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Some approaches to new antibacterial agents*

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Abstract: Bacteria use a number of resistance mechanisms to counter the antibacterial challenge, and one of these is the expression of transmembrane protein-based efflux pumps which can pump out antibacterials from within the cells, thus lowering the antibacterial concentration to nonlethal levels. For example, in *S. aureus*, the NorA pump can pump out the antibacterial alkaloid berberine and ciprofloxacin.

One general strategy to reduce the health threat of resistant bacteria is to block a major bacterial resistance mechanism at the same time as interfering with another bacterial pathway or target site. New developments of this approach in the context of dual-action prodrugs and dual-action (or hybrid) drugs in which one action is targeted at blocking the NorA efflux pump and the second action at an alternative bacterial target site (or sites) for the antibacterial action are discussed. The compounds are based on a combination of 2-aryl-5-nitro-1*H*-indole derivatives (as the NorA efflux pump blocking component) and derivatives of berberine. General design principles, syntheses, antibacterial testing, and preliminary work on modes of action studies are discussed

Keywords: antibacterials; efflux pumps; dual-action agents; prodrugs; berberine derivatives.

INTRODUCTION

Resistance to antibacterial agents by human pathogenic bacteria is an increasingly serious worldwide health issue [1–3]. This resistance is apparent, for example, in the Gram-positive bacteria *Staphylococcus aureus* and *Enterococcus faecium*, as well as in the Gram-negative pathogens *Escherichia coli* and *Pseudomonas aeruginosa*, amongst others. The most pressing concerns are particularly with regard to the problematic human bacterial pathogens *Acinetobacter baumanii*, ESBL-producing *Enterobacteriaceae*, vancomycin-resistant *E. faecium*, *P. aeruginosa*, and methicillin-resistant *S. aureus* (MRSA), as well as *Aspergillus* species of fungal pathogens [1]. The need for innovative and efficient approaches to tackle this resistance problem, together with the judicious use of antibacterials, is urgent.

Bacteria, no doubt due to exposure to natural antibiotics over millennia together with more recent exposures to semi-synthetic and synthetic antibacterials, have evolved a number of mechanisms to counter the antibacterial challenge. One major mechanism is based on enzymatic conversion of antibiotics into inactive forms, as typified by inactivation by the β-lactamases of the penicillins and cephalosporins through β-lactam hydrolysis. A second mechanism, highlighted by resistance to vancomycin, is the modification of the biological target site. Replacement of the terminal D-alanine residue in the cell wall peptidoglycan substrate for the cross-linking transpeptidase enzyme by D-serine or D-lactate confers moderate and full resistance to vancomycin, respectively. This is mediated through reduction in binding of this antibiotic to the modified substrate [3].

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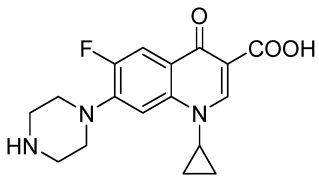
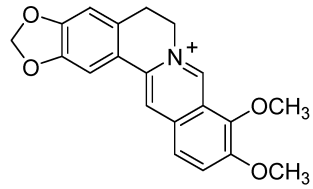
The third main mechanism involves reduction of intracellular antibacterial concentrations to sublethal levels. This may be mediated, for example, by reduced cell permeation or by active efflux mechanisms. Both Gram-positive and Gram-negative bacteria employ a range of such membrane pumps for this purpose and invest a major effort in their production. For example, in *S. aureus,* the NorA pump is a multidrug-resistance pump [4,5] which can extrude a range of antibacterial types, including the alkaloid berberine (**2**) and related quaternary salts, and fluoroquinolones, for example, ciprofloxacin (**1**). The latter group also carries a positive charge through protonation at physiological pH. Thus, amphipathic compounds are characteristic substrates for this protein pump.



Ciprofloxacin (**1**)



Berberine (**2**)

The effect of the pump can be demonstrated clearly using mutant strains of *S. aureus*. In a mutant strain of *S. aureus* (K 2361), which over-expresses this NorA pump, the MIC (minimum inhibitory concentration) value for berberine is >650 μM, while in the NorA-deleted mutant strain K1758, the corresponding value is much lower at 40 μM and in wild-type *S. aureus* (8325-4) it is 325 μM [6].

Gram-negative bacteria also employ a range of efflux pumps for protection against antibacterials, including the clinically relevant RND pumps expressed in *E. coli* and *P. aeruginosa* [7,8].

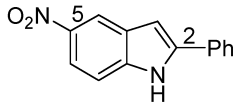
Overall, the currently known bacterial efflux pumps have been categorized in four major families as summarized in Table 1 [9]. These membrane-bound protein pumps vary in terms of size, numbers of transmembrane segments, and general mode of action, as well as in terms of substrates. For the majority of them, proton motive force provides the energy source, while a minority in the ATP binding cassette family use ATP hydrolysis. Limited detailed structural information is available on these pumps although this is changing as more X-ray structural elucidations come to hand [10,11]. Similarly, the detailed molecular mechanism involved in the operation of some of the pumps is starting to unfold [12,13]. A particularly elegant example of this involving the use of detailed X-ray single crystal data with the substrate antibiotic minocycline has been reported by Murakami and coworkers [12]. However, the X-ray crystal structure of the NorA pump has not as yet been reported, although it is the focus of much active research.

Table 1 Bacterial efflux pump classification.

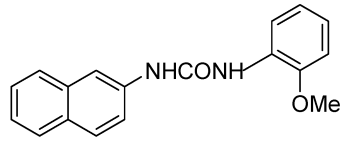
Class	Energy source	Substrate specificity	Number of amino acid residues
ABC superfamily	ATP hydrolysis	Specific, MDR	Variable
SMR family	PMF	MDR	ca. 110
MF family	PMF	Specific, MDR	400–600
MATE family	Na^+	MDR	ca. 450
RND family	PMF	MDR	Up to 1000

PMF: proton motive force; MDR: multidrug resistance; ABC: ATP binding cassette; SMR: small multidrug resistance; MF: major facilitator; MATE: multi antimicrobial extrusion; RND: resistance nodulation division.

In view of the importance of bacterial efflux pumps in mediating antibacterial resistance, they are also significant targets for inhibition studies. Small molecule inhibitors of the pumps are capable of reversing antibiotic resistance, thus rendering antibiotics more effective again [9,14]. The multidrug-resistance NorA pump in *S. aureus*, the main focus of this paper, can be inhibited by a structurally diverse range of small molecule inhibitors [9]. These inhibitor classes include indoles (e.g., INF55; [15,17]), flavonolignans [16], and ureas (e.g., INF271 (**4**); [15,17]).



(**3**) INF55



(**4**) INF271

While the NorA inhibitor 5-nitro-2-phenyl-1*H*-indole (**3**) (INF55) has essentially no antibacterial activity against wild-type *S. aureus* 8325-4 (MIC >525 μM), if given in combination with berberine it can decrease the MIC of the latter to 12.5 μM [6].

DUAL-ACTION ANTIBACTERIAL STRATEGIES

These and other related observations suggested to us that further development of dual-action approaches might result in a viable strategy for new antibacterial design, in which one action was specifically targeted at inhibition of an efflux pump. The second action could be directed to another bacterial target at the same time.

In terms of generalized actions, three types of dual-action approaches can be delineated. The first of these involve dual drugs, i.e., two drugs X' and Y' being administered with two different bacterial target sites A and B, respectively (Fig. 1). In the context of our studies, target site A is designated as an efflux pump.

1. Dual Drugs

X' + Y' → X'……A + Y'……B

A- bacterial target A (e.g. an efflux pump)

B- second bacterial target B (e.g. DNA;enzyme)

2. Dual Action Drugs (Hybrid Drugs)

[X-linker-Y] → [A……X-linker-Y] + [X-linker-Y…..B]

3. Dual Action Prodrugs

[X - cleavable linker-Y] → X' + Y' → X'….A + Y'…..B

Fig. 1 Generalized dual-action strategies.

The second type of generalized approach is that of dual-action drugs or hybrid drugs (Fig. 1). In this approach, one compound is involved, but with incorporated structural moieties of X and Y joined by an enzymatically or chemically stable linker group (potentially, the moieties X and Y could also be directly linked or structurally merged to afford chimeric molecules). Each moiety might then interact separately with biological targets A and B (Fig. 1). It should be stressed, however, that the format pictured in Fig. 1 is the simplest one, and one could have, for example, cooperative binding at one or both biological target sites A and B.

The third type of generalized approach is that of dual-action prodrugs. This is related conceptually to the hybrid design, but differs from it in that the linker would be designed to be cleaved enzymatically by bacterially specific enzymes to release both X' and Y', which could then interact with their respective targets A and B (Fig. 1). While such compounds are related to tripartite prodrugs with a drug-linker-carrier format, the carrier component is changed to be a second drug component, although it could also act as a carrier component as well. Once again, it should be stressed that this represents the simplest version of the dual-action prodrug approach, and more complicated variations on the theme can be envisaged, for example, where X and Y and the cleavable linker are incorporated initially in a cyclic skeleton.

Examples of all three general approaches in the antibacterial context have been reported in the literature (representative references include [18–20] for dual drugs, dual-action prodrugs, and dual-action drugs, respectively) but to our knowledge there were no specific examples prior to our reported work [6] of antibacterial dual-action (hybrid) drugs, or of dual-action prodrugs, which incorporate an efflux pump blocker as one component. Potential advantages of these approaches (over the administration of two separate drugs) include synchronous, or near synchronous, delivery to different bacterial target sites, as well as the possibility of a slower development of resistance. Disadvantages, however, include high molecular weights and potential problems with synthesis, permeability issues, and differences in the minimum efficacious concentrations required at the two different biological target sites. Another disadvantage of dual-action drugs is the increased probability for reduced activity at one or both biological target sites for steric or electronic reasons, unless these molecules are carefully designed.

The focus of our research has been on dual-action antibacterials and dual-action prodrug antibacterials. Both types have been centered on a NorA efflux pump blocker component and an antibacterial component, where the antibacterial would normally be a substrate for the NorA pump (Fig. 2).

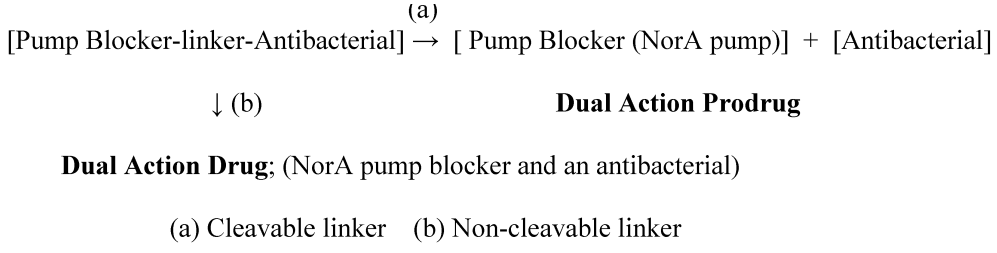
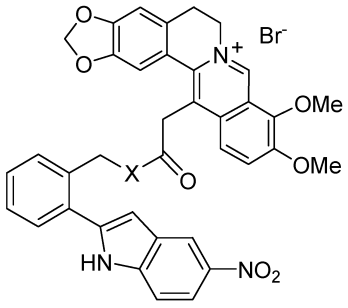


Fig. 2 General design principles for antibacterial dual-action drug/prodrugs incorporating NorA efflux pump inhibition.

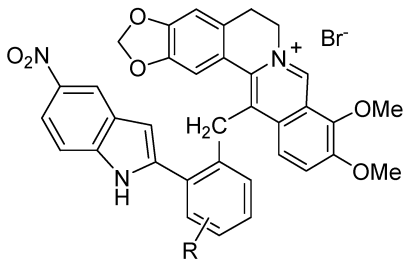
The initial realization of the design ideas was based on clues from nature. In a landmark study, Lewis and Stermitz and coworkers reported [21] the co-occurrence of a NorA pump inhibitor (5'-methoxyhydnocarpin) and an antibacterial substrate of the pump (berberine) in the North American plant *Berberis fremontii*. The plant, which was observed to be free of bacterial disease, had thus developed a dual-action strategy to combat bacterial attack. While linkage of the hydnocarpin inhibitor and berberine was considered, synthetic and stereochemical issues associated with the former compound made this combination more difficult to achieve.

Simpler synthetic inhibitor compounds based on the 5-nitro-2-aryl-1*H*-indole skeleton had also been reported to be NorA inhibitors [15], so our first-generation compounds were based on 5-nitro-2-phenyl-1*H*-indole (**3**) (INF55), a good inhibitor of the NorA pump, together with berberine (**2**).



(**5**) **a.** X = O

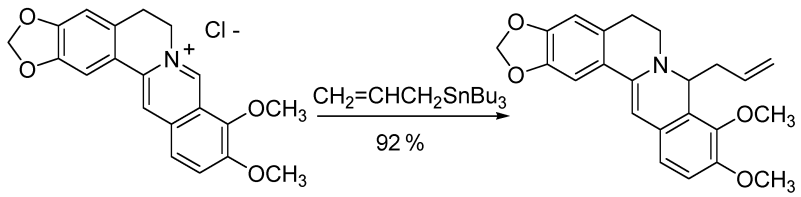
b. X = NH



(**6**)

The dual-action prodrugs were based on linkage of the indole with berberine via ester (**5a**) or amide (**5b**) linkages, while the dual-action drugs (**6**; R = H, 5'-OMe [Cl^- salt]) were based on a methylene group linkage with attachment to the 13 position of the berberine and the *ortho*-position of the 2-phenyl group.

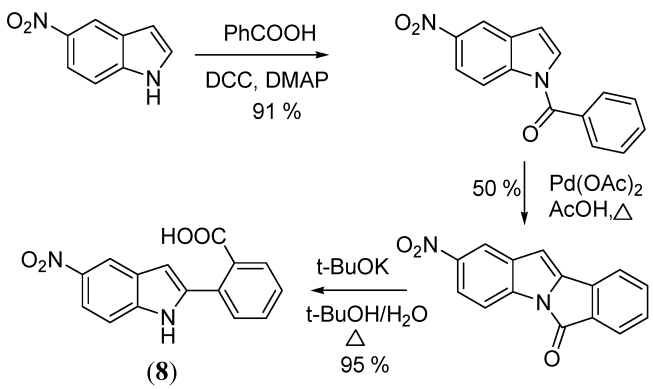
Synthetically, both these chemical targets (**5**) and (**6**) were to be approached at the penultimate stage using previously developed methodology [22] based on reaction of an appropriately substituted indole-based alkyl bromide (the pump blocker component) with 8-allyldihydroberberine (**7**) (to provide the berberine-based antibacterial component), the latter precursor component being accessed readily from a nucleophilic addition of allyltributyl tin to berberine chloride (**2**) (Scheme 1).



(**2**) (**7**)

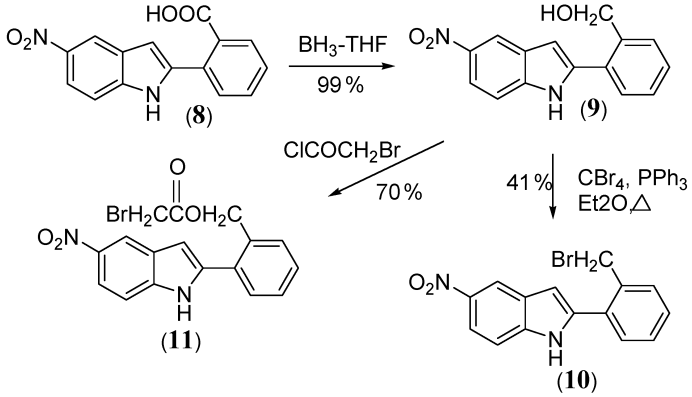
Scheme 1

The synthesis of the indole precursors with the appropriate *ortho*-substituted phenyl rings was based on a multistep synthesis, the first stage of which incorporated N-acylation of 5-nitroindole using direct coupling with benzoic acid [23], palladium-mediated oxidative cyclization following the method of Itahara [24], and hydrolytic ring-opening to give the acid (**8**) (Scheme 2) [17].

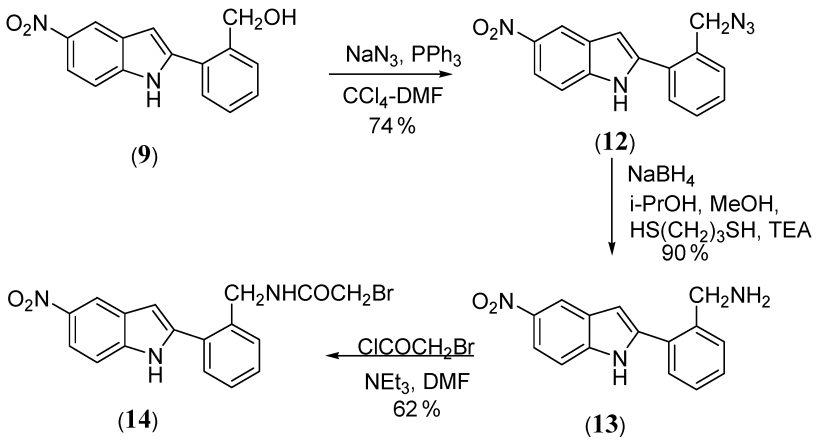


Scheme 2

Further functional group manipulations from the carboxylic acid (**8**) via the alcohol (**9**) provided the benzyl bromide (**10**) and the bromo ester (**11**) (Scheme 3), as well as the amine (**13**) (via the azide (**12**)), and the bromo amide (**14**) (Scheme 4), for the berberine coupling reaction as well as, for comparison purposes, the products to be expected from hydrolysis of the ester and amide prodrugs.

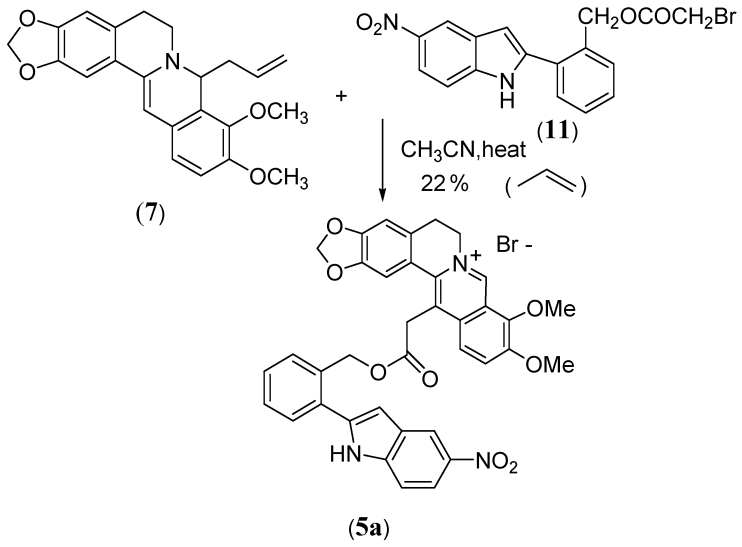


Scheme 3



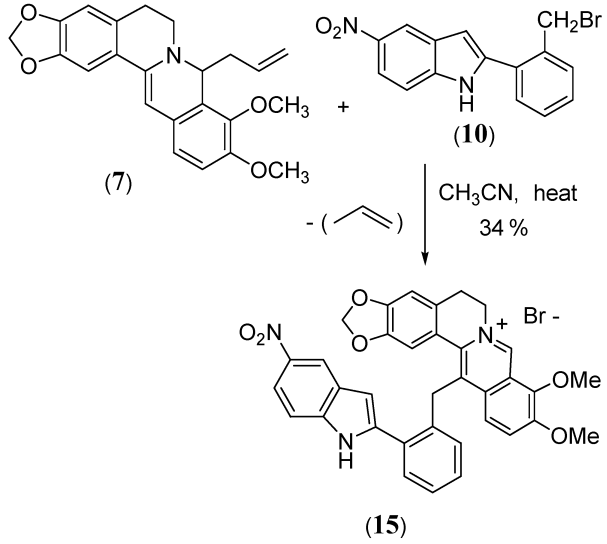
Scheme 4

The final coupling reaction to give the ester (**5a**) (Scheme 5) proceeded in low yield. However, it had the advantage of producing a final product at the oxidation level of berberine. The corresponding amide analog (**5b**) of (**5a**) could also be produced by the same reaction. The mechanism of the process probably follows that described previously [22] involving initial enamine C-alkylation in the dihydroberberine, followed by a [3,3]-sigmatropic rearrangement then a retro-ene reaction to give the isolated product and elimination of propene; direct elimination of propene from the enamine alkylation product cannot be excluded, however.



Scheme 5

The potential dual-action drug (**15**) was made analogously to the ester- and amide-linked derivatives and proceeded in better yield (Scheme 6) [6].



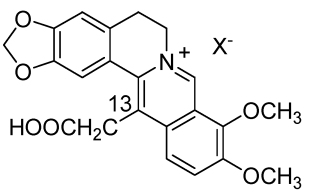
Scheme 6

INITIAL ANTIBACTERIAL TESTING RESULTS

Potential dual-action prodrugs

All the antibacterial testing was undertaken by Prof. K. Lewis, Mr. A. Ball, and Dr. G. Casadei from Northeastern University, Boston, MA, USA, and a selection of results are given in this paper. Initially, it was necessary to test the NorA pump inhibitory activity of the substituted indole alcohol (**9**) and the corresponding amine (**13**), as they were the expected hydrolysis products from the respective ester- or amide-linked prodrugs. The MIC values of (**9**) and of (**13**) in combination with a fixed sub-MIC concentration (80.7 μM) of berberine in each case in *S. aureus* 8325-4 were 46.6 and 1.5 μM, respectively; the MIC value for (**9**) alone in *S. aureus* 8325-4 was 186.4 μM, and for (**13**) it was 187.1 μM. These results were indicative of both (**9**) and (**13**) acting as pump inhibitors and thus potentiating the activity of berberine in this bacterium, with the amine (**13**) showing considerable inhibitory potency [17].

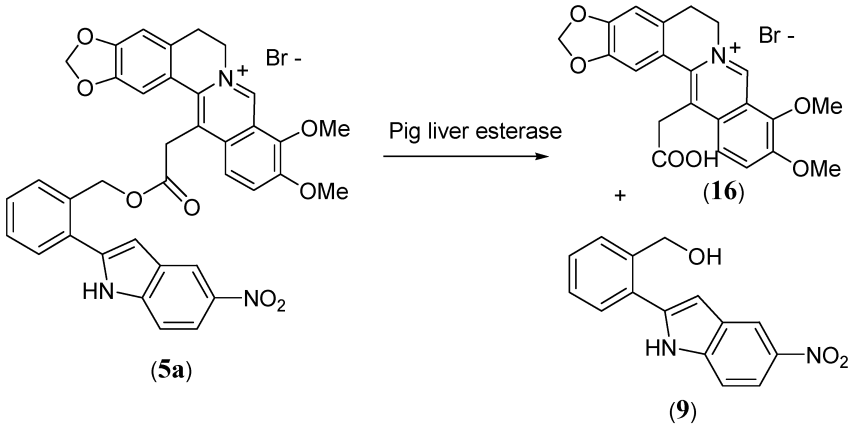
The other expected prodrug hydrolysis product, the berberine carboxylic acid (**16**), which was prepared separately from hydrolysis of the corresponding ethyl ester [25], was also assessed for direct antibacterial activity (as its chloride salt) and was shown to have mild activity with an MIC of 116.3 μM (*S. aureus* 8325-4).



(**16**)

With the potential dual-action ester prodrug (**5a**), moderate activity was shown against the wild-type *S. aureus* strain 8325-4 (MIC 22.1 μM), and, significantly, more potent activity was seen (MIC 4.3 μM) against an *S. aureus* mutant strain (K1758) in which the NorA pump is deleted. While these results are not inconsistent with (**5a**) acting as a dual-action prodrug, further evidence was needed that hydrolysis was feasible. To this end, the ester (**5a**) was exposed to pig liver esterase but little hydrolysis to the alcohol (**9**) was seen (Scheme 7). When the ester (**5a**) at a very low, sub-MIC level was incubated with *S aureus* 8325-4 in Mueller Hinton broth and then a dichloromethane extract of the broth, and of the bacterial pellet (after centrifugation of the culture broth), was analyzed by electrospray mass spectrometry (ESMS) (–) and MS/MS, evidence for the presence of the alcohol (**9**) in low concentration was obtained. In determining the concentration of (**9**) by ESMS, the related methyl-substituted derivative (**17**) was used as an internal reference compound; the limit of detection of the alcohol (**9**) was ca. 0.019 μM. The alcohol hydrolysis product was chosen as the target for analysis as it was more readily extracted from aqueous media. In a separate control experiment, some hydrolysis of the ester by the broth itself was also seen. Further work is thus necessary to assess the extent of bacterial hydrolysis of (**5a**), looking at both the alcohol (**9**) and acid (**16**) hydrolysis products.

With the potential dual-action prodrug amide analog (**5b**) of (**5a**), the MIC value against *S. aureus* wild-type 8325-4 was 4.3 μM.



Scheme 7

Potential dual-action antibacterial

High antibacterial potency was seen with the potential dual-action or hybrid agent (**15**; also designated SS14). For example, against the Gram-positive bacteria *S aureus* 8325-4, *E. faecalis* V-583, and *Bacillus cereus* T, MIC values of 3.1, 6.3, and 3.1 μM were obtained, respectively. The corresponding values for berberine itself were 325, >650, and 650 μM. In further studies on (**15**; SS14), it was shown [6] to rapidly accumulate in *S. aureus* cells, and also was considerably more potent than a mixture of berberine itself and the NorA pump blocker INF55 (Fig. 3; reproduced with permission from [6]). While (**15**) may not be a substrate for the NorA pump because of the bulky 13-indolyl substituent group, it may, alternatively, be blocking its own efflux as initially designed. Further studies are planned to determine this in the future.

Antibacterial activity for (**15**) has also been demonstrated in vivo in the worm curing assay developed by Ausubel and coworkers [26] using the nematode *Caenorhabditis elegans* infected with *E. faecalis*. In this assay, the berberine derivative (**15**) had a similar activity to tetracycline, and it was as active as vancomycin. Interestingly, berberine itself showed poor in vivo activity in this assay as did the pump blocker INF55, and the latter also showed toxic effects in the worms [6].

In initial studies on the potential mechanism of antibacterial action of (**15**), a comparison of DNA binding of (**15**) and berberine (**2**) has been undertaken. These studies, which were based on ESMS (negative ion mode) and were undertaken by Ms. K. Gornall and Dr. J. Beck at the University of Wollongong, confirmed that berberine (**2**) binds to double-stranded DNA [27], and also to triplex DNA, but (**15**) does not bind to either of these two DNA forms [28]. However, (**15**) was shown to bind to quadruplex DNA, as does berberine. The significance of this binding selectivity in the case of (**15**) is being pursued currently.

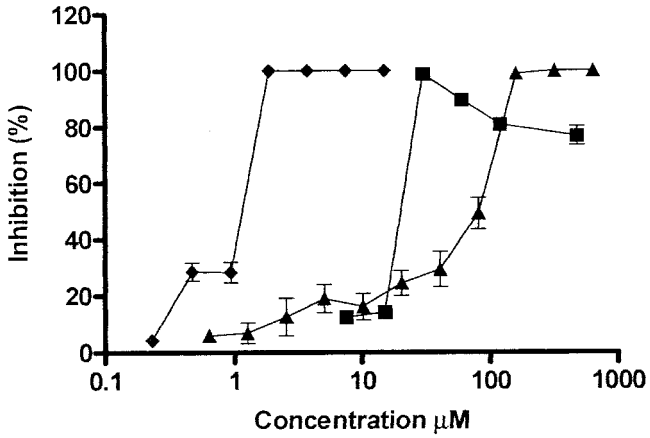


Fig. 3 Potentiation of berberine action against *S. aureus* [6]. Actively growing S. aureus cells were treated with berberine (▲) or hybrid SS14 (◆). Berberine at a fixed concentration (1.87 μM) was potentiated by varying amounts of INF_{55} (■).

CONCLUSION AND FUTURE PERSPECTIVES

Dual-action agents involving efflux pump inhibition hold considerable potential as agents to tackle certain drug-resistant human pathogenic bacteria. More work is needed in the area including the development of good ligand-based pharmacophores to inform more potent pump inhibitor design, as well as in the development of dual-action agents and dual-action prodrugs targeting, in part, RND pumps in Gram-negative bacteria. An X-ray structure on the NorA pump, the target of current work for example by Prof. P. Henderson in the U.K., would also greatly advance the design and implemention of new dual-action agents.

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