mmol) was then added and stirring continued for another 3-4 h at 70 ^{o}C. The reaction was monitored by TLC (EtOAc:Hexane, 1:4) to observe consumption of the chloride, and also by ESI-MS. Upon completion of the reaction the mixture was purified by silica gel column chromatography using EtOAC:CH_3CN:hexane (1:1:2) followed by

EtOAc:CH_3CN:MeOH (1:1:0.1). The semi-pure material was further purified by preparative RP-HPLC using a gradient from 0% A to 100% B over 30 min (R_t = 21.5 min). The fractions containing pure product were pooled and concentrated by freeze-drying to yield **9** as a yellow solid (118 mg, 40%). mp 186-189 ^oC; ^1H NMR (500 MHz, DMF-*d6*): δ 3.37 (s, 2H), 3.83 (s, 3H), 4.15 (s, 3H), 4.23 (s, 3H), 5.01 (s, 2H), 5.22 (bs, 2H), 6.20 (s, 2H), 6.63 (s, 1H), 7.10 (appt, *J* = 7.5 Hz, 1H), 7.20-7.25 (m, 3H), 7.48 (d, *J* = 7.5 Hz, 2H), 7.53 (d, *J* = 8.5 Hz, 1H), 7.62 (d, *J* = 7.5 Hz, 1H), 7.69 (d, *J* = 8.0 Hz, 2H), 8.04 (d, *J* = 7.5 Hz, 1H), 8.22 (d, *J* = 7.5 Hz, 1H) 9.42 (s, 1H); ^{13}C NMR (125 MHz, DMF-*d6*): δ 28.3, 31.5, 36.2, 57.4, 58.2, 62.6, 102.1, 103.1, 109.1, 109.2, 110.6, 120.2, 120.8, 121.1, 122.2, 122.4, 122.5, 126.8, 128.6, 129.2, 130.4, 131.1, 131.9, 134.0, 135.1, 138.5, 139.3, 139.9, 141.4, 145.5, 146.5, 147.6, 150.4, 151.2; ESI-MS *m/z* Calc. for $C_{36}H_{31}N_2O_4$+ [M-Cl^-] 555.2278; observed 555.2289.

Tert-butyl-2-(4-(methoxycarbonyl)phenyl)-1H-indole-1-carboxylate **12**

To a stirred solution of **11** (5 g, 9.9 mmol) in anhydrous 1,4-dioxane (50 mL) was added 4-iodomethylbenzoate (2.04 g, 7.78 mmol), palladium(II) chloride (43 mg, 0.24 mmol) and the reaction mixture was purged with nitrogen for 15 min. The reaction mixture was then heated to 100 ^oC and stirred for one 1h while monitoring by TLC (EtOAc:Hexane, 1:9). The reaction mixture was cooled, diluted with EtOAc (100 mL) and stirred over 15% aqueous potassium fluoride (300 mL) for 15 min. The precipitate was removed by filtration and washed well with EtOAc (200 mL). The organic layer was separated, washed with water and brine, dried over anhydrous Na_2SO_4 and concentrated *in vacuo.* The residue was purified by silica gel column chromatography (EtOAc:Hexane, 1.5:8.5) to afford **12** as a white semi-solid (2.3 g, 84%). mp 79-83 ^oC; ^1H NMR (500 MHz, $CDCl_3$): δ 1.32 (s, 9H), 3.94 (s, 3H), 6.6 (s, 3H), 7.2 (appt, *J* = 7.5 Hz, 1H), 7.35 (appt, *J* = 7.5 Hz, 1H), 7.50 (d, *J* = 7.5 Hz, 2H), 7.56 (d, *J* = 7.5 Hz, 1H), 8.07 (d, *J* = 7.5 Hz, 2H), 8.21 (d, *J* = 8.5 Hz, 1H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 27.6, 52.1, 83.8, 110.7, 115.2, 123.4, 125.1, 128.3,

128.8, 129.0, 129.2, 137.9, 139.4, 149.9, 166.86. ESI-MS *m/z* Calc. for $C_{21}H_{21}NNaO_4$ [M+Na^+] 374.1368, found 374.1368.

Methyl 4-(1H-indol-2-yl)benzoate **13**

To a stirred solution of **12** (2.3 g, 6.6 mmol) in anhydrous CH_2Cl_2 (7 mL) was added trifluoroacetic acid (7 mL) and the reaction mixture was stirred for 1-2 h. The mixture was concentrated *in vacuo* and the residue redissolved in ethyl acetate (100 mL). The EtOAc layer was washed with water and brine and dried over anhydrous Na_2SO_4 before concentrating *in vacuo*. The residue was purified by flash column chromatography (EtOAc:Hexane, 1:2) to afford **13** as an off-white crystalline solid (1.3 g, 79%). mp 204-205 °C; ^{1}H NMR (500 MHz, CD_3COCD_3): δ 3.90 (s, 3H); 7.029-7.059 (m, 2H), 7.14 (appt, *J* = 7.0 Hz, 1H), 7.45 (d, *J* = 8.5 Hz, 1H), 7.59 (d, *J* = 8.0 Hz, 1H), 7.96 (d, *J* = 8.5 Hz, 2H), 8.05 (d, *J* = 8.5 Hz, 2H), 11.01 (bs, 1H); ^{13}C NMR (125 MHz, CD_3COCD_3): δ 52.2, 101.8, 112.2, 120.6, 121.4, 123.3, 125.6, 129.3, 129.9, 130.8, 137.3, 137.8, 138. 8, 166.9. ESI-MS *m/z* Calc for $C_{16}H_{14}NO_2$ [M+H^+] requires 252.1025; found 252.1014.

Methyl 4-(1-methyl-1H-indol-2-yl)benzoate **19**

A stirring solution of (*N*-methylindol-2-yl)tributylstannane **18** (5.0 g, 11.9 mmol) in anhydrous THF was charged with methyl-4-iodobenzoate (2.18 g, 8.3 mmol) and $PdCl_2$ (97.6 mg, 0.832 mmol). The reaction mixture was purged with nitrogen for 30 min and then heated at reflux for 3-4 h with monitoring by TLC (EtOAc:Hexane, 1:5). The crude reaction mixture was adsorbed onto silica gel and purified by flash column chromatography with Pet. spirit:EtOAc (9.5:0.5 to 8:2) to yield **19** as an off-white solid (2.36 g, 75%). mp 105 ^{o}C; ^{1}H NMR (500 MHz, $CDCl_3$): δ 3.75 (s, 3H), 3.94 (s, 3H), 6.63 (s, 1H), 7.15 (appt, *J* = 7.5 Hz, 1H), 7.26 (appt, *J* = 7.5 Hz, 1H), 7.36 (d, *J* = 8.0 Hz, 1H), 7.58 (d, *J* = 8.5 Hz, 2H), 7.64 (d, *J* = 7.5 Hz, 1H), 8.12 (d, *J* = 9.8 Hz, 2H); ^{13}C NMR (125 MHz, ($CDCl_3$): δ 31.3, 52.1, 102.8, 109.7, 120.1, 120.7, 122.2, 127.8, 128.9, 129.2, 129.7, 137.2, 138.6, 140.1, 166.7; ESI-MS *m/z* Calc for $C_{17}H_{16}NO_2$ [M+H^+] 266.1181; observed 266.1173.

(4-(1-methyl-1H-indol-2yl)phenyl)methanol **20**

To a stirred solution of **19 (**250 mg, 0.94 mmol) in anhydrous THF was added $LiAlH_4$ (34 mg, 0.94 mmol) and the temperature gently raised to 40 ^oC. Another 3-4 eq of $LiAlH_4$ was added in portions over 20-30 min and the reaction mixture was stirred at 40 ^oC for a further 3-4 h. The reaction was monitored by TLC (EtOAc: Pet. spirit, 2:3) and upon completion was slowly quenched by dropwise addition of saturated aqueous NH_4Cl. After cessation of bubbling the mixture was diluted with water and extracted with EtOAc (3 x 10 mL). The organic layer was dried over anhydrous $MgSO_4$ and concentrated *in vacuo* to yield **20** as an off-white solid (190 mg, 85%). mp 97-99 ^oC; ^1H NMR (500 MHz, $(CD_3)_2SO$): δ 3.71 (s, 3H), 4.72 (s, 2H), 6.54 (s, 1H), 7.13 (appt, *J* = 7.5 Hz, 1H), 7.24 (appt, *J* = 15.5 Hz, 1H), 7.34 (d, *J* = 8.5 Hz, 1H), 7.43 (d, *J* = 8.0 Hz, 2H), 7.49 (d, *J* = 8.5 Hz, 2H), 7.62 (d, *J* = 8.0 Hz, 1H); ^{13}C NMR (125 MHz, $(CD_3)_2SO$): δ 31.1, 64.9, 101.6, 109.5, 119.8, 120.4, 121.6, 127.0, 127.9, 129.4, 132.1, 138.3, 140.4, 141.2; ESI-MS *m/z* Calc for $C_{16}H_{16}NO$ $[M+H^+]$ 238.1232; observed 238.1224.

2-(4-chloromethyl)phenyl)-1-methyl-1H-indole **21**

A solution of alcohol **20** (300 mg, 1.26 mmol) in CCl_4:CH_2Cl_2 (1:2, 7 mL) was stirred for 10 min at room temperature. PPh_3 (991 mg, 3.78 mmol) was then added and stirring continued for 1 h. TLC analysis (EtOAc:hexane, 1:4) indicated complete consumption of the alcohol. The product was filtered over a neutral alumina bed and washed with dichloromethane (3 x 5 mL). The combined filtrates were concentrated *in vacuo,* ensuring the water bath temperature stays below 40 ^{o} C. The residue obtained was triturated with pentane (3 x 4 mL) to afford crude **21** (210 mg, 65%). The crude **21** was used immediately and due to its instability was unable to be characterised.

MIC, Checkerboard, Uptake and C. elegans experiments

MIC, checkerboard and uptake measurements with 8325-4 wild-type, K1758 *norA*-knockout and K2378 NorA overexpressing *S. aureus* cells were obtained using the published methods.[2,9,10] MIC measurements with *S. aureus* MW2 and *E. faecalis* MMH594 were obtained as follows. Cultures of *S. aureus* MW2 and *E. faecalis* MMH594 were grown overnight in TSB or BHI broth, respectively, to stationary phase at 37°C with aeration. The cultures were diluted to an approximate density of 2 x 10^{-4} CFU/mL in the appropriate worm infection media. 12.5 µL of the bacterial culture dilutions were inoculated into 384-well plates containing 2-fold serial dilutions (in infection media) of the compounds being tested. Plates were incubated at 37°C for 15 h and scored by eye for bacterial growth. Checkerboard MIC experiments with MW2 and MMH594 were carried out in a similar manner as the MIC experiments with individual compounds as described above, with the exception that 2-fold serial dilutions of two compounds were arrayed in the 384-well plates in such a way that all combinations of dilutions between the two compounds were tested.

The *C. elegans*/MRSA MW2 live infection experiments were carried out according to our recently published methods.[18] The *C. elegans*/*E. faecalis* MMH594 experiments were carried out using the published procedure[19] with minor modifications. Briefly, a synchronous population of *glp-4(bn2); sek-1(km4)* worms were grown to the young adult stage on SK agar plates with *E. coli* HB101 lawns. The worms were washed off the HB101 SK plates and transferred onto BHI agar plates with *E. faecalis* MMH594 lawns and incubated for 15 h at 15**°.** Following infection, worms were resuspended in M9 and dispensed using a COPAS BioSort large particle sorter (Union Biometrica) into 384-well assay plates that contained compounds in 55 µl of infection assay media. Total volume per well was 70 µl with the final concentrations of components being 20% BHI, 60% M9 buffer, 19% sheath solution (Union Biometrica), 80 µg/ml kanamycin, 62.5 U/ml nystatin, and 1% DMSO. The plates were sealed with gas-permeable membranes and incubated at 26.3° with 85% relative humidity (RH) for 5 days without agitation. After 5 days, the plates were washed 5 times using a Biotek ELx405 plate washer, and Sytox Orange (Invitrogen) was added to a final

concentration of 0.7μM. The plates were sealed with gas permeable membranes and incubated at 20°, 80% RH for 16 h. After staining, bright field and red fluorescence images of the wells were captured using the ImageXpress Micro (Molecular Devices) and worm death was scored from the images using the image analysis software CellProfiler (www.cellprofiler.com).

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