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Translational deficiencies in antibacterial discovery and new screening paradigms

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An impending disaster is currently developing in the infectious disease community: the combination of rapidly emerging multidrug-resistance among clinically relevant bacterial pathogens, together with an unprecedented withdrawal from industrial dedication to this disease area, is jeopardizing human health on a societal level. For those who remain focused and dedicated to identifying solutions to this growing problem, additional challenges await when *in vitro* activity does not correlate with *in vivo* efficacy. Thus the development of more effective translational assays will greatly improve and streamline the process of identifying novel antibacterial agents that can stand the test of preclinical and clinical development. Here we describe recent examples of research that justify the need for such assays.

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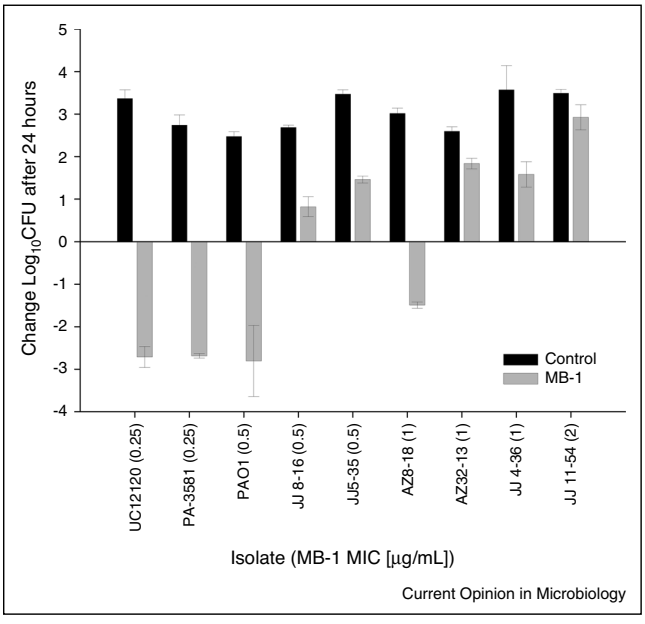
The need for new antibiotics that avoid the action of clinically relevant resistance mechanisms is continuing to grow. Sadly, it comes at a time when the gap between the spread of resistance and the pharmaceutical investment in developing novel antibacterial agents is widening at an alarming rate. The challenges of identifying novel chemotypes that offer safe and effective antibiotic activity, coupled with a limited return on investment relative to other therapeutic areas, has positioned us for a disastrous, pre-antibiotic era-type scenario. Further complicating matters, recent experiences that describe unexpected challenges during pre-clinical and clinical development have further dampened enthusiasm for novel antibacterial discovery, as unexpected failures are never rewarded and are rarely tolerated by large pharmaceutical organizations, particularly when operating under our current set of economic circumstances. Therefore avoiding such surprises should be considered vital to the continued interest and investment of large research groups. Here we describe multiple case studies that demonstrate the importance of having a fundamental understanding of the physiological context in which target bacterial pathogens exist during an infection. As these examples suggest, appreciating the *in vivo* environment by developing and utilizing relevant *in vitro* assays for screening and characterization will improve antibacterial drug discovery and development in two ways. First, through the reduction in the number of efficacy-related failures in preclinical and clinical studies, and second, by expanding our view of new antibiotic targets and pathways that could be exploited due to their importance *in vivo*, despite a lack of demonstrated importance *in vitro* when tested under traditional conditions.

Siderophore conjugation represents a recently revisited attempt by antibacterial researchers to outsmart target Gram-negative pathogens by tricking them into importing toxic compounds via outer membrane receptors used for normal iron acquisition processes. As iron is an essential micronutrient for bacterial survival, the rationale to target this pathway as a means to circumvent antibiotic resistance mediated by decreases in cell permeability via reduction in porin expression was well justified. Indeed, early [1,2] and more contemporary studies [3–5] demonstrating the *in vitro* activity of different siderophore conjugates provided convincing evidence of this strategy's potential, and multiple pharmaceutical companies made significant investment into this approach as a result. Unfortunately, these novel entities were screened in *in vitro* susceptibility assays using methods compliant with those established by the Clinical and Laboratory Standards Institute (CLSI; [6,7]), as is the standard practice for new antibacterial drug discovery and development. Included in these traditional methods, however, is the use of nutrient replete media (such as cation-adjusted Mueller Hinton broth), which do not mimic the physiological environments encountered by pathogenic bacteria during an infection in a mammalian host. This is a particularly critical disconnect when investigating the utility of siderophore conjugates, which require active iron acquisition for uptake and functionality. Since the concentration of free iron in these media is substantially higher (i.e. ~5 μM as reported in [8]) than the levels reported *in vivo* (reported as 10^{-24} M in [9]), the significant reduction in antibacterial activity associated with increases in endogenous siderophore production was not accounted for,

and the ability of native siderophores to shift the minimum inhibitory concentration (MIC) of lead siderophore conjugates was catastrophic to further development of these compounds [10•].

Figure 1 depicts the translational deficiencies for MB-1, a siderophore-monobactam conjugate that was in pre-clinical development at Pfizer for the treatment of multidrug-resistant (MDR) Gram-negative pathogens. With encouraging *in vitro* activity against organisms like *Pseudomonas aeruginosa* (MIC_{90} = 1 μg/ml), and a spontaneous resistance emergence frequency equivalent to those possessed by current, standard-of-care antibiotics (8.1×10^{-7} at 4X MIC), enthusiasm was high as this compound successfully passed pharmacokinetic (PK) and toxicity evaluations. It was after a comprehensive study for *in vivo* efficacy, however, that the liability of testing using iron-replete conditions *in vitro* became apparent. Using a panel of 9 contemporary *P. aeruginosa* clinical isolates, which varied in their antibiotic resistance profiles against marketed agents yet all appeared to be MB-1-susceptible in CLSI-compliant *in vitro* assays, a lack of widespread efficacy was demonstrated, despite the prediction of broad-spectrum activity across all 9 isolates (Figure 1). This issue with consistency prompted the evaluation of alternative *in vitro* assays, which strayed from the conventional testing guidelines, in an attempt to elucidate the cellular mechanism(s) by which these isolates resisted MB-1 activity. It was ultimately determined that elevations in the production of pyoverdine, one of the endogenous siderophores used by *P. aeruginosa* to acquire iron, was contributing to the transient recalcitrance of *P. aeruginosa* strains, and a predictive *in vitro* assay was developed to predict the performance of MB-1 *in vivo* [10•]. In hindsight, a more thorough appreciation for the contextual environment where MB-1 efficacy would be required, particularly as it pertains to free iron levels in this case, would have likely prompted the evaluation of native siderophore production on the activity of MB-1 prior to advancing to *in vivo* efficacy studies.

Figure 1



Efficacy of the siderophore-monobactam conjugate MB-1 against 9 different clinical isolates of *P. aeruginosa* using an immunocompromised mouse thigh model, as reported in [10]. Two hours after intramuscular infection, mice were subcutaneously administered a regimen of MB-1 (50 mg/kg) that resulted in free drug exposures of 4 μg/ml for the duration of the study. Mouse thighs were removed 24 hours after the initiation of therapy, bacterial burdens were determined, and changes from the initial bacterial burden are shown. *In vitro* MIC values, generated using standard, CLSI-compliant methods, are indicated in parentheses along the X-axis for each bacterial strain.

Another contemporary strategy for novel antibacterial drug discovery is the exploitation of the essentiality of lipid A biosynthesis for the production of lipopolysaccharide (LPS) by Gram-negative bacteria. LPS is involved in maintaining the cellular architecture of the outer membrane of these organisms, but also provides critical protection against host defenses such as complement deposition and phagocytosis. Basic research uncovered the genetic pathway responsible for the biosynthesis of lipid A, identifying the biochemical step mediated by LpxC, a UDP-3-*O*-(*R*-3-hydroxymyristoyl)-*N*-acetylglucosamine deacetylase, as the first committed step in the pathway. Small molecule LpxC inhibitors have since been identified and characterized [11–13], with several showing broad-spectrum antibacterial activity against multiple Gram-negative pathogens, including those that are multidrug-resistant. Interestingly, *Acinetobacter baumannii* strains, which display similar levels of resistance and susceptibility to comparator antibiotics as other Gram-negative bacteria tested, have shown complete recalcitrance to the activity of various LpxC inhibitors. It should be noted here, however, that these *in vitro* susceptibility assays were performed in accordance with the same CLSI guidelines described earlier. While inclusion of *A. baumannii* is important in a novel antibacterial agent's spectrum of activity, failure of LpxC inhibitors to affect this pathogen was overshadowed by their coverage of *P. aeruginosa* and multiple members of the *Enterobacteriaceae*.

An examination of the inhibitory activity of lead LpxC compounds on the isolated enzyme revealed significant differences in the IC_{50} for the *A. baumannii* enzyme versus that for the *P. aeruginosa* ortholog [14], prompting researchers to believe that this was the primary reason for poor antibacterial activity. Others postulated that efflux-mediated resistance could be contributing to this phenotype, and susceptibility assays were performed in the presence of efflux pump inhibitors such as phenyl-Arg-β-napthlyamine (PAβN) to test this, but their results did not support this hypothesis (unpublished data). It was