Potentiation of antibacterial activity of the MB-1 siderophore-monobactam conjugate using an efflux pump inhibitor

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Running title: Impact of efflux on siderophore conjugate activity
ABSTRACT

Preliminary enthusiasm over the encouraging spectrum and \textit{in vitro} activity of siderophore conjugates such as MB-1 has recently been tempered by unexpected variability in \textit{in vivo} efficacy. The need for these conjugates to compete for iron with endogenously-produced siderophores has exposed a significant liability for this novel antibacterial strategy. Here, we have exploited dependence on efflux for siderophore secretion in \textit{Pseudomonas aeruginosa}, and provide evidence that efflux inhibition could circumvent this \textit{in vivo}-relevant resistance liability.
The extent and severity of antibiotic resistance in Gram-negative pathogens requires the development of innovative therapeutic interventions that not only circumvent existing clinically-relevant resistance mechanisms, but will also result in limited spontaneous resistance emergence. Although standard in vitro methodologies exist for predicting resistance development, translational deficiencies that can stem from their lack of in vivo physiological relevance are becoming increasingly apparent. We have previously described the siderophore-monobactam conjugate MB-1 (Figure 1, (1)), which has broad-spectrum in vitro activity against multi-drug-resistant (MDR) Gram-negative pathogens, including P. aeruginosa, when tested using standard methods established by the Clinical and Laboratory Standards Institute (CLSI; (1)). Unfortunately, we have also demonstrated that despite this encouraging in vitro activity, MB-1 efficacy in a neutropenic murine infection model is highly variable and not directly predictable from traditional in vitro activity assays (2). Instead, the development of a modified in vitro resistance frequency assay, which relies on the utilization of more in vivo-relevant iron conditions, has proven to be more predictive of MB-1 in vivo efficacy. Additionally, this assay has demonstrated a role for endogenous siderophores such as pyoverdine in mediating the adaptive cellular response that enables P. aeruginosa to transiently resist MB-1 activity (2). While these results suggest that the use of MB-1 as monotherapy is not a viable option, we have speculated about alternative strategies that could mitigate this adaptive response and resurrect MB-1 as a legitimate antibacterial agent. Among these potentiation-type approaches, we considered the idea that inhibiting endogenous siderophore functionality should circumvent the adaptation phenotype mediated by the competition for iron by native siderophore systems. Siderophore secretion into the extracellular milieu has previously been shown to be dependent on functional efflux pumps in Gram-negative bacteria (3-5). In the case of P. aeruginosa, the secretion of newly-synthesized pyoverdine is accomplished by the ABC transporter-type...
efflux system encoded by \textit{pvdR}, \textit{pvdT}, and \textit{opmQ} (6). Additionally, this system is responsible for pyoverdine recycling after successive rounds of iron acquisition and delivery (7).

To prove that inhibiting the activity of this pump could potentiate the activity of MB-1, we generated a \textit{pvdRT-opmQ} deletion mutant in \textit{P. aeruginosa} JJ4-36, which is a clinical isolate that has demonstrated a significant propensity for adaptation to MB-1. This mutant was constructed by replacing the C-terminus of PvdR (amino acids 68-691), all of PvdT, and the majority of OpmQ (amino acids 1-450) with a gentamicin-resistance cassette from pPS856 (8), subcloning into pEX100T (9), and delivering it to recipient cells via tri-parental mating as described previously (10). This derivative (heretofore referred to as $\Delta$RTQ), along with the parental strain and isogenic pyoverdine biosynthesis-deficient \textit{pvdA} deletion mutant, was tested in the MB-1 adaptation assay using methods described previously (2). Briefly, Luria-Bertani cultures of each \textit{P. aeruginosa} strain were grown to mid-log-phase, and ~1x10^7 cells were spread onto chelexed, dialyzed, Mueller Hinton Broth (CDMHB, solidified with 1.5% agarose) plates containing increasing concentrations of MB-1 and incubated at 37°C for 40 hours. While the wild-type JJ4-36 strain showed substantial growth at MB-1 concentrations as high as 32 µg/ml, both of the mutants were limited to growth of a few, if any, colonies on the CDMHB plate with 0.25 µg/ml MB-1 (Table 1, “Unsupplemented” row).

One potential interpretation of these results could be that the PvdRT-OpmQ system was responsible for directly effluxing MB-1 from the cell, rather than mediating resistance indirectly through the transport of pyoverdine. To test this, the adaptation assay was performed by supplementing CDMHB with conditioned media from either wild-type JJ4-36 or the isogenic $\Delta$pvdA mutant. Conditioned media, described as the cell-free supernatants from overnight-grown CDMHB cultures, was previously demonstrated to restore the growth of $\Delta$pvdA cells in the presence of MB-1 if it was prepared from pyoverdine-producing strains, whereas conditioned medium prepared from pyoverdine-deficient
mutants did not provide this restoration (2), demonstrating the importance of this endogenous siderophore in mediating the adaptive response. Here, if PvdRT-OpmQ is responsible for the physical removal of MB-1, then supplementation with wild-type JJ4-36 conditioned medium should not promote the growth of JJ4-36 ΔRTQ cells on MB-1-containing plates. As shown in Table 1 (compare rows 2 and 3), when 1% conditioned medium from wild-type JJ4-36 was added to CDMHB plates, these mutant cells were capable of growing in the presence of up to 4 μg/ml MB-1 (i.e. the presence of pyoverdine reduced MB-1 activity). In contrast, plates that were not supplemented, or were supplemented with 1% conditioned medium from JJ4-36 ΔpvdA, did not have any detectable growth after 40 hours of incubation. These data suggest that this efflux pump is not mediating MB-1 resistance through the active efflux of this siderophore conjugate.

These preliminary results demonstrated the importance of this particular efflux pump in mediating adaptation to MB-1, prompting the hypothesis that an efflux pump inhibitor (EPI) that functionally inhibits the PvdRT-OpmQ system would alleviate this liability for MB-1 efficacy. A variety of compounds that have demonstrated activity against either resistance-nodulation-division (RND) pumps or ABC transporter-type systems were tested. Specifically, phenyalanine-arginine β-naphthylamide (PAβN) (11), 1-(1-naphthylmethyl)-piperazine (NMP) (12), reserpine (13), probenecid (14), verapamil (15), and gemfibrozil (16) were all tested for their ability to potentiate MB-1 activity in the adaptation assay. Using concentrations that have previously been shown to affect the activity of various efflux pumps, improvements in MB-1 activity were not observed when it was combined with PAβN, NMP, probenecid, verapamil, or gemfibrozil. The combination of MB-1 with 25 μg/ml reserpine, however, showed at least an 8-fold reduction in the emergence of adaptively-resistant colonies (Table 2). Lowering the reserpine concentration to 6.25 μg/ml decreased the degree of MB-1 potentiation, although a clear beneficial effect was still detected. Attempts to increase the reserpine concentration to 100 μg/ml did not yield any significant improvements relative to those seen when 25 μg/ml was used, although this may have
been due to apparent solubility issues at this elevated concentration. In the absence of MB-1, however, none of the EPIs tested at the indicated concentrations had any detectable effect on *P. aeruginosa* growth in this assay (data not shown).

Although reserpine alone was not found to cause any growth inhibition in this assay, the possibility that it could be acting synergistically with MB-1 by affecting a distinct cellular target was assessed in two separate assays. First, the adaptation assay was repeated using JJ4-36 ΔRTQ cells and MB-1 concentrations lower than those used initially (0.03-0.5 µg/ml), both in the presence and absence of 25 µg/ml reserpine. After 40 hours of incubation, the growth patterns of reserpine-treated versus untreated plates were indistinguishable (Table 2), suggesting that reserpine was not killing cells through an alternative mechanism. The second approach involved conducting the adaptation assay with other antibacterial agents, again both in combination with reserpine or alone, using wild-type JJ4-36 cells. Table 2 shows that in both cases the degree of JJ4-36 growth was unaffected by the inclusion of 25 µg/ml reserpine, suggesting that its ability to potentiate MB-1 was specifically caused by PvdRT-OpmQ inhibition.

To determine the translatability of the *in vitro* results generated with this combination, MB-1 efficacy against wild-type JJ4-36 was tested in the presence and absence of reserpine using an MB-1 humanized dosing regimen, which required multiple doses to maintain free drug concentrations at 4 µg/ml, in a neutropenic mouse thigh model of infection described previously (2). The JJ4-36 ΔRTQ mutant was also included to confirm the hypersusceptibility witnessed with it in the *in vitro* adaptation assay. MB-1 susceptibility of the ΔRTQ mutant was confirmed, as evidenced by the recoverable number of mutant cells being 4.25-log lower relative to that of wild-type JJ4-36 after 24 hours of infection (Figure 2, white
Interestingly, MB-1 treatment against this mutant showed a 1.99-log further decrease in bacterial density when compared with the JJ4-36 ΔpvdA mutant, a phenotype which may be explained by fitness costs associated with efflux deficiency, as suggested by the 1.58-log reduction in bacterial burden recovered from the corresponding control groups (Figure 2). A 3.67-log decrease in wild-type JJ4-36 bacterial burden was also observed when MB-1 treatment was combined with 30 mg/kg reserpine (relative to MB-1 monotherapy), despite the fact that treatment with each of these compounds individually had a considerably weaker impact on bacterial growth after 24 hours (Figure 2). It should be noted that only a single dose of reserpine was administered during the course of this study, and the resulting exposure of this regimen was not evaluated. With a more thorough understanding of reserpine pharmacokinetics and safety, it is possible that additional doses would have increased the amount of bacterial killing even further.

In an effort to definitively demonstrate reserpine-mediated inhibition of pyoverdine secretion, wild-type JJ4-36 cells were grown in CDMHB and supernatant samples were collected to assess pyoverdine secretion over time. Unfortunately, absorbance measurements of reserpine-treated cultures at 405 nanometers, which is a routinely used method to detect pyoverdine production in *P. aeruginosa* cultures, did not demonstrate a significant difference from mock-treated control samples (data not shown). In addition, even though the reserpine combination with MB-1 in the adaptation assay did result in a reduction in the concentration of MB-1 in which wild-type JJ4-36 cells could grow (Table 2), those colonies that did emerge on plates containing lower MB-1 concentrations still produced the typical fluorescent-green halo that has previously been demonstrated to represent pyoverdine production (2). Taken together, these data prevent reserpine from being classified as a *bona fide* inhibitor of the PvdRT-OpmQ ABC transporter in *P. aeruginosa*. Based on the *in vitro* and *in vivo* results with the ΔRTQ mutant, however, it still seems feasible that specific inhibition of this efflux pump could impart the necessary effects to potentiate the activity of siderophore conjugates such as MB-1.
In this report we have provided early insight into an innovative approach to circumventing adaptation-mediated resistance to MB-1, and demonstrate significant improvements in \textit{in vivo} efficacy for MB-1 when combined with the EPI reserpine. This plant alkaloid has previously been described to function as an EPI in various Gram-positive pathogens such as \textit{Staphylococcus aureus} (13, 17) and \textit{Streptococcus pneumoniae} (18), aiding in the restoration of antibiotic susceptibility amongst MDR strains of these clinically-prevalent bacteria. Our data suggest that reserpine potentiates MB-1 activity, both \textit{in vitro} and \textit{in vivo}, although the exact mechanism(s) behind this potentiation is still unclear. From data generated previously, we proposed a model in which a subpopulation of \textit{P. aeruginosa} cells that hyper-produced pyoverdine could provide adaptive protection to neighboring cells by enabling them to acquire iron more rapidly than MB-1 bactericidal activity could be achieved (2). With that in mind, we have speculated that reserpine is acting to prevent both pyoverdine hyper-production and the downstream protective effect for nearby cells, even though it does not appear to affect normal pyoverdine production in the absence of MB-1. As a result, this would limit the amount of adaptive resistance observed in the absence of this EPI, although this hypothesis requires additional investigation to confirm or refute. And while reserpine itself may not be an acceptable combination partner for safety and tolerability reasons, we are encouraged that the efforts described here provide mechanistic proof-of-concept for this novel resistance mitigation strategy.
REFERENCES


Gibbons S, Udo EE. 2000. The effect of reserpine, a modulator of multidrug efflux pumps, on the in vitro activity of tetracycline against clinical isolates of methicillin resistant *Staphylococcus aureus* (MRSA) possessing the tet(K) determinant. Phytotherapy research : PTR 14:139-140.

Table 1. Adaptive resistance to MB-1 by *P. aeruginosa* JJ4-36 wild-type and isogenic pyoverdine-biosynthesis and secretion mutants.

<table>
<thead>
<tr>
<th></th>
<th>Highest MB-1 concentration (in µg/ml) with growth</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>WT JJ4-36</td>
</tr>
<tr>
<td>Unsupplemented</td>
<td>32</td>
</tr>
<tr>
<td>1% WT CMa</td>
<td>NTa</td>
</tr>
<tr>
<td>1% ΔpvdA CM</td>
<td>NT</td>
</tr>
</tbody>
</table>

a - CM - conditioned medium; defined as cell-free, spent medium harvested from wild-type (WT) or pyoverdine-deficient (ΔpvdA) *P. aeruginosa* JJ4-36 grown overnight in CDMHB at 37°C

b - NT - not tested
Table 2. Determination of MB-1 potentiation capabilities by various EPIs in the CDMHB adaptation assay.

<table>
<thead>
<tr>
<th>Compound (µg/ml)</th>
<th>[MB-1]</th>
<th>[Aztreonam]</th>
<th>[Ciprofloxacin]</th>
<th>[MB-1]</th>
</tr>
</thead>
<tbody>
<tr>
<td>No combination</td>
<td>≥32</td>
<td>32</td>
<td>2</td>
<td>0.25</td>
</tr>
<tr>
<td>PAβN (50)</td>
<td>≥32</td>
<td>8</td>
<td>0.5</td>
<td>NT</td>
</tr>
<tr>
<td>NMP (100)</td>
<td>≥32</td>
<td>32</td>
<td>1</td>
<td>NT</td>
</tr>
<tr>
<td>Reserpine (6.25)</td>
<td>8</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>Reserpine (25)</td>
<td>4</td>
<td>32</td>
<td>2</td>
<td>0.25</td>
</tr>
<tr>
<td>Probenecid (712.5)</td>
<td>≥32</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>Verapamil (50)</td>
<td>≥32</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>Gemfibrozil (50)</td>
<td>≥32</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
</tbody>
</table>

* – NT – not tested
Figure 1. The chemical structure of the MB-1 siderophore-monobactam conjugate.
Figure 2. In vivo efficacy* of MB-1, either alone or in combination, against wild-type *P. aeruginosa* JJ4-36 and its isogenic pyoverdine biosynthesis (*pvdA*) or secretion (RTQ) mutants in a neutropenic mouse thigh model of infection. Numbers above each data point represent the mean change in CFU after 24 hours relative to 0 hour control animals. *Using, where appropriate, a Students T-test or Analysis of Variance followed by Tukey Test for multiple comparisons, all treatments were statistically different than control (p<0.001); for WT JJ4-36, combined
treatment with MB-1 and reserpine was also statistically better ($p<0.001$) than reserpine or MB-1 monotherapy, which were not statistically different from one another ($p=0.06$).