

## Review

## The future of antibiotics begins with discovering new combinations

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Antibiotic resistance is a worldwide and growing clinical problem. With limited drug development in the antibacterial space, combination therapy has emerged as a promising strategy to combat multidrug-resistant bacteria. Antibacterial combinations can improve antibiotic efficacy and suppress antibacterial resistance through independent, synergistic, or even antagonistic activities. Combination therapies are famously used to treat viral and mycobacterial infections and cancer. However, antibacterial combinations are only now emerging as a common treatment strategy for other bacterial infections owing to challenges in their discovery, development, regulatory approval, and commercial/clinical deployment. Here, we focus on discovery—where the sheer scale of combinatorial chemical spaces represents a significant challenge—and discuss how combination therapy can impact the treatment of bacterial infections. Despite these challenges, recent advancements, including new *in silico* methods, theoretical frameworks, and microfluidic platforms, are poised to identify the new and efficacious antibacterial combinations needed to revitalize the antibacterial drug pipeline.

**Keywords:** antibiotics; antibacterials; drug combinations; antibiotic resistance; microfluidics

### Introduction

Antibiotic resistance is a worldwide and growing clinical problem. Infections caused by multidrug-resistant bacteria contribute to an increasing number of deaths each year, with an estimated 35,000 deaths in the United States and as many as 700,000 deaths globally.<sup>1,2</sup> The Organization for Economic Cooperation and Development predicts that the occurrence of resistance to last-line-of-defense antibiotics will double from 2005 to 2030.<sup>3</sup> In an alarming contrast, the rate at which new FDA-approved antibacterials are entering the mar-

ket has steadily decreased over the past 50 years, and recent approvals are dominated by analogs of old drugs.<sup>4,5</sup> The rediscovery of known scaffolds has plagued recent efforts to discover new antibiotics, especially for the treatment of Gram-negative pathogens, where we observe the highest need for new drugs but almost no innovation in the clinical development pipeline.<sup>6,7</sup> These obverse rates of drug resistance and drug development will soon leave modern medicine with few and poor options for treating severe multidrug-resistant bacterial infections. Even when new antibacterial drugs are introduced, bacterial organisms will continue to evolve and develop resistance mechanisms to circumvent antibiotic activity. Thus, we need not only novel therapeutic agents to meet the near-term

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challenges, but also a reinvigorated discovery and development pipeline that can be sustained far into the future.

The most historically successful single-agent antibiotic classes engage multiple cellular targets or targets encoded by multiple genes—a phenomenon known as multitargeting. The multitargeting hypothesis of long-term antibiotic efficacy posits that bacteria must acquire multiple mutations to develop resistance to multitargeting drugs, thus slowing the acquisition of resistance.<sup>4,8</sup> Multitargeting is exemplified by the fluoroquinolones, which inhibit two topoisomerase enzymes and the  $\beta$ -lactams, which target a group of enzymes called the penicillin-binding proteins.<sup>4</sup>

The success of multitargeting antibiotics reflects multifactorial antimicrobial strategies recognized in nature. For example, microbes in natural communities compete with one another for resources in “chemical warfare” involving arrays of agents, while the innate immune system wards off pathogens with a combination of physical barriers, environmental pressures, antimicrobial peptides, antibodies, and direct cellular action.<sup>9,10</sup> Ironically, multitargeting has not been a common therapeutic design strategy, with multitargeting compounds derided as “dirty binders” that fly in the face of Ehrlich’s magic bullet (one drug, one target) ideal.<sup>11,12</sup> However, the high resistance frequencies afflicting many single-targeted agents is stimulating discussion about multitargeting as a central aim for new discovery efforts to recapitulate the success of golden age multitargeting antibiotic scaffolds.<sup>4,8</sup> Since the discovery of new multitargeting scaffolds has shown limited success to date, combinations of agents are a rational alternative strategy for multitargeting antibiotic therapeutic design. Already, several existing single-targeted antibiotics, including rifampicin and trimethoprim, are no longer commonly provided as monotherapies, but instead are used in combination with other antibiotics as a *de facto* combination multitargeting approach.<sup>13</sup>

Combination therapies are a treatment modality in which a patient receives two or more drugs to treat a single disease. Combinations or cocktails of drugs open up a tremendous variety of multitarget strategies inaccessible to individual pharmacophores. In addition, combinations can improve treatment efficacy, reduce resistance,

extend antibacterial coverage, or reduce toxicity to the host.<sup>14</sup> Drug combinations have famously been used to thwart drug resistance and provide new treatment options for many cancers, tuberculosis, malaria, and HIV/AIDS.<sup>15–18</sup> However, combinations have yet to be extensively exploited in the antibacterial space, with the notable exception of direct inhibition of antibiotic-degrading  $\beta$ -lactamases.<sup>19</sup> The discovery of  $\beta$ -lactamase inhibitors came out of an enormous effort made primarily by the pharmaceutical industry to rescue the activity of penicillins from a known target-independent resistance mechanism.<sup>19,20</sup>

Many of the drugs used in clinical combinations today were discovered as single agents with previously known activities or mechanisms of action, and combined post-hoc. This approach precludes access to some interaction types in which at least one component has little to no independent antibiotic activity and no strong hypothesis for interaction is in focus. Here, we argue for the use of phenotypic combinatorial (multiple-agent) screening to discover new drug interactions in which one or more compounds may lack antibiotic activity or exhibit a previously unknown antibiotic activity in a novel interacting compound set. In particular, we see great promise in novel adjuvants that may revive the efficacy of compounds with known antibiotic activity against multidrug-resistant pathogens.<sup>21–23</sup>

The discovery of such interactions requires phenotypic screening of huge combinatorial chemical spaces, challenging the available screening capacity and compound stock quantities. For example, many labs can test each compound in a modest 5000-compound library individually, but the number of assay conditions balloons to a daunting 12.5 million in a pairwise combination screen. Nonetheless, we believe that new technology, particularly the combination of efficient *in silico* and micro-scale empirical methods, renders screening for novel drug interactions tractable and makes a solid first step toward a new era of antibiotics development that can keep effective treatments in providers’ hands for many years to come. In this review, we briefly describe strategies for antibacterial combination therapy and discuss in greater detail the leading screening approaches that can enable the discovery of novel combination hits.

## Balancing therapeutic efficacy and drug resistance in antibacterial combinations

Antimicrobial resistance is driven by the replication of pre-existing resistant organisms, the acquisition of resistance elements through horizontal gene transfer, and *de novo* acquisition of resistance through new genetic changes within an organism's genome. As the latter two mechanisms entail some probability per cell per time, the establishment of large pathogen populations over extended periods of time increases the likelihood that drug resistance develops. Hence, antibiotic treatments should focus on highly efficacious therapies that can rapidly reduce the pathogen population before drug resistance spawns and spreads within the population.

Antibacterial combinations potentially combat resistance through prevention or improved efficacy of infection clearance. Combinations of single-targeted therapeutics can repress the emergence of resistance, just like multitargeting single agents, by requiring multiple mutations for increased pathogen fitness under treatment. Naturally, single mutations causing cross-resistance against multiple drugs could still occur in principle, such as generally improved efflux. Overall treatment efficacy has complex effects on resistance, including multidrug resistance against combinations. On one hand, increased efficacy can reduce the timeframe and pathogen population size, thus reducing the likelihood of acquired resistance. On the other hand, increased efficacy may apply strong selective pressure that enables resistant variants to more rapidly outcompete sensitive variants and subsequently dominate the pathogen population.<sup>24</sup> Additionally, if this resistant population persists, it becomes poised to acquire higher level resistance to the given drug and/or additional resistance to other drugs leading to multidrug resistance. Furthermore, synergistic combinations may be formulated in lower doses to reduce host toxicity.<sup>25</sup> Taken together with imperfectly overlapping compound distribution in a patient, exposure of the pathogen to sublethal drug conditions likely increases the opportunity for pathogens to acquire resistance. Therefore, such synergistic combinations raise the risk that single resistance develops, creating a starting point for multidrug resistance against additional low-dose drugs.<sup>26</sup> Many factors interact to drive such resistance dynamics, demanding that

resistance be assessed empirically. Combination therapies present new risks, but also new opportunities; we argue that it is time to invest in leveraging these opportunities, assessing the residual risk, and learning the best ways to move combinations forward against bacterial infections.

## Opportunities to leverage drug interactions against bacterial infections

Drugs used in combination exhibit independent, antagonistic, or synergistic effects (Table 1). Independence occurs when the combined effect is equal to the sum of the individual effects. Antagonism or synergy occurs when the combined effect is less or greater than the sum of the individual effects, respectively. A special case of antagonism is hyperantagonism, also known as suppression, in which the combined effect is even less than at least one drug's individual effect.<sup>27</sup> Antibacterial combinations can leverage each of these interactions to offer a variety of interesting therapeutic strategies, for example, improving efficacy, preventing the emergence of antibiotic resistance, reviving legacy antibiotics, increasing the target spectrum, or achieving high specificity for target pathogens.

While drug synergy is a favored topic, independent drug combinations may prove efficacious in some use cases, such as treating acute and severe infections when limited diagnostic information is available. Independent drug combinations can combine activities against a single organism or extend coverage across multiple bacterial species where at least one drug is active in each species. An example of the latter type in the clinic today is co-fluampicil, which comprises ampicillin, a moderate-spectrum penicillin active against streptococcal infections, and flucloxacillin, a narrow-spectrum antibiotic active against *Staphylococcus aureus*.<sup>28</sup> In the absence of a differential diagnosis, co-fluampicil can increase the chance of successful treatment by broadening the treatment coverage. Bacterial infections often progress rapidly; thus, employing two or more antibacterials can be life-saving by improving the chance that the initial therapy is efficacious, especially for patients at risk of severe respiratory infections or septic shock.<sup>29,30</sup>

Recent research suggests that antagonistic and hyperantagonistic combinations can repress resistance evolution.<sup>31</sup> While no hyperantagonistic combination has yet been translated to the clinic, their

**Table 1.** Summary of antibacterial interaction types

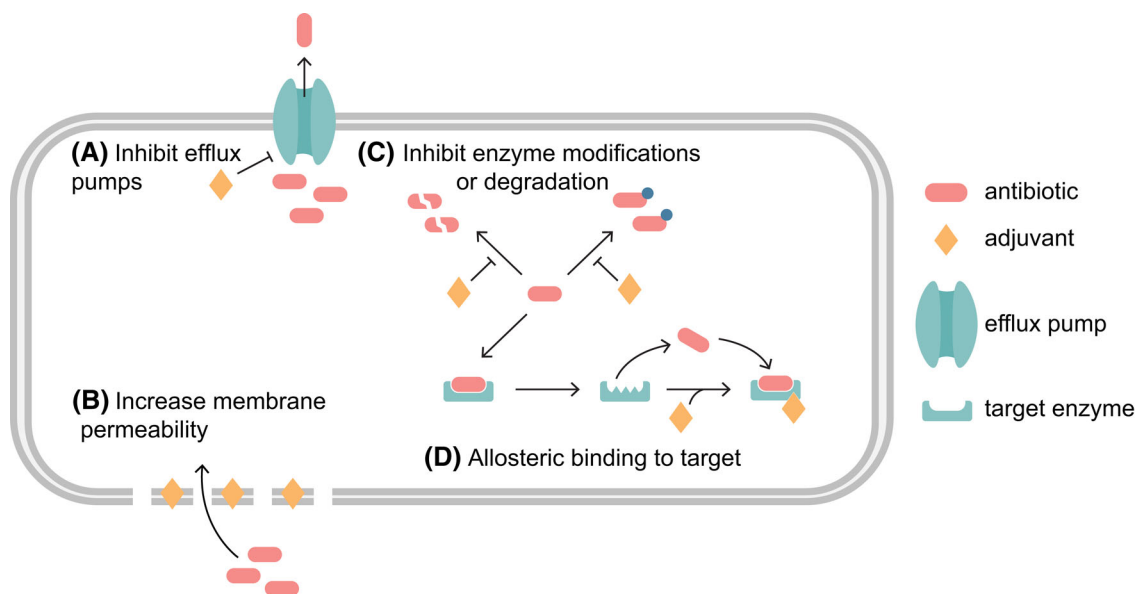
Interaction types	Component 1 independent activity	Component 2 independent activity	Combined effect is...
Independence (additivity)	Yes	Yes	equal to the sum of the individual effects
Antagonism	Yes	Yes	less than the sum of individual effects
Hyperantagonism (suppression)	Yes	Yes	less than at least one component's individual effect
Synergy: congruous	Yes	Yes	greater than the sum of the individual effects
Synergy: syncretic	Yes	No	
Synergy: coalism	No	No	

theoretical utility merits attention. Antagonism and hyperantagonism have been shown to reduce the selective advantage of resistant strains across a range of dosing regimens. For example, hyperantagonistic or suppressive pairs can reverse the selective advantage of resistant strains. When one drug suppresses the activity of a second drug, susceptible bacteria can grow in the presence of the first drug if a high concentration of the second drug is present. Acquisition of resistance to the first drug then removes the suppressive protection from the second drug, thereby creating a concentration regime in which susceptible bacteria will grow better than resistant bacteria in the presence of both drugs.<sup>31</sup> While this method of suppressing resistance is of mechanistic interest, deploying hyperantagonistic combinations would require well-tolerated compounds to avoid serious host toxicity at the higher concentrations needed and potentially a third drug to further inhibit the growth of susceptible bacteria. Additionally, antagonistic drugs that fail to kill susceptible bacteria can become synergistic if the bacteria acquire certain resistance mutations.<sup>32</sup> Here, a resistance mutation drives the synergistic interaction between two compounds, and all three components (the mutation and two mutually antagonistic compounds) must be present to enable effective killing.

Finally, resistance acquisition to one drug can increase the bacteria's sensitivity to a second drug independent of the interaction between the two drugs, a phenomenon known as collateral sensitivity. Because collateral sensitivity is characterized by an evolutionary tradeoff, it can occur without coadministration of antagonistic drug combinations. For example, altered bacterial membrane potentials can decrease both the uptake of one drug and the efflux of another drug.<sup>33</sup> Thus, one can conceive alternating drug treatments in which treatment

with the first drug generates a resistance mutation that sensitizes the bacteria to the second drug, allowing for greater inhibitory activity and total clearance of the infection. However, the development of antagonistic drug combinations requires a precise understanding of both drugs' pharmacokinetics and toxicity and still faces serious clinical challenges, such as ongoing monitoring for the presence of resistance mutations and a less favorable risk-reward ratio for individual patients. Such challenges render antagonistic drug combinations less attractive development candidates.

Synergistic drug interactions have a range of potential benefits for antibacterial therapy, including bypassing resistance mechanisms and reducing toxicity to the host. Three classes of synergistic pairwise combinations exist: congruous, syncretic, and coalistic combinations. Congruous combinations are based on compounds that each has antibacterial activity toward the target organism. The activities might target different pathways or the same pathway and can exhibit high joint efficacy to enable faster killing and overcome resistance to one of the antibiotics.<sup>34,35</sup> Zheng *et al.* recently reported an innovative strategy for congruous combination therapies by pairing metabolism-dependent and metabolism-independent antibacterials.<sup>36</sup> Such combinations resulted in a synergistic effect that enabled persister cell growth inhibition at dose-sparing levels. Specifically, they observed the sterilization of persister cell populations when pairing the metabolism-dependent antibacterial ampicillin with the toxic metabolism-independent antibacterial colistin at a fourfold lower concentration of colistin than is required for sterilization with colistin alone. The increased potency of congruous combinations not only reduces the toxicity of antibacterials to the host when reduced dosing is enabled but



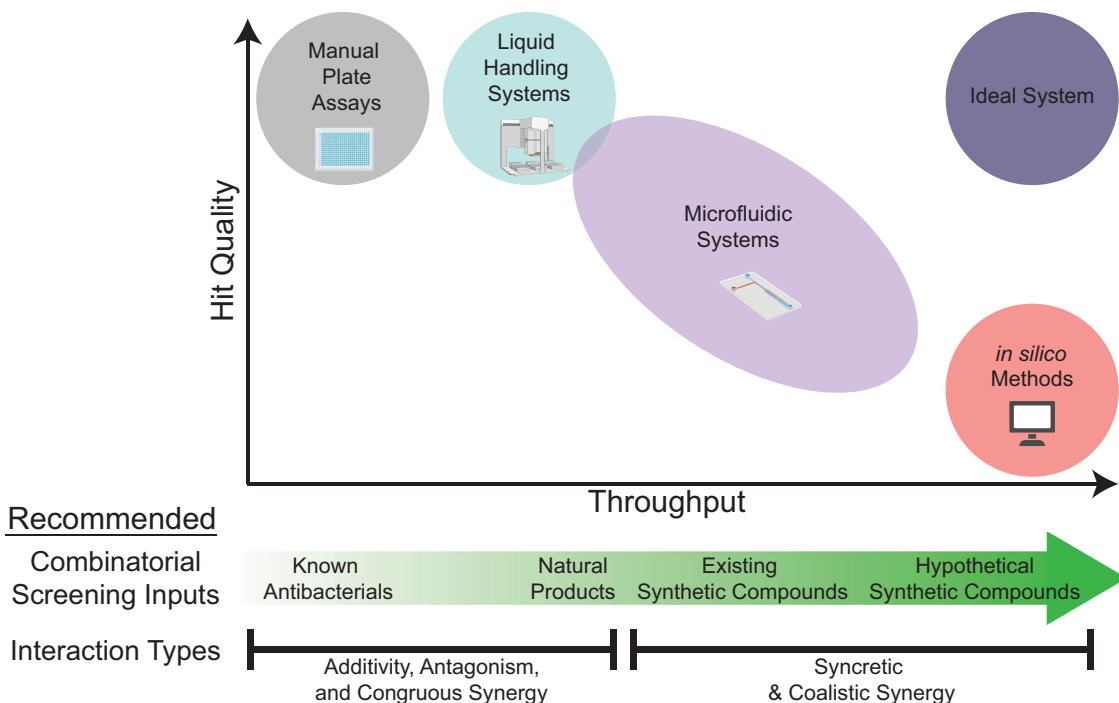
**Figure 1.** Synergistic interactions of synthetic combinations can bypass antibiotic resistance mechanisms. Nonantibacterial adjuvant compounds can synergize with known antibiotics through a variety of mechanisms. Adjuvant compounds can inhibit efflux pumps (A) or increase membrane permeability (B), leading to the accumulation of the antibacterial compound. Additionally, adjuvant compounds can inhibit enzyme modification or degradation (C) or allosterically bind to the target enzyme (D) to protect the activity of the antibacterial compound.

also decreases the time to clear an infection. Considering such benefits, congruous combinations should be a high priority for antibacterial therapeutic development and can arise from combining known antibacterials, or even novel candidates from monotherapeutic discovery pipelines. Understanding which congruous combinations are effective against which organisms, at least *in vitro*, is critical for improving the clinical success rates of such treatments and can still require large-scale, combinatorial screens of known antibacterials, particularly for exploration of high-order combinations.

Syncretic combinations consist of an antibacterial and a nonantibacterial adjuvant. The latter increases the activity of the antibiotic, for instance, by overcoming a drug-specific efflux resistance mechanism to increase its accumulation in cells, or altering the state of the pathogen to increase its sensitivity to the antibiotic, as depicted in Figure 1. For example, sulbactam, a  $\beta$ -lactamase inhibitor, and PA $\beta$ N, a well-studied efflux pump inhibitor, promote the accumulation of  $\beta$ -lactams or other antibiotics (e.g., fluoroquinolones) in the periplasm of Gram-negative bacteria, and thereby increase their efficacy.<sup>37</sup> Therefore, syncretic com-

binations are useful for extending the lifetime of existing antibiotics and amplifying their therapeutic effects.<sup>21,22</sup> Syncretic adjuvant discovery is a common objective of combination screens today since they may require the discovery, evaluation, and approval of only one additional novel compound.

Finally, coalism arises for compound sets where each compound lacks individual antibacterial activity but the combination exhibits “emergent” antibacterial activity when combined by driving a “synthetic lethal” interaction.<sup>14</sup> While the FDA has not yet approved such a combination for treating bacterial infections, synthetic lethal pairs were recently established as a paradigm for cancer therapy.<sup>38–40</sup> Synthetic lethal therapy may have lower toxicity in oncology as one or more compounds may exploit tumor-specific mutations, reducing the impact of therapy on noncancerous cells. Similar principles have been explored in antibacterial drug discovery, albeit with less success.<sup>41,42</sup> These benefits of congruous, syncretic, and coalistic synergy remain the primary motivation for antibacterial combination discovery and development efforts.



**Figure 2.** Recommendations for navigating the hit quality and throughput tradeoff for combinatorial screening efforts. Hit quality is defined as the likelihood of a predicted or estimated drug interaction being true *in vitro*. Here, we represent these factors as a two-dimensional space in which manual plate assays, liquid handling systems, microfluidic systems, and *in silico* methods occupy certain niches. We highlight that while the ideal system for screening combinations does not yet exist, microfluidic systems most closely approach an ideal system considering hit quality and throughput. Additionally, we note combinatorial screening inputs and drug interactions that become discoverable as throughput capacity increases.

High-order combinations comprising more than two drugs can amplify or combine interaction effects, for example, to broaden spectrum or overcome high-level or multiple resistances in a single organism. High-order combinations have already proven necessary and successful in the treatment of cancer, and tuberculosis and HIV infections. For example, high-order combinations are the standard of care for both drug-sensitive and resistant tuberculosis.<sup>43</sup> Mycobacterial infections require a much longer treatment course than many other bacterial infections and the acquisition of resistance through spontaneous mutations in individual patients is more likely than other bacterial infections.<sup>44,45</sup> Thus, multiple drugs (independent and synergistic) are used to suppress the expansion of resistant mycobacteria during treatment.

Similarly, the development of highly active antiretroviral therapies, comprising three or more drugs, transformed HIV/AIDS treatment by greatly

improving treatment efficacy and reducing the probability of resistance development.<sup>46</sup> Highly active antiretroviral therapy for HIV infection uses independent and synergistic combinations of antivirals to prevent and overcome resistance mutations during long-term therapy.

Horn *et al.* reported that at least fourth-order congruous combinations were necessary to kill some highly drug-resistant colorectal cancer cell lines.<sup>47</sup> Additionally, drug combinations have been shown to be beneficial even in the absence of additivity or synergy to cover heterogeneous patient populations through independent mechanisms.<sup>48,49</sup> Although the antibacterial combinatorial space has primarily focused on pairwise combinations for nonmycobacterial infections, the demonstrated clinical success of high-order combinations warrants more attention as increasing resistance raises the challenge of delivering efficacious antibacterial therapy.



## Challenges for combinations beyond the discovery pipeline

While the potential impact of antibacterial combinations is significant, their additional complexity results in challenges pertaining not only to their discovery, but also to their development, regulatory approval, and deployment. Here, we focus on the challenges beyond the combination discovery pipeline and touch upon emerging solutions.

If efficacious drug combinations are found, it is necessary to appropriately overlap the drugs in time and space during treatment. Differences in pharmacokinetic and pharmacodynamic properties can significantly complicate multidrug coadministration. One approach to address this requirement is to synthetically conjugate two or more pharmacophores to form a single chemical agent as a hybrid antibiotic that tightly links the pharmacodynamics of the different pharmacophores.<sup>50</sup> Fluoroquinolone-oxazolidinones are promising examples of multitargeting hybrid antibiotics that inhibit DNA and protein synthesis. A 4-hydroxypiperidine-linked fluoroquinolone-oxazolidinone prodrug, DNV3837, has progressed to clinical trials.<sup>51,52</sup> Another hybrid antibiotic, a ciprofloxacin-neomycin conjugate, has also demonstrated delayed resistance.<sup>53</sup> In addition to slowing drug resistance, hybrid antibiotics have shown success in preventing enzymatic degradation and increasing binding specificity for their targets.<sup>54,55</sup> While few examples have yet been disclosed, the hybrid antibiotic concept offers a logical path to advance combinations of pharmacophores as multitargeting agents.

Combination therapies present new challenges for clinical development and regulatory approval, which should be considered in an integrated discovery and development strategy as the chosen technical approach has implications for late-stage development and approval. For example, a strategy to sensitize pathogens to an approved antibiotic that alone represents standard of care may not require a factorial trial design, but could bring commercial constraints if the antibiotic is under patent protection. Strategies advancing multiple novel compounds may have or be perceived as having a large number of possible failure points and require more complex clinical trials.

Finally, postmarketing antimicrobial susceptibility testing and resistance monitoring remain highly

active areas in infectious disease research with a large focus on tool development for resistance monitoring in the clinical setting.<sup>56</sup> Much of the field's progress on single-agent susceptibility testing can likely be applied to understanding and predicting the effects of antibacterial combinations on patient isolates in clinical laboratory settings.

## Models to predict drug interactions

Researchers use several mathematical frameworks, including Bliss independence and Loewe additivity, to quantify drug interactions.<sup>57,58</sup> The Bliss independence model estimates the deviance from a model of independent drug action. In a pairwise interaction, the bliss score is determined by the difference between the expected individual effects of each drug and the observed combined effect.<sup>59</sup> With limited measurements needed to compute bliss scores, Bliss independence is useful when evaluating a large number of drug combinations at specific concentrations. Only four measurements are needed to determine a pairwise interaction at a single concentration of each component: growth inhibition of one drug, growth inhibition of the second drug, the combined growth inhibition, and the growth value without drugs. However, the Bliss independence model does not satisfy some intuition about interactions. For example, a sham mixture—a drug in combination with itself—deviates from Bliss independence and is not cleanly categorized as a particular interaction type.<sup>60</sup>

The Loewe additivity model describes the dose-dependency of a drug interaction with additivity defined as a drug's interaction in combination with itself.<sup>57</sup> The Loewe additivity model is an effect-based approach to describe the combined effect of drugs at specific concentrations relative to the independent effects of the drugs, which is summarized as the fractional inhibitory concentration (FIC) index, whose values can be used to classify drug interaction types. Conservatively, FIC values below 0.5 correspond to synergy, and values above 4 correspond to antagonism.<sup>61</sup> While the Loewe additivity model is a useful way to summarize complex dose dependencies, it depends on reliable characterization of the drugs' individual and combined dose–response curves.<sup>60</sup>

## Combinatorial screening technologies and their place in antibacterials discovery

Combinatorial drug discovery demands efficient use of compounds and incredibly high screening throughput to address explosive combinatorial statistics. To illustrate, the number of pairwise combinations balloons with the number of library compounds: 10 compounds yield 45 possible pairwise combinations; 100 compounds, 4950 combinations; and 1000 compounds, 499,500 combinations (where  $C_{(n=10, 100, \text{ or } 1000; r=2)} = \frac{n!}{(n-r)!r!}$ ). Additionally, estimating the interactions among compounds further requires interrogation of the independent activity of each component in the combination, adding to the number of required measurements.

Combinatorial drug screening requires phenotypic assays as each compound may target multiple known or unknown pathways in a cell, with the compounds' interactions likely based on incompletely understood relationships among or within the pathways. Novel phenotypic hits require additional work to identify the mechanism(s) of action and may be particularly challenging where one or more novel compounds lack independent antibacterial activity.

Checkerboard growth suppression assays carried out in 96- or 384-well plates liquid cultures are the standard experimental format for drug interaction assessment. However, checkerboards are resource-intensive, especially when assessing the interaction of more than two drugs. Automated liquid handling systems have been used to improve the throughput and reliability of checkerboard assays, but checkerboard implementations do not provide adequate throughput for combinatorial screening and consume tremendous quantities of compound.<sup>62,63</sup> Tekin *et al.* screened over 20,000 different pairwise and high-order combinations among  $N$  compounds in standard 384-well plates by estimating Bliss independence from a small number of drug concentrations rather than full checkerboards.<sup>64</sup> To decrease compound consumption, inkjet- and aerosol-based deposition have been used to miniaturize drug-screening from microliter- to picoliter-scale volumes.<sup>65,66</sup> However, these approaches still require complex engineered systems to formulate compound combinations and are burdensome at large scales.

New approaches and technologies are needed to support combination screening at the scales required to address the unmet need for new antibiotic therapies, which we estimate as interactions among libraries of thousands of compounds at a minimum. In the following subsections, we discuss advancements in antibacterial combination screening technologies that predict or assess growth inhibitory activity, including *in silico* methods, DIAMOND (a theoretical framework to evaluate combinations), and microfluidic platforms that do not require directed formulation of each combination.

### *In silico prediction methods*

Computational methods can greatly expedite the discovery process by predicting interactions to enable the most relevant regions of combinatorial chemical search spaces to be targeted. Several groups have developed computational models trained on chemogenomic datasets to predict synergistic or antagonistic drug interactions among small molecules in bacteria. Chemogenomic datasets comprise fitness scores derived from relative growth values for sets of gene-deletion strains grown under the treatment of small molecule libraries. These models test the hypothesis that compounds with similar genetic interaction profiles are more likely to interact than randomly selected compound sets.<sup>67</sup> For example, the Overlap2 Method (O2M) identified a subset of gene deletion strains showing a similar pattern for a synergistic antibacterial combination and leveraged this mutant set to predict additional small molecules from a set of 2000 to synergize with each compound in the known synergistic pair.<sup>68</sup> These putative hits were then tested in combination with trimethoprim for synergistic growth inhibitory activity against *Escherichia coli*. In particular, azidothymidine was validated to synergize with trimethoprim in *E. coli*. The authors were able to extend this interaction to other compounds disrupting nucleotide biosynthesis, such as hydroxyurea and floxuridine, and show combination efficacy against trimethoprim/sulfamethizole-resistant clinical isolates of *E. coli* and *Klebsiella pneumoniae*.

Inferring Drug Interactions using Chemogenomics and Orthology (INDIGO) utilized chemogenomic datasets of known synergistic and antagonistic compound combinations to train a



random forest classifier.<sup>69</sup> This classifier was then used to predict the interactions among drugs with known chemogenomic profiles. INDIGO outperformed O2M and generalized to organisms not represented in the original training dataset, such as *Mycobacterium tuberculosis* and *S. aureus*. Of the top 10 synergistic and antagonistic drug pairs, six synergistic and seven antagonistic predictions were experimentally validated in checkerboard assays evaluating *E. coli* growth inhibition. Since metabolic perturbations are known to impact drug susceptibility, the developers of INDIGO hypothesized that drug interactions are similarly impacted, resulting in the development of another approach, Metabolism And GENomics-based Tailoring of Antibiotic regimens (MAGENTA).<sup>70</sup>

MAGENTA was developed to predict synergistic or antagonistic drug interactions specific to different microenvironments and applied to identify synergy in 19 combinations robust to nine distinct growth conditions.<sup>70</sup> Interestingly, MAGENTA revealed microenvironment-dependent drug interactions and predicted changes in drug combination efficacy in glycerol- versus glucose-supplemented media, which were validated experimentally. The predicted and experimental interaction scores for 55 drug combinations had a rank correlation of  $R = 0.69$  in glycerol-supplemented media. These computational methods depend on the availability and scope of chemogenetic data but allow rapid *in silico* screening and prioritization of compound combinations for empirical testing.

Notably, Stokes *et al.* recently developed a machine learning approach for predicting the bacterial growth inhibitory activity of individual compounds on the basis of their chemical structures, allowing for *in silico* screening of uncharacterized molecules and molecules that have never been synthesized.<sup>71</sup> This model was trained on thousands of empirically tested repurposing compounds (of which only 120 exhibited growth inhibitory activity) and applied to more than a hundred million novel compounds and additional repurposing candidates in just 4 days. This *in silico* screen yielded a curated list of 23 potential candidates for empirical testing, which resulted in eight empirically validated antibacterial compounds. Additionally, the authors identified broad range antibacterial activity by halicin, a drug from the repurposing library. This type of approach could

be valuable to prioritize bioactive compounds for inclusion in empirical combination screens.

To our knowledge, all reported approaches to predict combinations with antibiotic activity require a basis in empiric, compound-specific data. The generation of large combinatorial datasets encompassing wide chemical and pathogen coverage could make *in silico* methods even more powerful and broadly applicable. Some combination therapies may be more organism-specific than single-agent therapies and thus more challenging to predict, requiring larger training datasets to acquire sufficient relevant coverage. Depending on the specificity of *in silico* methods for predicting antibiotic combinations, substantial combinatorial screening capacity may also be required to identify and validate true hits among the prediction set. Thus, further development and employment of both *in silico* and empirical *in vitro* screening technologies are needed to establish a robust discovery pipeline for antibacterial combinations.

While the aforementioned *in silico* methods focus on predicting the growth inhibitory activity of single compounds or combinations of compounds, *in silico* methods have been developed to evaluate the resistance potential of antibacterial combinations as well. Torella *et al.* mathematically modeled drug pairs under *in vivo* infection dynamics.<sup>24</sup> The results from this model caution that optimizing for maximal synergy can be undesirable. Under some conditions, such as strong competition for resources, highly synergistic combinations disproportionately increased the risk of multidrug resistance without a compensating improvement in infection clearance owing to the increased selective pressure for resistance mutants. As screening efforts continue to discover hit combinations and estimate their efficacy through growth inhibition assays, we should carefully study their resistance potential as they progress along the development pipeline.

### DiaMOND

The DiaMOND (diagonal measurement of  $n$ -way drug interactions) concept enables interaction assessment using fewer measurements. For example, calculating an FIC score with standard checkerboards requires  $D^N$  measurement conditions for  $N$  drugs at  $D$  doses, while DiaMOND requires only  $N \times D + D$  measurement conditions, a particularly big difference when  $N$  is large.<sup>72</sup> DiaMOND is not

specific to any hardware platform and achieves its efficiency gain by selectively measuring the single-drug dose responses and the “diagonal” of a multidrug checkerboard assay where the drugs are concomitantly titrated and drug interactions cause the greatest change in growth response. In this way, DiaMOND captures the dose dependency of drug interactions without sampling the full checkerboard array. Additionally, lower-order interactions underlying the high-order interaction can be sampled simultaneously using DiaMOND to dissect the contributions of each lower-order interaction and identify high-order “emergent” interactions. Using the DiaMOND assay, Cokol *et al.* screened pairwise interactions for nine first- and second-line antibiotics for tuberculosis treatment and three-, four-, and five-way drug interactions for five of the original nine antibiotics that exhibited synergy in the pairwise screen. In this dataset, pairwise interactions were predictive for the majority of high-order interactions with notable exceptions, including third- and fourth-order antagonism and synergy. In all, the DiaMOND assay provides a framework for efficient and rigorous quantification of drug interactions amenable to standard laboratory equipment and some miniaturized screening systems. We imagine that applying the DiaMOND approach in miniaturized assay formats could support significant throughput for important drug interaction screening and analysis tasks.

### *Microfluidic-based platforms*

Microfluidic automation technologies can obviate the need for complex liquid handling procedures, and their small assay volume reduces compound consumption. Miniaturized bioanalytical systems are commonly prototyped or manufactured in polydimethylsiloxane (PDMS), an elastomeric, optically clear, and inexpensive polymer compatible with biomolecules and cells.<sup>73–75</sup> Such microfluidic devices have successfully miniaturized (1) the detection and separation of macromolecules, (2) the interrogation, sorting, and manipulation of prokaryotic and eukaryotic cells, and (3) large-scale, combinatorial experiments.<sup>74,76</sup> PDMS-based microfluidic arrays using fluid–fluid diffusional contacts, or Quake valves, wherein a pressurized channel can restrict fluid flow in an adjacent microchannel, enabled some of the first multi-

plexer tools supporting large-scale combinatorial experiments within microfluidic devices.<sup>77–79</sup>

Droplet-based microfluidics, using surfactant-stabilized water-in-oil emulsions, have also been shown to facilitate large-scale experiments in microdevices, permitting massively parallel enzymatic and cell-based morphological and transcriptomic screening.<sup>80–82</sup> However, small molecule crosstalk among small droplets, caused by surfactant-mediated interdroplet exchange, has almost entirely prevented the application of droplet-based microfluidics for drug screening.<sup>83,84</sup>

Microwell array formats block small molecule crosstalk by physically separating each assay. Here, we focus on the droplet-in-microwell array system, referred to as bChip, kChip, and mChip, which spontaneously organizes nanoliter-sized droplets in a high-density array of tens to hundreds of thousands of microwells fabricated in parylene-coated PDMS or another material.<sup>85–88</sup> The droplet-in-microwell array system has facilitated a wide variety of large-scale combinatorial experiments, including antibiotic sensitizer screening. In this type of system, droplets carrying different assay components are merged to formulate assays combining multiple components in each microwell, robustly facilitating high-throughput applications by loading and merging droplets in parallel rather than one or a few at a time. The droplet-in-microwell system is flexible across assay types and combinatorial configurations, as the microwell geometry is configurable to set the desired number of droplet inputs per assay. After the random assembly of droplet combinations in the arrayed microwells, droplets are sealed in their microwell compartments using hydrophobic-treated glass or transparent adhesive film sealing. Each class of droplet inputs is encoded with a combination of fluorescent dyes to track their contents via low-magnification fluorescence imaging. A high-voltage AC electric field rapidly merges the droplet set within each sealed microwell across the entire array to initiate all the microwell-level assays in one step. To date, the platform has been deployed with a range of readouts, including fluorescent reporters, white-light imaging, and single-cell RNA sequencing (N. Hacoheh and M. Reyes, personal communication, December 1, 2020).

The spontaneous self-assembly of droplets into microwells is fast, scalable, robust, and eliminates the need for robots or engineered devices to

deterministically formulate each combination of assay components. However, there are two principal tradeoffs: the need to reidentify the assay component inputs to each microassay using a fluorescent or other code and statistical coverage of the combinatorial assay space. Rather than a fixed number of replicates per assay condition as typical for screening in microtiter plates, the combinatorial space is sampled statistically as random droplet sets come together in each microwell, resulting in a distribution of replicate counts for each possible combination of inputs. This is analogous to sequence coverage statistics in shotgun genome sequencing, wherein an operator specifies a target average coverage level (e.g.,  $30\times$  genome coverage), and actual coverage varies across genomic loci with a chance that some loci are not sampled. In the context of droplet array assays, average coverage levels of  $5\text{--}15\times$  produce good results in practice, with most drug combinations sampled with many replicates.

Kulesa *et al.* first demonstrated the effectiveness of droplet arrays in a sensitizer screen of 4000 repurposing compounds across 10 antibiotics in an *E. coli* growth assay.<sup>85</sup> Drug interactions were estimated following the Bliss Independence model with candidate sensitizers at a single dose and each antibiotic at three doses. Twenty-eight compound–antibiotic combination hits were called, and 14 of the 17-hit compound–antibiotic pairs tested scored as synergistic in a secondary screen performed in 96-well plate liquid cultures following a similar analytical procedure for estimating drug interaction. Applying a more stringent FIC synergy criterion to eight-dose checkerboard datasets, six compounds demonstrated synergy with at least one antibiotic from the original screen. In this sensitizer screen, over four million microwell assays were carried out in just 10 days by loading 64 inputs (or 2016 pairwise combinations) per chip, yielding a median of 13 replicates per combination or a  $13\times$  median coverage level. The dose-resolution of the combinatorial screen is a direct tradeoff with the number of unique combinations interrogated. Here, the throughput capacity of the screen, or the number of sensitizer molecules screened, was prioritized by applying the Bliss Independence model to estimate drug interactions at relatively few doses. Full checkerboard assays (but not selective diagonal measurements like DiaMOND) can also be run on the existing platform configura-

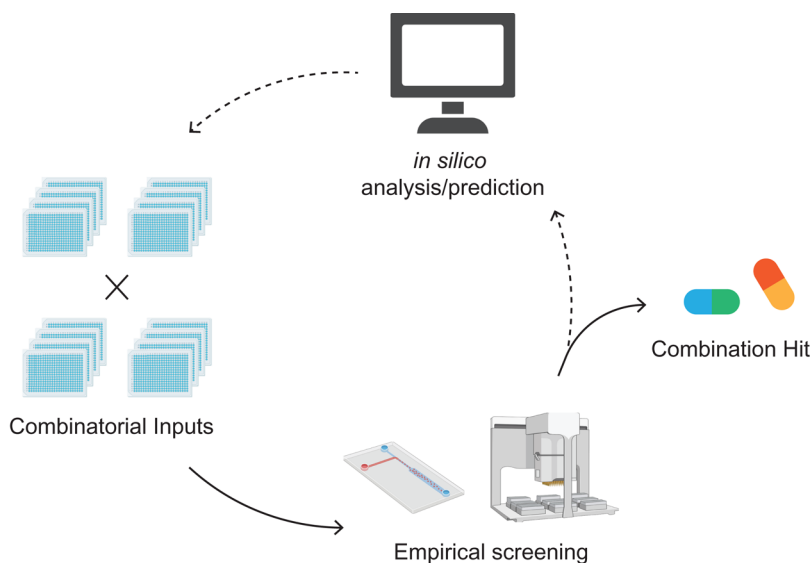
tion to enable more precise interaction assessments, albeit at lower throughput of combinations. The droplet array platform has demonstrated the ability to merge up to 19 droplets per microwell, accept up to 1050 unique inputs per chip, and utilize arrays as large as 177,000 microwells.<sup>86,87</sup> Given this flexibility and scalability, droplet arrays are well suited for many types of combinatorial compound screens. For example, ultra-high throughput screening technologies can be deployed for unbiased screening of novel compound combinations for sets of targeted screens testing more focused hypotheses about how to fight resistance against specific antibiotics, and also further into the development pipeline where it may be desirable to test combinations of derivatives across large pathogen strain panels.

With the immediate imperative to extend the life of today's antibiotic drugs and a growing appreciation for the importance of multitargeting antibacterial therapies, efficient combinatorial screening technologies should be advanced in earnest. We argue that these technologies can support assay approaches with real potential for discovering progressable antibacterial combinations whose translation to the clinic should be incentivized and supported by the biomedical establishment.

## Future directions

The history of antibiotics is ancient, long predating humans, and is a story of ongoing molecular innovations and resistance responses. While combination therapies promise to expand the antibiotic toolset for medicine, resistance development is inevitable as bacterial pathogens continue to evolve and escape the grasp of our therapeutic arsenal. Thus, rapid and adaptable discovery pipelines for a wide range of therapeutic strategies, including combination treatments, are crucial to keep the rate of discovery ahead of pathogen resistance and maintain the benefits of modern medical practice for patients. This review discussed several *in silico* and empirical screening technologies that are advancing our capability for discovering antibacterial combinations and the tremendous potential in their expanded deployment, particularly the integration of *in silico* and empirical methods.

We envision a tight iterative feedback cycle between *in silico* and empirical screening methods to propel antibacterial combinations into the drug development pipeline, illustrated in Figure 3.



**Figure 3.** A proposed combinatorial drug discovery pipeline. Combinatorial inputs should be assessed using microfluidic technologies for larger combinatorial chemical spaces or automated liquid handling systems for smaller spaces to measure and empirically define drug interactions. At the same time, *in silico* methods can be deployed to actively learn from these datasets to better select screening inputs and nominate combination hits.

Empirical data from large-scale, combinatorial screens can support prediction model training. Training can and should be reinforced continuously as compound families with predicted activity are synthesized and validated *in vitro* to close the feedback loop. Hie *et al.* demonstrated the utility of such an interactive cycle using an uncertainty-guided active learning approach to identify novel compounds for tuberculosis with multiple rounds of model-guided experimentation to explore unknown regions of the chemical space.<sup>89</sup> Employing a similar framework for compound combinations will be even more impactful when applied to address combinatorial spaces that exceed the reach of purely empirical approaches.

Empirical screening methods, which have largely relied on growth assays, are also poised for major advances. Information-rich readouts have the potential to contribute to combination drug discovery as well. Such information-rich readouts include high-resolution imaging to monitor morphological changes, RNA-sequencing readouts characterizing the molecular responses of bacterial cells, and DNA sequencing to monitor mutations.<sup>90–92</sup> Such information-rich and -omic readouts can also contribute to compound “profiling” to support

mechanism of action prediction and a further basis on which to predict compound interactions. Collection of these data in the discovery process can better streamline the drug development pipeline by supporting early and confident lead selection. Several sequencing and imaging approaches for rich data collection at a large scale are emerging, with high-resolution imaging assays appearing especially promising.<sup>93</sup> These parallel technological developments can also accelerate antibacterial combination discovery and contribute to solving the antibacterial resistance crisis.

### Concluding remarks

Combination therapies are promising strategies to combat multidrug-resistant bacteria. Since certain antibacterial combinations, including nonantibacterial components, can show synergy and other potentially beneficial drug interactions, we must look beyond the small number of antibacterial drugs being identified in monotherapeutic phenotypic and biochemical assay discovery pipelines. However, screening large combinatorial chemical spaces presents a major challenge to traditional screening methods, even with automated liquid handling systems. The integration of generalizable *in silico* prediction methods and high throughput

empirical methods is needed to identify efficacious drug combinations within vast combinatorial chemical spaces. While a new generation of *in silico* prediction methods allows for incredibly high throughput, these methods depend strongly on empirical (combination) datasets and much remains to be learned about their performance. We envision iterative loops between *in silico* prediction methods and high throughput microfluidic platforms to efficiently explore uncharted combination chemical spaces across a range of pathogens. This approach can be developed to initiate a robust pipeline for combinatorial drug discovery.

### Acknowledgments

We are grateful to Bree Aldridge, Jared Kehe, and Samuel Goldman for thoughtful manuscript feedback and other Blainey lab members for insightful discussions. All authors contributed to writing the manuscript. Figures 2 and 3 contain icons created at BioRender.com. The authors' research in this area has been supported by the NIH NIAID Grant 5U19AI42780, as well as funds from the Broad Institute of MIT and Harvard, the MIT Deshpande Center Innovation Award, the Merkin Institute for Transformative Technologies in Healthcare, the Koch Institute for Integrative Cancer Research at MIT, the Defense Advanced Research Projects Agency, and a Burroughs Wellcome Fund CASI Award (to P.C.B.). M.Z. and M.W.T. were supported by the National Science Foundation Graduate Research Fellowship under Grant No. 1745302.

### Competing interests

P.C.B. is a coinventor on patent applications concerning droplet array technologies and serves as a consultant and equity holder of companies in the microfluidics and life sciences industries, including 10x Genomics, GALT, Celsius Therapeutics, Next Generation Diagnostics, Cache DNA, and Concerto Biosciences.

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