Expanding the search for small-molecule antibacterials by multidimensional profiling

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Abstract

New techniques for systematic profiling of small molecule effects can enhance traditional growth inhibition screens for antibiotic discovery and change how we search for new antibacterial agents. Computational models that integrate physicochemical compound properties with their phenotypic and molecular downstream effects can not only predict efficacy of molecules yet to be tested, but also reveal unprecedented insights on compound Modes of Action (MoAs). The unbiased characterization of compounds that themselves are not growth inhibitory but exhibit diverse MoAs, can expand antibacterial strategies beyond direct inhibition of core essential functions. Early and systematic functional annotation of compound libraries thus paves the way to new paradigms in the selection of lead antimicrobial compounds. In this Review, we discuss how multidimensional small molecule profiling and the ever-increasing computing power are accelerating the discovery of unconventional antibacterials capable of bypassing resistance and exploiting synergies with established antibacterial treatments and with protective host mechanisms.

Introduction

Empirical susceptibility screening, wherein new antimicrobial compounds are chosen for their ability to inhibit bacterial growth, catalyzed the initial formidable success in antibiotic discovery. However, screening of soil microorganisms for antibiotic production quickly yielded the rediscovery of the same common antibiotics, and the output of novel antibiotic classes rapidly decreased[1]. The danger raised by the evolution of antimicrobial resistance urges the development of novel paradigms in the search for small-molecule antibacterials with new mechanisms of action (**Box 1**) that do not cross-resist with existing classes of antibiotics[2]. While new genetic and genomic approaches have enabled the genome-wide discovery of essential genes and revealed a plethora of new and unexploited antibacterial targets[3 4], target-based drug discovery has struggled to deliver new classes of antibacterials. Molecules identified to bind and interfere with promising targets often fail to fulfill subsequent key requirements, such as permeability, toxicity or pharmacokinetics. Hence, the field of antimicrobial discovery is still largely dominated by *in vitro* susceptibility screening assays (Figure 1A) allowing to directly single out compounds that produce the desired growth inhibitory activity. This has restricted the search for antibacterials to compounds inhibiting core growth-related processes, mainly identifying inhibitors of macromolecule biosynthesis, like cell wall-, protein- and DNA/RNA synthesis, eventually leading to the rediscovery of the same antibiotics over and over again[1]. Furthermore, the use of *in vitro* conditions that largely differ from *in vivo* conditions[5], and biases in physicochemical properties of synthetic chemical compound libraries [6] (e.g. optimized for permeability in eukaryotic cells) contributed to the lack of success in recent antibiotic discovery campaigns. As if it wasn't enough, the almost inevitable evolution of resistance poses additional concerns to evaluate the effectiveness of newly discovered antibacterial molecules. Hence, there is an urgent need for alternative and efficient strategies for the experimental identification of starting points to produce the next generation of antibacterials.

In the traditional susceptibility-based approach, only compounds exhibiting measurable growth inhibitory activity, typically with a Minimum Inhibitory Concentration (MIC) below 10 µM, are selected. Moreover, lead compounds are chosen without knowledge of their targets or Modes of Action (MoAs) (**Box 1**). These are major bottlenecks hindering the identification of promising antibacterial molecules that have poor bioactivity or penetration[7 8], properties which could subsequently be rationally improved. Similarly, classical susceptibility screens overlook compounds that target processes not essential *in vitro* but that could be attractive strategies for *in vivo* treatment and may associate with a lower selective pressure for resistance [9 10]. Meanwhile, new computational and experimental approaches have emerged to not only predict compound growth inhibitory activity, but also characterize the broader functional impact of small molecules on *in vivo* pathogen

physiology[11 12 13]. Multidimensional and high-throughput technologies, like molecular profiling [14 15 16] or high-content imaging [17 18 19], in combination with novel genetic tools like gene editing (e.g. CRISPR) [20], enable the prediction of drug targets, MoAs, secondary effects, tolerance mechanisms and *in vivo* efficacy at large scale and already at early stages of drug discovery[14 21 22]. Such a multiparametric characterization can guide the selection of the most promising bioactive compounds, mitigate the problem of rediscovering compounds with the same MoAs (i.e. dereplication problem) and empower computational approaches like Artificial Intelligence (AI) [22 23] that can learn new and fundamental principles in target-based design, chemical optimization and rational combination therapies.

In this review, we focus on experimental technologies (Figure 1B-F) that, with the aid of mathematical modeling, can augment classical phenotypic screens and provide a more comprehensive characterization of the consequences of drug treatment (Figure 1G), beyond growth inhibition. First, we review recent technological advances that allow multidimensional characterization of drug effects and thereby of drug action. Next, we examine computational strategies to perform *in silico* antibacterial screening and extract functional information from multidimensional profiling of small-molecule effects. Finally, we discuss how the systematic characterization of virtually any type of bioactive molecule can expand the search of antimicrobials to molecules that don't directly inhibit bacterial growth but exploit synergies with the host or with conventional antibiotics.

Beyond growth inhibition

Early identification of antimicrobial targets

Because essentiality is condition-dependent[5 24], the translational relevance of *in vitro* susceptibility screens (Figure 1A and 2A) hinges on the availability of experimental models that can mimic *in vivo* environments and interactions with the host. Environmental conditions can be so important that clinically relevant antibiotics, while potent antibacterials *in vivo*, exhibit poor activity in standard laboratory conditions. An emblematic example is daptomycin, for which the use of synthetic media with physiological concentrations of calcium is sufficient to reduce its Minimal Inhibitory Concentration (MIC) almost by 100 fold with respect to standard Mueller Hinton broth (MHB) [25]. Conversely, compounds with promising *in vitro* activity, such as pyrimidine–imidazole derivatives, can target processes that become dispensable *in vivo*[5]. Thus, standard *in vitro* growth assays don't adequately evaluate the *in vivo* antimicrobial potential of bioactive small molecules, and screening for growth inhibitory compounds in rich laboratory conditions that fail to reproduce host environments can miss attractive antibiotic candidates or potential strategies to potentiate the effect of classical

antibiotics [5] [26]. Nevertheless, mimicking the complexity of *in vivo* conditions and host-pathogen interactions with scalable *in vitro* systems remains challenging[27].

Shifting the focus from merely characterizing growth inhibitory activity to identifying drug binding partners can reveal properties of small molecules that are largely condition-independent and hence provide an orthogonal starting point from which to assess their potential as antibacterials. Classical biochemical approaches to directly probe drug-target binding and mechanisms of action (Box 1), such as affinity purification, chromatography or *in vitro* enzyme assays, are not only low-throughput, but typically focus on a small number of protein targets at a time, making these techniques inadequate to systematically identify new drug targets. Conversely, new mass spectrometry approaches for the profiling of protein conformational changes (i.e. chemical proteomics, Figure 1B) such as thermal proteome profiling (TPP)[28] and limited proteolysis (LiP-MS) [29], while still limited in throughput, can probe drug-target interactions proteome-wide (Figure 2B). Both technologies detect the stabilization of protein structures as the drug binds its target, leading to higher thermal stability (e.g. melting temperature), measured by TPP, or protection from proteolytic cleavage, measured by LiP-MS, allowing the *de novo* identification of direct drug-protein binding events and indirect effects on the interaction of drug targets with other proteins. TPP can be performed in intact cells, allowing confirmation of drug penetration and of whether the interaction occurs within the cell, while LiP-MS is able to report on binding sites, potentially facilitating chemical optimization. Hence, these two technologies are complementary to each other and can become key to train new *in silico* structural models[30] [31] to predict ligand-target binding at an unprecedented scale.

While chemical proteomics methods are attractive technologies to characterize putative targets of candidate antibacterials, physical interactions don't directly report on the functional consequences of drug treatment. Hence, binding assays are typically performed together with functional (i.e. phenotypic) assays that inform on cellular processes affected by the drug treatment. An emblematic example is the characterization of the mechanisms of action underlying the bactericidal activity of a novel antibiotic SCH-79797 [32]. Only by combining TPP with phenotype-based assays were the authors able to reveal that SCH-79797 not only binds and inhibits dihydrofolate reductase, but simultaneously disrupts membrane potential, similar to nisin or polymyxin B[32].

Complementing phenotypic with molecular descriptors

An effective approach for the systematic and mechanistic characterization of large compound libraries and to accelerate the discovery of antimicrobials with potentially new MoAs is to combine genome editing technologies with scalable *in vitro* phenotypic assays. Versatile genetic tools like RNAi, antisense RNA-, haploinsufficiency or CRISPR-Cas9 technology allow systematically generating

genome-wide mutant-, knockout-, knockdown- or overexpression libraries, in virtually any organism. In arrayed or pooled formats, these collections are invaluable resources enabling high-throughput growth profiling of genetically modified bacteria against compound libraries (i.e. chemogenetic screens) to query for genes that enhance or suppress antimicrobial activity [3 4 20 33]. Genes associated with drug action can encode direct or proximal drug targets, such as downstream enzymes in the same pathway of the drug target, or cellular functions crucial to endure drug treatment (Figure 1C). When interpreting chemogenetic screening data, it is important to consider that changing gene expression, such as with RNAi or CRISPR-based modulators, is not always sufficient to reveal functional dependencies between gene function and the drug MoA. For instance, proteins might be in excess, such that overexpression or mild down-regulation might not be sufficient to induce measurable changes in drug susceptibility. Similarly, if a drug target is regulated mainly at the post-translational level, protein activity can be largely invariant to protein abundance. Moreover, an increased expression of a drug target does not always lead to an inversely proportional change in susceptibility to an antimicrobial. For example, antibiotics like ciprofloxacin that don't directly act on the catalytic activity of the targeted protein but rather modify its function, can exhibit a counterintuitively higher toxicity when the target (e.g. gyrase complex) is overexpressed[34].

Testing changes in susceptibility for all gene-drug combinations remains laborious, and classical chemogenetic screening is limited to compounds directly or indirectly affecting growth-related processes (Figure 2C). Advances in imaging technologies have bypassed this limitation and made it possible to monitor several cytological features in a single-pass screen (Figure 1D). Such features are largely orthogonal to growth inhibition, like cell/colony morphology (e.g. shape, area, length, width) [18 19 35] or fluorescent labels reporting on transcriptional activity or metabolic states [17 36] (Figure 2D and Table 1). A key advantage of imaging-based approaches is that parameters can be measured at the single-cell level, offering unique insights into the heterogeneity of bacterial responses and drug efficacy, potentially unraveling key factors contributing to tolerance and evolution of resistance [37].

While imaging-based profiling augments conventional phenotypic screening from one (e.g. growth) to dozens of features (e.g. morphological parameters), predicting and characterizing new drug MoAs based on low-dimensional descriptors remains challenging. Molecular profiling technologies, by generating multiparametric signatures encoding drug-induced changes in the abundance of thousands of individual proteins [15], transcripts [16] or metabolites [38] (Figure 1E and Table 1), can fill this gap and enable unbiased and *de novo* characterization of compounds with potentially unconventional MoAs[39]. Thanks to advances in recent years, technologies like metabolomics, proteomics and transcriptomics [16 15 38 40] now achieve the necessary throughput and scalability to rapidly generate large compendia of high-dimensional molecular profiles (Figure 2E), increasingly also at the single-cell level[41] (Figure 1F).

Remarkably, molecular profiling methods are often able to detect subtle adaptive changes even in the absence of measurable phenotypic effects [38]. Such high sensitivity enables deriving functional insights also for molecules or drug concentrations that exhibit poor penetration or result in low activity, respectively, maximizing the information collected from single-pass screening (Figure 1G).

Several characteristics define the potential and limitations of technologies used for the characterization of small molecule effects. There are inevitable tradeoffs between throughput, number of measured parameters, level of resolution – i.e. from phenotypic to molecular descriptors or from averaged population measurements to single cells –, and the possibility to make comparative or *de novo* predictions of compound MoAs. Currently, no single technology outperforms the others in all aspects (Figure 2). Therefore, the characterization of *in vitro* small molecule effects by both phenotypic and molecular profiling is crucial for mechanistic and predictive modeling of antimicrobial efficacy in more complex environments, and for identifying possible synergies with the host immune response or with classical antimicrobial treatments.

From *in silico* activity to functional annotation

Identifying bioactivity and MoAs early in the discovery pipeline aids the selection of the most promising next-generation antibacterials, e.g. excluding compounds with conventional MoAs and widespread (cross-)resistance, while prioritizing compounds exhibiting novel and promising MoAs. Computational frameworks able to integrate heterogenous small molecule-associated profiles are key to maximize the utility of multidimensional data[42] [38] [43] [22], providing experimentally testable hypotheses on drug efficacy, MoA and molecular targets (Figure 1G).

In silico prediction of inhibitory activity and effects

The size and complexity of drug profiling data has fostered the adoption of automated analysis strategies able to extract non-obvious patterns and to learn new fundamental rules (Figure 3A). Machine- and deep learning algorithms [22] [23] are particularly efficient approaches to mine high-dimensional and heterogeneous chemoinformatics-, phenotypic- and molecular drug profiling data and to address problems like activity prediction[44] [22] [12]. For example, machine learning algorithms enabled the prediction of mouse treatment outcomes based on growth inhibitory activities of drug combinations measured in multiple *in vitro* models of *M. tuberculosis* infection[11]. Similarly, when trained on *in vitro* growth inhibitory activity of 2335 molecules, a deep learning approach allowed *in silico* prediction of the inhibitory activities of more than 100 million molecules in only few days [12]. This approach identified molecules that, while structurally dissimilar from already known antibiotics, exhibit growth inhibitory activity *in vivo* against a broad bacterial spectrum – a remarkable result considering that deep neural networks typically require more data for training compared to classical

machine learning algorithms. Strikingly, the authors found a human kinase inhibitor, renamed Halicin, to be a potent antibacterial[12], emphasizing the importance of considering compound diversity[45] in screening for new antibacterial compounds.

AI models trained on *in vitro* growth inhibitory activities can overlook molecules with promising antibacterial targets, but poor bacterial penetration. To overcome this problem, measuring the accumulation of 180 diverse compounds in the Gram negative bacterium *Escherichia coli* uncovered new practical rules on how to optimize the physicochemical properties of small molecules to increase their accumulation inside bacteria[8]. Thus, high-throughput assays that inform on drug accumulation/penetration[46] could provide invaluable information in the training and application of machine- and deep learning algorithms for the simultaneous discovery and optimization of new antibacterial molecules. Unfortunately, AI approaches are generally "black-box" systems, and rationalizing the AI decision-making process to derive causal and mechanistic biological insights from complex drug profiling data is a daunting task. Combining multiscale modeling such as genome-scale metabolic networks with drug profiling data can render AI approaches more transparent and interpretable [47]. In a recent approach, instead of directly training a machine-learning model on growth inhibitory data, a genome-scale model of *Escherichia coli* metabolism and constraint-based modeling was used to map measured screening data into network representations of changes in metabolic fluxes[47]. This network model-driven machine-learning approach revealed a previously unappreciated causal role of purine biosynthesis in mediating the bactericidal activity of diverse antibiotics. Hence, machine- and deep learning frameworks, by incorporating additional molecular characteristics (e.g. metabolic changes) in the analysis of growth inhibitory activities, could expand the scope of virtual screening to a broader characterization of the functional impact of small molecules.

Comparative annotation of small molecules MoAs

Despite recent advances, *in silico* antibiotic discovery still suffers from the lack of mechanistic insights on the MoAs of newly identified compounds. To enable rapid and systematic MoA predictions, molecular profiles of small molecules can be directly compared to those of compounds with known MoAs, following the "guilt-by-association" principle [14 15 48] – i.e. drugs that exhibit similar profiles can have similar MoA and/or efficacy (comparative prediction, Figure 3B). One key aspect in such a comparative analysis is the metric used to assess distance or similarity between compound profiles. The choice depends on the specifics of the problem and the nature of the data. Correlation metrics like Pearson correlation, estimated on the full set of measured features, favor global relationships of a specific form (e.g. linear), and could be better suited to find drugs with almost identical properties. Other metrics testing entropy-based similarity (e.g. mutual information) or conditional independence (e.g. graphical Gaussian models), or techniques to decompose a signature into

individual components that are maximally independent from each other, like principal- or independent component analyses, are able to better exploit the complexity of high-dimensional data and can capture local similarities, revealing more subtle commonalities between compounds MoAs[38] [14] [49]. A key premise of comparative approaches is that different classes of antibiotics exhibit distinct physicochemical properties or induce characteristic changes in cell morphology, transcriptome, proteome or metabolome profiles [14] [15] [20]. Using drug profiling signatures at multiple scales and in combination is likely to maximize the predictive power of comparative analysis in chemically and functionally diverse compound libraries and to reveal crucial differences between compounds with similar MoAs [50] [51] [32] [52]. While the primary scope of comparative approaches is limited to the search against antimicrobials with already known MoAs, finding unique signatures that differ from those of conventional antimicrobials can lead to molecules with new MoAs, thereby alleviating the problem of rediscovering compounds with the same MoAs [14] [19]. Moreover, maximizing diversity in multidimensional drug profiling signatures can represent an attractive alternative to maximizing chemical diversity [45] [38], which alone is not sufficient to yield compound sets with diverse biological actions [19].

As an alternative to the comparison between drug profiles, profiling of large-scale libraries of genetically modified bacteria[3] [20] [33] has opened the door to systematically search for genetic perturbations that can mimic the effect of small-molecule perturbations[38] [53] [54] . Differently from chemogenetic screens in which each mutant is tested against all compounds, comparisons of molecular profiles in response to genetic *vs* chemical perturbations requires each perturbation to be tested only once. Hence, multidimensional profiling of genetic perturbations can generate a set of reference profiles to speed up the search for inhibitors of specific gene functions, and simultaneously accelerate the functional annotation of large compound libraries[55]. By using this principle and comparing metabolic changes induced by knocking down essential genes in *E. coli* with drug-induced metabolic changes, we discovered that tegaserod, a drug commonly used to treat irritable bowel syndrome, interferes with the essential structural anchoring between peptidoglycan and lipoproteins [55]. It is worth noting that modulating the expression of one individual gene is often not sufficient to completely mimic drug effects, because drugs can engage multiple targets without necessarily inhibiting their catalytic functions, and the response to drug treatment can be radically different from compensation for gene downregulation. In spite of these limitations, this approach has been used successfully in a number of diverse drug discovery applications, from antibiotics[14] to anticancer agents[40] [55], offering new opportunities to combine target- and phenotype-based drug discovery and ultimately expanding the chemical space of small molecules for emerging therapeutic targets.

Network modeling enables *de novo* predictions of MoAs

Molecular profiling of small molecule effects offers a sensitive and high-dimensional readout, able to detect effects of treatment also for molecules that fail to inhibit bacterial growth[38]. Exposure to antibacterials that have poor penetration, bioactivity[22], or interfere with non-essential functions, can trigger adaptive molecular changes, such as target overexpression, without inducing macroscopic phenotypes (e.g. growth inhibition, morphological changes). If detected, such adaptive changes can still reveal key aspects of drug action. However, formulating experimentally testable hypotheses of drug targets, MoAs and response mechanisms is less straight-forward than with phenotypic assays. Differently from chemogenetic screening or mutational profiling of antibiotic-resistant strains, which directly point to genes interfering with the compound's ability to inhibit growth, interpreting drug molecular profiles requires integration with prior knowledge and mathematical models, like gene regulatory-, signaling- or metabolic networks. In return, model-based analysis of molecular profiles allows differentiating drug targets from downstream effects and key processes mediating the response to drug treatment [43 51 56 57 58] (Figure 3C), also in the absence of measurable phenotypic changes [52]. For instance, combining dynamic metabolic profiles with kinetic modeling unraveled how *E. coli* can robustly cope with fluctuations in the expression of essential genes, such as carbamoyl phosphate synthetase (CarAB) [56]. The authors showed that the allosteric activation of CarAB by ornithine allows maintaining constant fluxes in arginine and pyrimidine nucleotide biosynthesis even when CarAB is knocked down. Network analysis of genetic perturbations can offer invaluable insights on the potential efficacy of drug targeting essential gene products, and which innate or potentially evolvable compensatory mechanisms can bypass and neutralize drug effects.

Often, the large number of unknown parameters and variables limits the scope of these models to small-scale systems [51 56 57]. A powerful approach is the linear approximation of dynamic systems operating at near-steady state, which enables formulating tractable models of complex non-linear regulatory and biochemical networks at genome-scale [58 43 59]. For example, gene regulatory networks can be parametrized using large compendia of steady-state transcriptional profiles across largely diverse conditions[59]. These relatively simple models are able to identify genes encoding drug targets by estimating a set of additional external inputs that simulate drug-target interactions, and thereby improve fitting of drug-induced transcriptional changes [43] (Figure 3C). The potential of this approach applied to drug-induced gene expression changes was shown by inferring that 1-phenyl-1H-tetrazol-5-ylsulfonyl-butanenitrile (PTSB), a potent antifungal and anticancer drug, interferes with redox balance and specifically inhibits the thioredoxin/thioredoxin reductase system[43]. Additionally, integrating the dynamics of the drug-induced changes, while adding an additional level of complexity in the analysis, can provide crucial information to resolve direct (typically early-onset) from indirect drug effects[51 57 56].

Exploiting unconventional antimicrobial targets

The difficulties in finding antibiotics with new MoAs might also be a consequence of a limited number of druggable proteins being essential in nutrient-rich laboratory conditions. The number of essential genes can vary between 300 to 600, depending on genome size and bacterial species[60]. Of these, only less than 10% (i.e. 30-60) is predicted to be druggable [61], raising the question whether, with 50 non-redundant antibiotic targets[61], we have already discovered most MoAs that act by targeting core essential bacterial proteins. Expanding the systematic characterization of the chemical space also to molecules with no *in vitro* antimicrobial activity can facilitate the discovery of compounds targeting cellular processes fundamental for *in vivo* survival and colonization (Figure 4A), epistatically interacting targets (Figure 4B) or mechanisms to survive antimicrobial treatment (Figure 4C). In this section, we will focus on how multidimensional profiling and computational tools could facilitate and enable the systematic exploration of unconventional antibacterial strategies.

Systematically interfering with regulators of virulence.

Anti-virulence therapies such as interfering with pathogen quorum sensing systems, *in vivo* adaptation, biofilm formation or bacterial toxins [62] [63], are promising unconventional antibacterial strategies that, without killing or inhibiting bacterial growth, can fight and curb the emergence and spread of resistant pathogens. Transcriptome and proteome analyses of bacterial physiology at the infection site can reveal regulatory strategies of pathogens during colonization and infection and thereby, expose a plethora of new potential targets for antivirulance agents [82] [64] [65]. For example, dosR-mediated transcriptional regulation (DosRST) is essential in *Mycobacterium tuberculosis* (Mtb) to adopt a dormant state and survive in granuloma [9]. By screening a large library of small molecules on a DosRST-dependent fluorescent Mtb reporter strain, new inhibitors of DosRST signaling have been discovered. Among them artemisinin, which was shown to induce transcriptional profiles largely similar to those of a *dosR* deletion mutant strain[36]. Because modulating key transcriptional regulators of virulence, like *dosR* in Mtb or toxT in *Vibrio cholerae*, induces unique transcriptional signatures [66] [67], transcriptome profiling of small molecule effects could be a valid alternative to monitoring individual gene reporters[36] [10] and speed up the search for compounds that selectively interfere with or revert the downstream effects of virulence factors. While monitoring individual gene reporters would require the genetic insertion of a new construct and to re-screen the compound library for each gene target of interest, reference databases of genome-wide transcriptome profiles of chemical and genetic perturbations together with similarity-based analysis could allow searching for molecules able to interfere with virtually any regulator. This strategy has been used to find selective inhibitors of human kinases [40], or to identify compounds potentially interfering with important *E. coli* regulators for *in vivo* cellular adaptation, such as *rpoS* and *fur* [38].

Targeting host-pathogen interactions

Profiling of pathogen-induced gene expression changes in the host revealed that host-derived metabolites can play a key role in dictating the intracellular lifestyle of pathogens and in establishing host-pathogen interactions essential for virulence and survival [68 69]. Mounting evidence suggests that the host can unintentionally promote virulence and pathogen survival [68], but also employ strategies that limit the access of bacteria to key nutrients like amino acids or cofactors [70 69]. Pathogens in such hostile environments will likely exhibit vulnerabilities that cannot be discovered in standard phenotypic *in vitro* assays. Using human serum to mimic physiological conditions such as wound exudate or human blood revealed key differences in gene expression regulation of the *arn* operon, mediating arabinosaminylation of lipopolysaccharides in *Pseudomonas aeruginosa* [26]. The authors found that downregulation of the *arn* operon is not only likely responsible for the increase in the susceptibility to azithromycin in host-like media with respect to standard MHB, but also for augmenting the synergy between azithromycin and synthetic host-defense peptides.

Metabolic profiling of supernatants and analysis of uptake/secretion rates of nutrients and by-products[71] can be used to constrain genome-scale models of metabolism and predict attractive and selective targets essential *in vivo* [72 73 74] (Figure 4A). For example, a genome-scale metabolic model of *E. coli* was able to identify conditionally essential enzymes in the human blood, macrophage and urinary tract, predictions which could subsequently be validated[13]. Overall, molecular profiling techniques able to measure the composition of complex nutritional environments or monitor *in vivo* pathogen physiology[75] can aid the characterization[68 76]and *in vitro* reconstitution of conditions in infected tissues[77 78] and enables directly searching for molecules that can target mechanisms to circumvent host-induced nutrient limitation, to scavenge essential nutrients [70 79 80], or further potentiate the host immune response[81].

Leveraging epistatic interactions between small molecules

Large-scale di- and trigenetic screens have unraveled a large space of gene pairs or triples that, if simultaneously inhibited, could provide an alternative solution to the search for antibacterial monotherapies. Instead of single drugs targeting essential proteins, small molecules could be co-administered to target synthetic lethal proteins (Figure 4B), or potentiate the action of classical antibiotics (Figure 4C) [82 83]. Exploiting combinations of two or more compounds could dramatically expand the range of targets and molecules that could be selected from large-scale screening[84]. However, due to the explosion in the combinatorial complexity, the discovery of combination therapies remains a major challenge. To bypass testing all possible combinations of small molecules, multidimensional profiling of the effects of individual molecules has opened new opportunities in the

data-driven prediction of effective drug combinations[85 38 86]. For instance, chemogenetic profiles were shown to be a powerful training data source for a machine learning algorithm able to predict epistatic interactions between antibiotics[85]. Combining metabolomic- or transcriptomic profiling of small-molecule effects with chemical-genetic interaction data not only enables to predict pairs of epistatically interacting compounds, but also reveals mechanistic insights underlying drug-drug interactions[38 87]. Hence, combining classical phenotypic with multidimensional profiling of chemically diverse compound libraries can rationalize and expedite the search for unconventional combination therapies.

Hampering resistance and tolerance

The rapid emergence of antimicrobial resistance represents a major challenge to the discovery and development of new antibacterial treatments. A growing body of genome-wide association studies has unraveled global molecular signatures, like gene expression or proteome changes, that associate, explain and could be predictive of resistance [28 88 89 90]. A particularly comprehensive study demonstrated that proteome composition analysis of more than 300.000 clinical isolates enables machine learning to predict antimicrobial resistance across clinically relevant pathogens[90]. Even if the most obvious approach would be to directly target the element responsible for increased resistance (e.g. inhibiting β-lactamases, efflux pumps), the comprehensive characterization of the indirect effects of resistance elements uncovered additional ways to hamper and select against evolution of resistance, or predict collateral susceptibility[28 91 92], i.e. increased susceptibility to antibiotics different from the one selecting for resistance. The fact that different resistance mechanisms associate with specific molecular signatures [93 94] offers the opportunity to search against reference databases, and reevaluate the profiles of small molecules with no or little antibiotic activity. Inhibiting non-essential cellular processes that are nonetheless key to evade antibiotic killing actions, such as those mediating metabolic homeostasis[88] or compensating for the cost of resistance mutations[91] can not only potentiate the efficacy of classical antibiotics, but also hamper evolution of resistance [91 92]. A high-throughput competitive assay identified two compounds, both with metal-chelating properties, that invert the selective advantage of an increased expression of tetracycline-resistance efflux pump (TetA) in *E. coli* [92]. Remarkably, the authors found that both compounds are able to select for null insertions or deletions of the *tetA* gene suggesting for the possibility that compounds that select against resistance genes can be used in a two-phase treatment regimen. The first phase can revert resistant pathogens back to sensitive thereby improving the efficacy of a second-phase treatment with a conventional antibiotic.

Major obstacles in implementing such strategies in a clinical setting are the possible emergence of mutations conferring cross-resistance and the ability to evolve strategies to survive the exposure to

lethal antibiotic concentrations longer (i.e. tolerance) [95 96] (Figure 4C). The possibility to broadly profile the consequences of drug treatment has revealed indirect but nevertheless critical effects of classical antimicrobial treatments, as well as unexpected pathways allowing bacteria to tolerate treatment for longer periods, thus facilitating evolution of resistance[96 97 98 99]. Single point mutations in central metabolic genes, such as proton-pumping NADH:ubiquinone oxidoreductase (Nuo complex)[96], were shown to be sufficient to achieve high levels of multidrug tolerance in *E. coli*. Consistent with these findings, it has been suggested that secondary effects altering the homeostasis of potentially toxic metabolites act as key mediators of antibacterial activity of antibiotics with largely different MoAs [97 98 55]. For example, Korormicin is a small molecule that selectively kills gram-negative bacteria expressing the Na^+-pumping NADH:quinone oxidoreductase. Interestingly, it was shown that the antibiotic's action is not directly caused by inhibiting enzyme activity, but rather results from indirect promotion of the production of reactive oxygen species[98]. Hence, targeting cellular processes that catalyze or mitigate indirect toxicity of antibiotics, such as homeostatic regulation of metals, cofactors, purine biosynthetic intermediates or reactive oxygen species, represents an attractive strategy to potentiate conventional antibiotics (Figure 4C) [98 47 55]. Altogether, multidimensional profiling of small-molecule effects opens new opportunities in the rational design of combination therapies that can preserve the efficacy of conventional antibiotics and expands the scope of lead selection to small molecules with no growth inhibitory activity.

Conclusions/Perspective

Discovering new antibacterial strategies is a daunting and urgent challenge. Over the past decade, antibiotic discovery has been transformed from screening for growth inhibitory activity to the characterization of several alternative phenotypes and molecular characteristics of small-molecule effects. Moving the characterization of lead compounds MoAs up in the drug discovery pipeline has fostered an iterative learning process to expand the functional diversity of compound libraries and the spectrum of molecules with potential as antimicrobials (Figure 5). A new promising strategy in the selection of unconventional antibacterial molecules is to search for molecules that induce similar or synergistic signatures to downstream toxic antibiotic effects or that are able to interfere with key adaptive mechanisms to establish *in vivo* infection, without the requirement for growth inhibitory activity *in vitro*. Moreover, the unbiased characterization of small-molecule effects will enable assembly of compound libraries modulating diverse bacterial functions, providing an entirely new chemical probe toolbox to gain insights in fundamental aspects of microbiology.

While new computational tools like AI algorithms are able to deal with levels of complexity that other methods cannot handle, they not only necessitate large amounts of data for training. Carefully designed experiments generating high-quality data that avoid potential biases, like enrichment for

specific physicochemical compound properties, or confounding elements such as batch effects, are of utmost importance. This raises the demand for generating data repositories and standards in data acquisition that can adequately support the development of computational approaches able to tackle long-standing challenges in antibacterial discovery and development. This is an already ongoing effort in other research fields, like anticancer research, where large multiscale data resources are being assembled and shared (e.g. https://depmap.org/broad-sanger/). Consortia involving academia and pharma companies engage the scientific community to develop and test new *in silico* approaches, such as new strategies to predict drug efficacy or combinations from gene expression profiling (e.g. DREAM Challenges), with the prospect of more rapidly advancing our understanding of how to extract biological insights from multidimensional data[40 100]. However, while in the cancer field there is a strong support from the private sector, this is lagging behind in antibacterial discovery, where most efforts are supported by government programs. The more mature experience in anticancer research could serve as a blueprint for establishing similar consortia to accelerate translational research in antibacterial discovery.

With ever-increasing throughput and financial incentives to expedite antibacterial research, ultimately the bottleneck will move from data acquisition to data analysis. Combining artificial intelligence with mechanistic models of genetic and biochemical networks will not only increase the success rate and thereby reduce the cost of antibacterial discovery. We will also learn fundamental lessons in basic and pathogenic microbiology, opening new opportunities beyond antibacterial/drug discovery and potentially fostering new directions in synthetic biology, sustainability and biomedical applications.

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Competing interests

The authors declare no competing interests.

Boxes

Box 1:

Mode of action: reports on the functional consequences of drug treatment and how the drug achieves its intended therapeutic effects. For example, most current antimicrobials can be grouped into 4 classes with distinct modes of action: interference with the cell wall (e.g. penicillins), inhibition of nucleic acid metabolism and repair (e.g. fluoroquinolones), inhibition of protein synthesis (e.g. macrolides) and interference with folate metabolism (e.g. sulfonamides).

Mechanism of action: the mechanism of action of a drug is more specific than its mode of action. It defines the targets and specific biochemical interaction (e.g. competitive *vs* non-competitive, agonist *vs* antagonist) through which a drug produces its pharmacological effect. For example, the mechanism of action of penicillins is the irreversible binding of the β-lactam ring to the active sites of penicillin-binding proteins (i.e. transpeptidase and acylates), ultimately preventing the formation of cross-links between peptidoglycans.

Tables

Table 1. Platforms and readouts for the screening and multidimensional characterization of small molecule effects. Different screening platforms (Figure 1) make use of different measurements and techniques to characterize drug effects on target organisms. As methods differ in the respective readouts they generate, functional and/or molecular effects of small molecules on target organisms can be profiled along multiple dimensions.

Figures

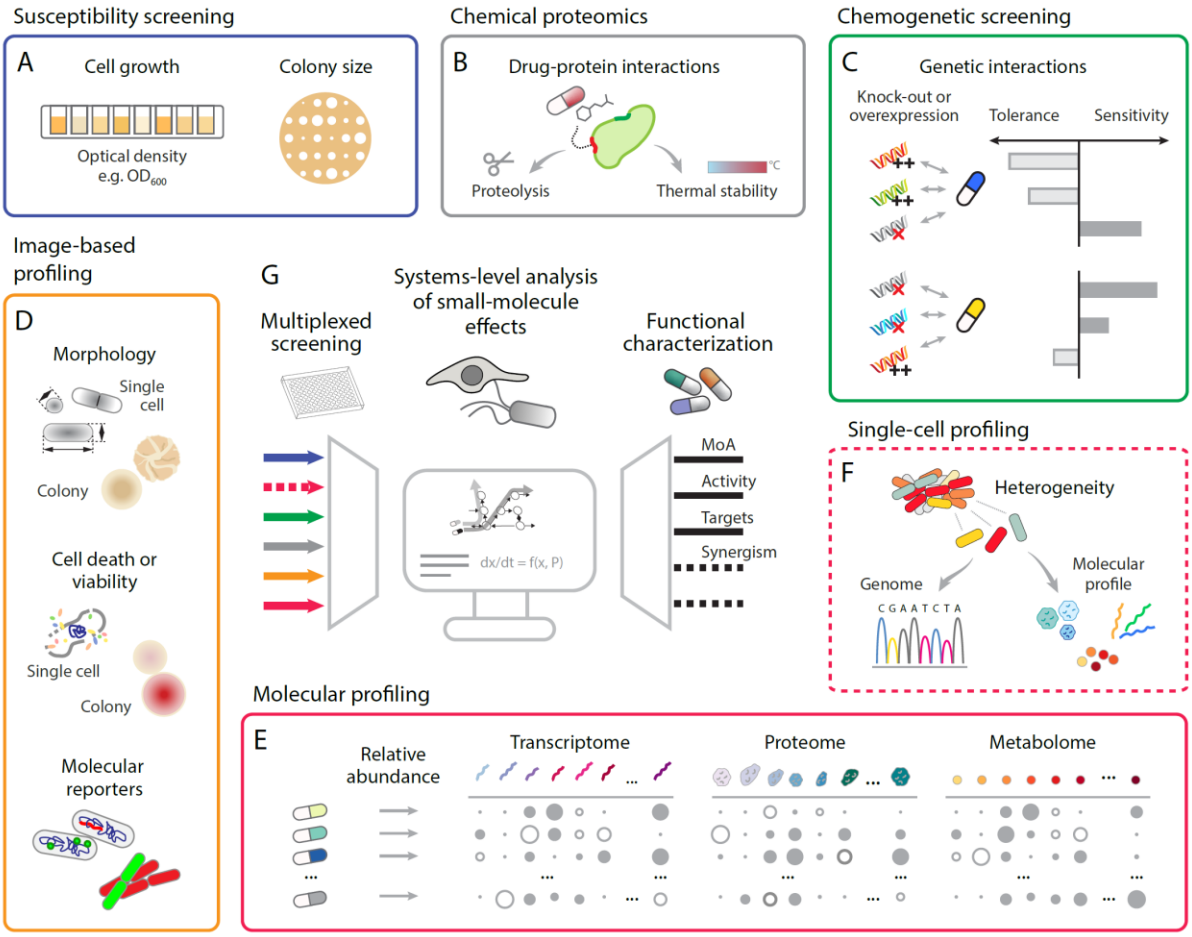

Figure 1. Multidimensional and multiplexed characterization of small-molecule antimicrobials. Techniques for the systematic characterization and multidimensional description of small molecule effects are grouped in five major approach types. **(A)** Susceptibility screening: measures the ability of antimicrobial candidates to inhibit bacterial growth, typically by measuring cell density in planktonic cultures or colony size on solid media. **(B)** Chemical proteomics: alternative to classical biochemical approaches, by measuring stabilization of protein structural features and protection from proteolytic cleavage and melting (i.e. thermal stability), advanced mass spectrometry-based proteomics methods allow detecting direct and proximal physical interactions of small molecules with proteins. **(C)** Chemogenetic screening: identifies genetic perturbations, like gene deletion (x) or overexrepssion (++), that enhance or reduce susceptibility (i.e. sensitivity or tolerance). **(D)** Imaging-based phenotypic profiling: automated imaging platforms enable large-scale monitoring of additional cellular characteristics, such as cell- or colony morphology, cell shape transitions (e.g. bulging, cell lysis), reporters of gene expression or metabolic activity. **(E)** Molecular profiling: measures drug-induced changes in the abundance of intracellular biomolecules, like transcripts, proteins and metabolites, yielding high-dimensional signatures of drug action independent of growth inhibitory activity. While gene expression is typically profiled using DNA microarrays or RNA sequencing, proteomics and metabolomics rely mostly on mass spectrometry-based approaches. **(F)** Adaptations for single-cell profiling are emerging, allowing single-cell measurements of bacterial responses to candidate antibacterials to characterize rare sub-populations (e.g. persisters) and heterogeneity in the treatment response. **(G)** The reviewed technologies for the profiling of small-molecules effects are complementary to each other and can be multiplexed in drug screening. With the aid of models to predict and investigate how chemical and genetic perturbations propagate through biochemical and genetic networks and thereby affect pathogen physiology and its interactions with the host, phenotypic and molecular profiling of small molecules effects can unravel unique mechanistic insights on their antibacterial activity, MoAs or potential synergism with other compounds.

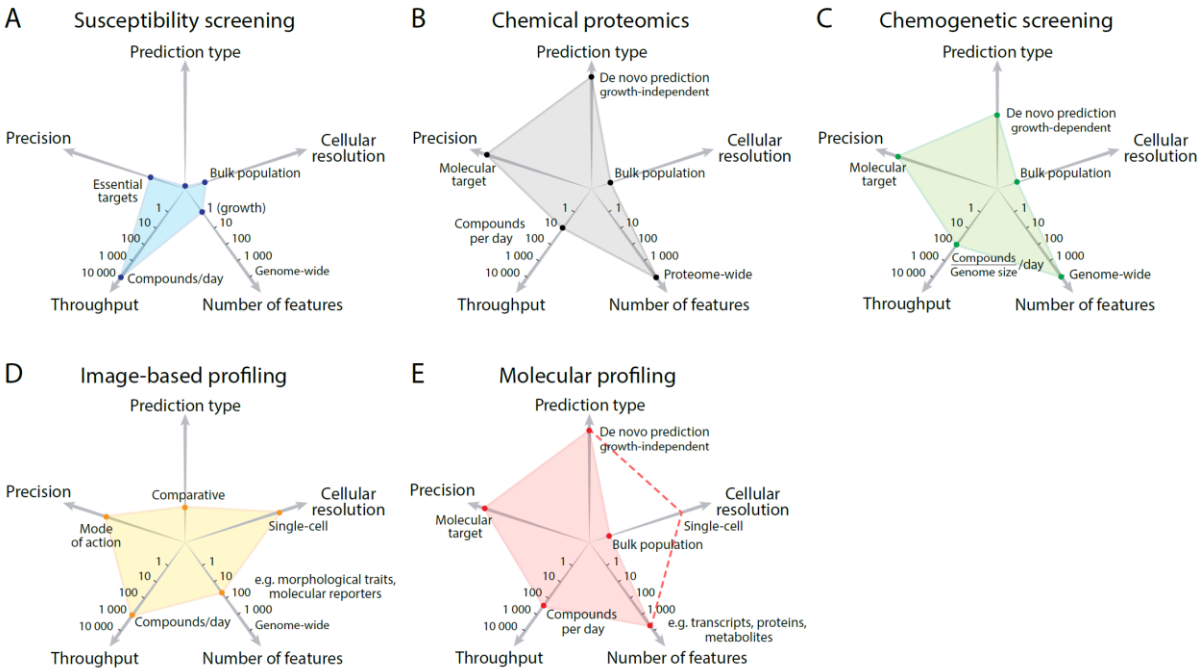

Figure 2. Different scopes of small-molecule antibacterial profiling technologies. (A-F) Approaches for the profiling of small molecule effects reported in Figure 1 are here described by five major characteristics. Throughput: How many compounds can be profiled per day - susceptibility screens (panel A) are scalable and offer the highest throughput, while chemogenetic screens (panel C) necessitate that the growth inhibitory activity of each compound is tested against multiple genetically modified strains, typically genome-wide mutant libraries, reducing the effective throughput. Number of features: dimensionality (number of measured parameters) of the small-molecule descriptors. Cellular resolution: from bulk measurements reporting the average behavior of bacterial populations to single-cell measurements in image-based profiling (panel D), and more recently molecular profiling technologies (panel E, dashed line). Prediction type: from the comparative analysis of uncharacterized compounds against a reference set of antimicrobials to *de novo* predictions of new antimicrobial MoAs even for compounds without growth inhibitory activity. Precision: the ability to discriminate direct targets and mechanisms of action from drug MoA and indirect/general effects of small molecules.

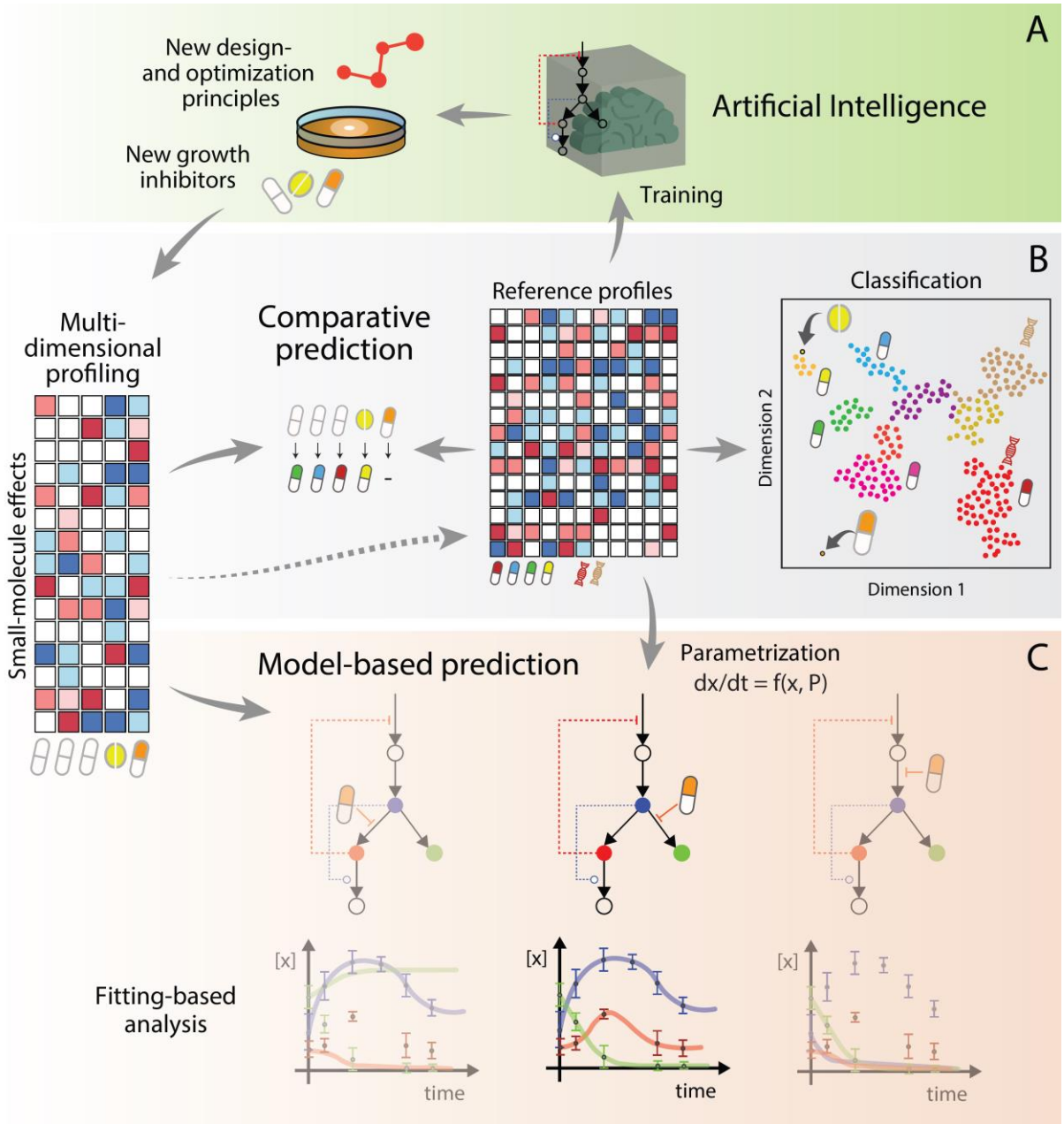

Figure 3: Computational extraction of functional insights from multidimensional profiles of small-molecule effects. (A) AI algorithms for the *in silico* discovery of new and yet to be profiled antibacterials use multidimensional profiling data to train and improve interpretability, unraveling novel principles guiding compound optimization and development. **(B)** Profiles of uncharacterized molecules are compared to antibacterials with an already known MoA or with genetic perturbations, such as knockout or overexpression. Such comparative analysis enables the systematic functional annotation of compounds and their classification in already known classes of antimicrobials or as potential new and unconventional antibacterials. **(C)** Frameworks for the de novo prediction of MoAs integrate molecular profiles and a priori knowledge in mechanistic or probabilistic models of genetic and/or biochemical networks to simulate different possible scenarios, like the effects of inhibiting different drug targets. Model-based simulations are compared to experimental data to find the most plausible parameters and drug targets.

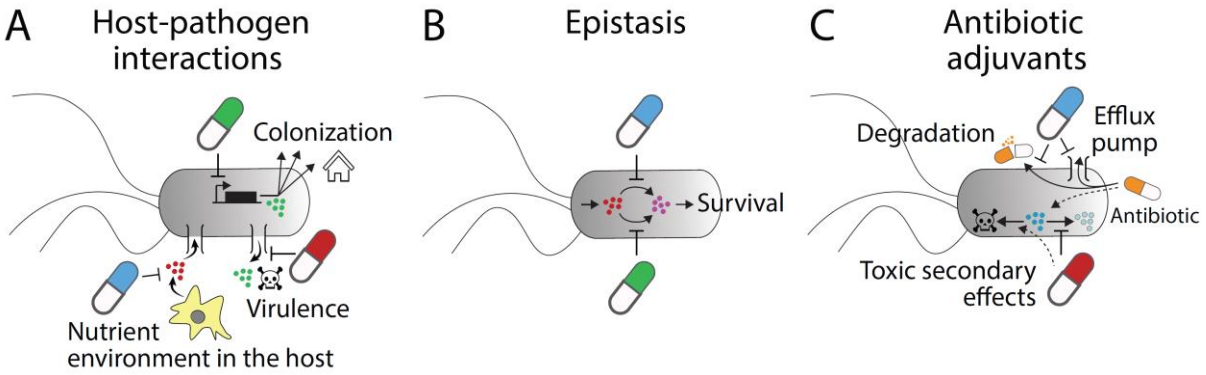

Figure 4: Leveraging potential synergies within the host and bacteria. Molecules that per se have little or no growth inhibitory activity *in vitro* could still be effective antibacterials: **(A)** by interfering with virulence factors (red), bacterial adaptation to the host (green) or synergizing with the host immune response, like inducing nutrient restrictions (blue). **(B)** By targeting synthetic lethal proteins, i.e. targeting proteins that become essential only when their activity is simultaneously inhibited (green + blue). **(C)** By interfering with antibiotics (orange) resistance mechanisms (e.g. efflux pumps) (blue) or potentiating the side effects of antibiotics action, like the production or remediation of toxic intermediates (red).

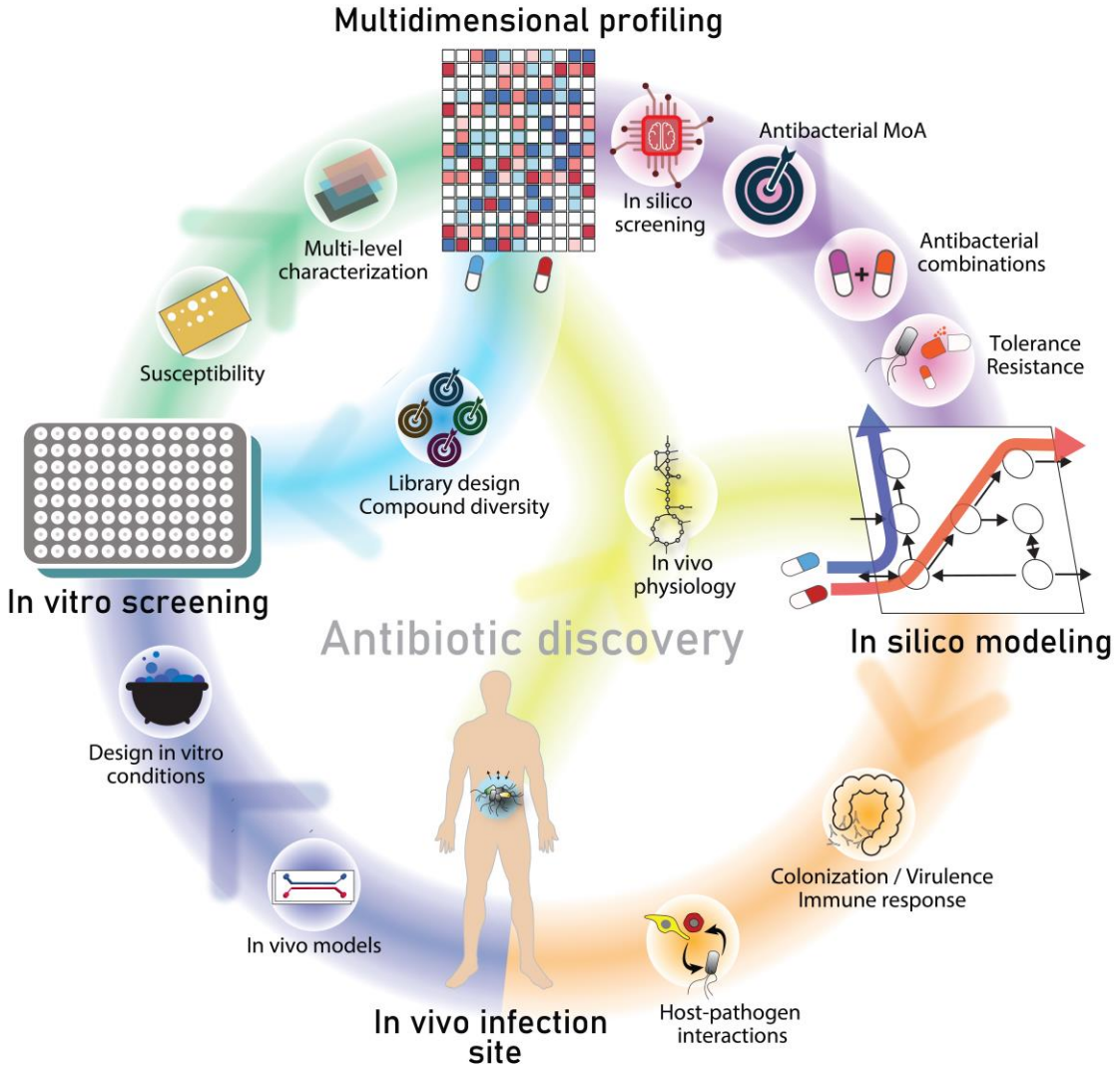

Figure 5: New iterative cycles in the discovery and validation of potential antibacterial compounds. Multidimensional characterization of large compound libraries, beyond susceptibility, inform on drug action

already in the first screening phases (green). Multidimensional characterization of small molecules effects can be directly used to assemble libraries of diverse bioactive compounds (light blue). With the aid of computational tools, like AI or genome-scale models of metabolism or transcriptional regulation, small molecules profiles can provide systematic predictions of MoA, epistatic drug interactions, mechanisms of resistance/tolerance (purple). Model-based analysis of small molecules profiles can also make predictions of drug efficacy, before experimental *in vivo* testing. Moreover, model-based predictions can repurpose molecules that are not themselves growth inhibitory, but can target processes fundamental for virulence, colonization or can interfere with host-pathogen interactions, such as nutrients restrictions at the infection site (orange). In turn, the same technologies used to profile drug effects could be used to gain quantitative insights on physiology of bacteria at the infection site, providing additional information to refine model-based predictions (yellow) or foster the development of *in vitro* systems that better mimic naturalistic *in vivo* conditions (purple).

Highlighted references

[3] Systematic titration of gene expression and analysis of fitness cost in Mycobacterium tuberculosis reveals genes vulnerability and quantify essentiality of bacterial functions.

[4] Elegant strategy to screen large chemical libraries against hypomorph pools of strains depleted of essential bacterial targets. This strategy named PROSPECT increase the probability of hit compounds discovery and provide mechanistic insights on compound functions.

[14] Combination of high-throughput metabolic profiling of chemical libraries and limited proteolysis reveal the modes of action of new antituberculosis compounds.

[15] By combining SWATH technology with high-flow chromatography the authors developed an ultra-fast proteomics approach reducing sample acquisition measurement to 60 seconds opening new opportunities in drug mode-of-action screening.

[22] This study comprehensively demonstrates the power of combining multiparametric high content screening and genomic approach, beyond classical susceptibility screening, to guide lead compound selection and their functional annotation.

[38] Comparing metabolic changes induced by genetic and chemical perturbations can be used to characterize MoA of compounds that target non-essential processes and thereby enable searching for unconventional antibacterial compounds.

[12] The authors developed a deep learning model trained to predict antibiotics based on structure and in vitro growth inhibitory activity. AI was able to predict a molecule that while structurally different from classical antimicrobials exhibits broad-spectrum antibiotic activities in mice.

[87] The authors used a machine learning approach to screen in silico more than 1 million potential drug combinations using Mycobacterium tuberculosis transcriptomic profiles of individual drug effects revealing mechanistic insights on the mechanisms of drug-drug interactions.

[56]Model-based regulatory analysis of metabolic adaptive changes upon gene knockdowns revealed regulatory mechanisms that can buffer fluctuations in enzyme protein levels.

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