Assessing evolutionary risks of resistance for new antimicrobial therapies

New antibiotics are urgently needed to combat rising rates of resistance against all existing classes of antimicrobials. We highlight key issues that complicate the prediction of resistance evolution in the real world and outline the ways in which these can be overcome.

Michael A. Brockhurst, Freya Harrison, Jan-Willem Veening, Ellie Harrison, Grace Blackwell, Zamin Iqbal and Craig Maclean

The rising global rates of antimicrobial resistance against all existing antibiotic classes combined with a near-empty pharmaceutical pipeline for new antibiotic classes have produced renewed urgency in drug discovery efforts[1]. Recent high-profile discoveries based on novel screening methods for natural products (for example, teixobactin[2]) or modified natural products (such as arylomycin[3]) are encouraging, but the evolution of resistance remains a serious concern in maintaining the long-term efficacy of new antibiotics. The standard approach for assessing the risk of resistance evolution in such studies is to measure the rate at which spontaneous resistance mutations arise using fluctuation tests: assays in which bacterial cultures are spread onto agar containing an antibiotic, and then the number of colony-forming units (CFU) is compared with that of antibiotic-free control cultures[4]. Whole-genome or targeted sequencing of resistant mutants can then identify the mutated genetic locus (or loci) and the degree of resistance provided by different mutations. While these data provide a valuable snapshot of the potential for the tested bacterium to evolve resistance via spontaneous mutation, resistance evolution in the real world is often more complex, as is evident from older antibiotics that induce high rates of in vitro resistance but remain clinically useful (such as rifampicin). Our recent conversations with research scientists in a small antibiotic discovery firm and a multinational pharmaceutical company suggest that the pharmaceutical industry now takes resistance evolution very seriously and implements stringent cut-offs for in vitro resistance mutation frequencies that are deemed acceptable for new antibiotics. An oversimplified view of resistance evolution combined with stringent in vitro targets for resistance risks halting the development of potentially useful compounds if in vitro tests overestimate the evolutionary success of resistant genotypes (by ignoring high in vivo fitness costs, for example). Conversely, underestimating the in vivo evolutionary success of resistant genotypes will lead to wasted efforts in antibiotic development. How, then, can we bridge the gap between existing practice and a more realistic assessment of the risk of resistance evolution to ensure the long-term utility of new antibiotics?

Mind the reality gap

Here we identify four key issues that complicate the prediction of resistance evolution in the real world and outline the ways in which these can be overcome (Fig. 1).

1. Population ecology of infections. All else being equal, resistance by spontaneous mutation is more likely in larger bacterial populations[5]. Bacterial population size varies widely between different kinds of infection, although accurate estimates of population sizes are surprisingly limited. For example, the total number of bacteria in bloodstream infections caused by *Escherichia coli* and *Staphylococcus aureus* varies between approximately 10^3 and 10^8 cells[6], and pathogen densities during ventilator-associated pneumonia can exceed 10^6 CFU per mL of sputum[7]. However, invasion by resistant genotypes can also be affected by differential clearance of resistant versus susceptible genotypes. For example, the number of *E. coli* cells in a urinary tract infection can reach 10^{10} CFU, guaranteeing the presence of thousands of resistant mutants, but resistance rarely evolves during infection because of the high rate of turnover of bladder contents[8]. In contrast, chronic biofilm infections may sustain large population sizes, lower rates of population turnover and elevated mutation rates, giving rise to high levels of standing genetic variation that are available for natural selection to act upon[9–11]. Further, physiological tolerance to antibiotics can allow sufficient time for resistance mutations to arise in vivo[12], which may not be observed in vitro. Improving resistance prediction requires better data on the basic population ecology parameters of different infections.
2. Pre-existing resistance. Standing genetic variation (SGV) for resistance is likely to exist for new antibiotics targeting cellular processes that are already targeted by existing drugs, or that are derived from natural antimicrobials (such as antimicrobial peptides[13]). For example, *S. aureus* acquired the staphylococcal cassette chromosome *mec* (SCC*mec*) element years before the clinical introduction of methicillin, and methicillin use resulted in the very rapid spread of the Methicillin-resistant *S. aureus* (MRSA) lineages[14]. Selection by non-antimicrobial drugs[15] and non-clinical antimicrobial agents (like triclosan[16,17]) also has the risk of collateral evolution of antibiotic resistance. The vast database of genome sequences of pathogenic bacteria enables genome mining, a powerful method to prospectively test for SGV at genes involved in resistance to new antibiotics. For instance, for arylomycin, seven resistance mutations were identified in the *lepB* gene[3]. We scanned the European Nucleotide Archive (ENA) for mutations in *lepB* using the search tool BIGSI[18] ($n = 447{,}833$ whole-genome sequence datasets). We found 45,009 datasets contained a *lepB* gene for which >97% of DNA was identical to the *lepB* gene in the *E. coli* strain ATCC 25922, harbouring a total of 98/324 codons with at least one non-synonymous mutation. We did not detect any of the seven variants that were found to confer arylomycin resistance in the original study[3], but we did find

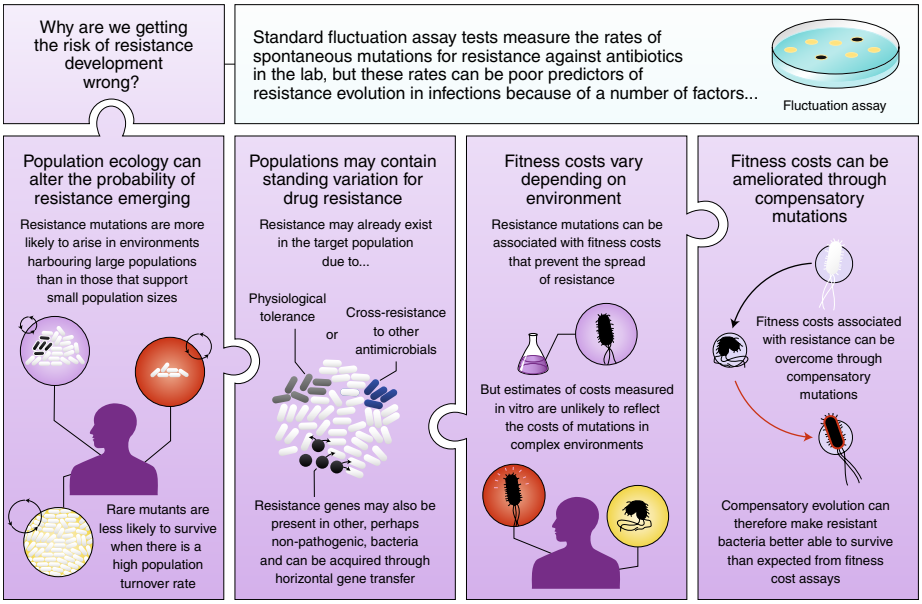

Fig. 1 | Real-world resistance. Predicting the evolutionary risk of resistance against new antibiotics is complicated by the complexity of real-world infections.

82 datasets containing another amino acid substitution at one of those seven positions (p.L142I). This demonstrates that it is feasible to estimate whether resistant variants are circulating among the bacterial population before clinical use of an antibiotic if the mutations that confer resistance are known. Alternatively, resistance determinants against new antibiotics can be detected using functional metagenomics[19]. Here, fragments of metagenomic DNA are expressed and screened for their effect on resistance. This approach is especially useful for detecting unknown resistance genes present in environments where natural-product-derived antibiotics are naturally expressed[20,21] or in animal or human microbiomes[22,23]. Pre-existing resistance is especially problematic if these genes become mobilised on mobile genetic elements (MGEs), such as transducing phages and plasmids[24]. Horizontal gene transfer via MGEs or bacteriophages, or via competence for transformation, can strongly contribute to the rise and spread of resistance[25,26]. Experiments that will estimate rates of gene mobilisation in relevant environments[27] are urgently required.

3. Fitness costs and interplay with the host. Population genetic models suggest that fitness costs associated with resistance limit its persistence upon removal of the antibiotic[5]. Costs arise because resistance mutations may impair the normal function of the target gene or through physiological costs of expressing resistance genes or harbouring MGEs[28]. Fitness costs can be easily quantified using growth or competition assays in lab media and animal infection models, and these should be provided in all reports of new antibiotics[28]. Although there is some degree of agreement between in vivo and in vitro measures of fitness cost[28,29], in vitro assays can underestimate the fitness costs of resistance that manifest in more complex infection environments[30]. For example, mutations conferring resistance to bacteriocin-derived avidocin antibiotics cause loss of the surface layer of *Clostridium difficile*. This has no effect on in vitro growth rate but makes resistant cells highly susceptible to innate immune effectors and avirulent in an in vivo infection model[31]. A high in vitro rate of mutations conferring resistance against a new antibiotic can deter further development of a new drug, even if high fitness costs in vivo effectively limit the success of these resistant mutants in patients because of competition or immune clearance. For example, the development of mecillinam would currently be halted by pharmaceutical companies because of high resistance mutation frequencies (ranging from 8×10^{-8} to 2×10^{-5} mutations per cell), but resistance in the clinic is rare because of the low in vivo fitness of mutants[32]. Accurately modelling within-host bacterial metabolism and physiology by using more realistic experimental environments, such as host-mimicking media[33,34], in vitro biofilm models[35,36], animal models[37] or ex vivo tissue models[38,39], will offer a more accurate picture of fitness costs.

4. Compensatory evolution. Over time, second-site mutations that compensate for the fitness cost of the resistance mutation while leaving the resistant phenotype intact can allow persistence of resistant genotypes in vivo and in environmental reservoirs in the absence of antibiotic selection[28,40]. The targets of compensatory mutations can be determined using evolve-and-resequence experiments in which resistant mutants are serially passaged without antibiotic until they recover ancestral-level fitness[41–43]. For example, mutations in *Mycobacterium tuberculosis* that compensate for the fitness cost of rifampicin resistance in vitro are found in clinical isolates and contribute to the tuberculosis epidemic[44]. The extent to which lab studies provide an unbiased view of compensatory evolution in the real world is unclear[45], but one promising solution to this problem is to use genome-wide association analyses of bacterial genomic datasets to link known resistance mutations with subsequent compensatory mutations. For example, acquisition of multidrug resistance plasmids in *E. coli* sequence type 131 has been shown to be associated with specific regulatory mutations that may compensate the fitness cost of plasmid carriage[46].

Bridging the gap

Ideally, evolutionary analysis of resistance should accompany in vivo experimental and clinical trials of new antibiotics. The following changes to existing practices would allow far better understanding of the potential for resistance evolution:

- Appropriate choice of infection models during research and development. Consideration of the physicochemical environment at infection sites and its effects on bacterial growth, gene expression and physiology will provide better estimates of likely rates of resistance emergence and associated fitness costs, which may differ substantially from those observed in standard laboratory growth media[34]. For example, the leucyl-tRNA synthetase inhibitor GSK2251052 showed limited resistance development in vitro but failed in clinical trials because of rapid emergence of resistance in treated

patients[47]. Improved estimates of the risk of in vivo resistance would allow drug candidates to be better prioritised for progression to clinical trials and could therefore directly benefit the pharmaceutical industry by enabling more effective targeting of resource investment during antibiotic development.

- Dosing to minimise resistance selection. Massive experimental and modelling efforts are made to quantify the pharmacokinetics/pharmacodynamics (PK/PD) of new antibiotics before a clinical trial, with the goal of maximizing antibiotic efficacy. Minimizing selection for resistance should also be a goal of PK/PD studies[48], which should employ appropriate infection models.
- More intensive sampling of infections during clinical trials. Current approaches sample single colonies, ignoring the diversity present in most infections and therefore losing the opportunity to understand evolutionary processes occurring in infections[49,50]. More extensive culture-based sampling should be combined with deep sequencing to quantify resistance allele frequencies and capture hard-to-culture organisms (such as persister cells and polymicrobial infections). Providing access for scientists to the samples from completed clinical trials would allow the development of methods and analyses for efficiently characterizing the evolutionary mechanisms leading to resistance in patients. Furthermore, development of culture conditions that allow maintenance of diverse bacterial populations and/or communities in the lab would facilitate understanding of how resistance evolution is a function of microbial community interactions.
- Microbiome sampling in clinical trials. Antibiotic treatment often has collateral effects on the microbiome, including driving lateral transfer of resistance elements at other body sites[51]. Combining new bioinformatics tools for resistance prediction[52] with long-read or proximity-ligation sequencing technologies allows localisation of resistance genes to their genomic context and the tracking of gene mobilisation and transfer[53,54].

These new approaches will increase the scale and cost of clinical trials in the immediate term and will require that medical funders and pharmaceutical companies embrace eco-evolutionary thinking. However, there are likely to be direct benefits in terms of more effective targeting of investment and fewer potentially useful drug candidates being discarded owing to misleading in vitro resistance estimates. It is therefore possible that taking a more realistic view of resistance evolution could help rejuvenate the antibiotic pipeline. ❐

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Competing interests

The authors declare no competing interests.