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Current Opinion in Microbiology

Antimicrobial interactions: mechanisms and implications for drug discovery and resistance evolution

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Combining antibiotics is a promising strategy for increasing treatment efficacy and for controlling resistance evolution. When drugs are combined, their effects on cells may be amplified or weakened, that is the drugs may show synergistic or antagonistic interactions. Recent work revealed the underlying mechanisms of such drug interactions by elucidating the drugs' joint effects on cell physiology. Moreover, new treatment strategies that use drug combinations to exploit evolutionary tradeoffs were shown to affect the rate of resistance evolution in predictable ways. High throughput studies have further identified drug candidates based on their interactions with established antibiotics and general principles that enable the prediction of drug interactions were suggested. Overall, the conceptual and technical foundation for the rational design of potent drug combinations is rapidly developing.

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Current Opinion in Microbiology 2015, **27**:1–9

This review comes from a themed issue on **Microbial systems biology**

Edited by **Eric D Brown** and **Athanasios Typas**

For a complete overview see the Issue and the Editorial

Available online 1st June 2015

http://dx.doi.org/10.1016/j.mib.2015.05.008

Introduction

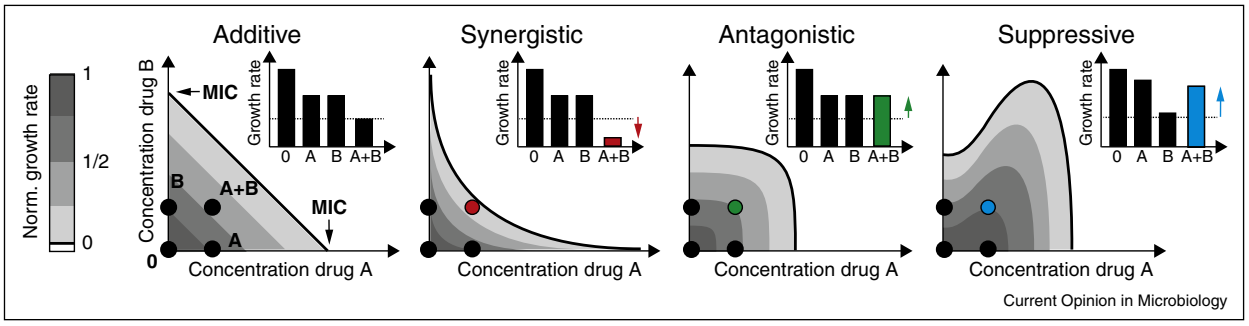
Drug combinations are increasingly used in the treatment of many conditions and diseases including tuberculosis and cancer [1,2]. The interaction between two drugs is synergistic if the joint effect of the drugs is stronger than an additive expectation and is antagonistic if it is weaker [3,4] (Figure 1). Suppression is an extreme kind of antagonism in which one drug alleviates the effect of the other (Figure 1). Synergistic antibiotic pairs such as the well-known combination of trimethoprim with sulfonamides have been applied for decades as they can reduce side-effects and increase the potency of drugs that are ineffective alone [5]. Despite notable exceptions [6], the discovery rate of new antibiotics is in decline while antibiotic resistance in pathogens is rapidly increasing [7–9]. Drug combinations offer potential strategies for controlling the evolution of drug resistance [10–12,13•,14,15••,16••,17,18]. They are further used in basic research as a means of perturbing multiple cell functions to reveal relationships in cell physiology [19,20], analogous to genetic epistasis measurements [21].

Despite their growing biomedical relevance, fundamental questions about drug interactions remain unanswered. In particular, little is known about the underlying mechanisms of most drug interactions. A strategy for designing drug combinations that can slow resistance evolution also remains elusive. Still, our understanding of how antibiotic combinations affect microbes has advanced considerably in recent years. Specifically, networks of pairwise interactions for large numbers of drugs were quantified and in a few cases, the underlying mechanisms of drug interactions were characterized. Furthermore, new drug discovery strategies identified candidates for drugs that synergize with established antibiotics. Several studies provided insights into the effects of drug combinations on resistance evolution. Finally, general principles that hold across drugs and target organisms promise the possibility to predict drug combination effects. This article summarizes new developments in the investigation of antimicrobial drug combinations and focuses on basic research from the last three years; additional aspects and earlier work in the field have been reviewed elsewhere [22,23•,24–27].

Drug interaction networks and the underlying mechanisms of drug interactions

Systematic measurements of drug interaction networks revealed that drug interactions occur frequently and are partly predictable. First, the entire network of pairwise interactions between 20 antibiotics representing the main modes of action was measured in *Escherichia coli* [28]. This drug interaction network is highly structured: the mode of action of the drugs that are combined largely determines the interaction that occurs between them (Figure 2). In principle, this property enables the identification of a new drug's mode of action by simply measuring its interactions with other drugs [28]. Analysis of the interaction network of antifungal drugs in *Saccharomyces cerevisiae* revealed that certain drugs tend to form network hubs, that is they have synergistic or antagonistic interactions with many other drugs [29–31]. Multiplexed screening of ~500 000 drug pairs against HIV identified new synergistic pairs [32]. While such high-throughput techniques are powerful, the systematic investigation of all possible combinations of large numbers

Figure 1

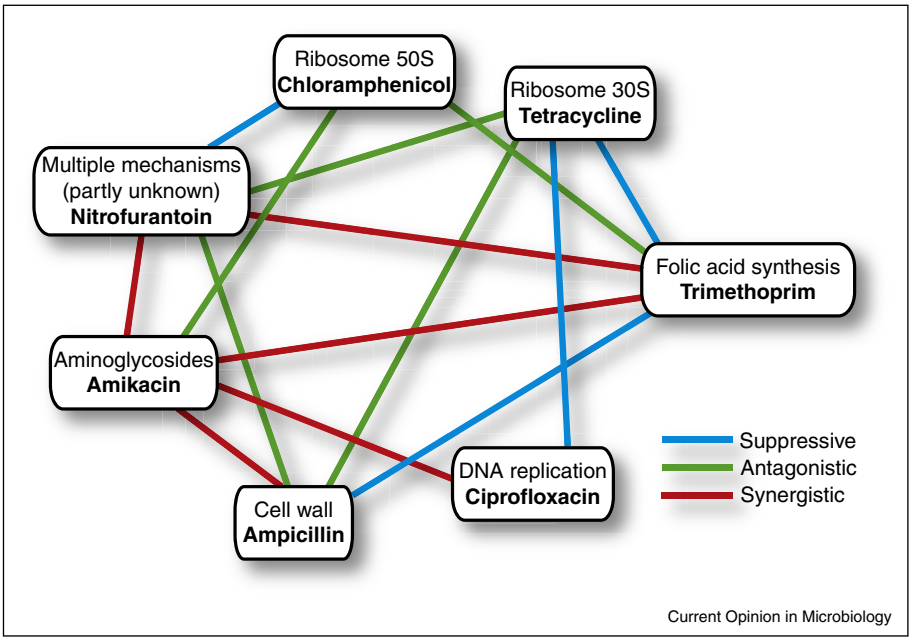


Drug interactions are defined by the shape of lines of equal effect in two-drug concentration space. Schematics showing growth rate (grayscale) and minimal inhibitory concentration (MIC) line (black, line of zero growth) in the two-dimensional concentration space of drugs A and B. The additive reference is given by linear interpolation of the MICs of the individual drugs [3]. For synergistic and antagonistic drug interactions the MIC line lies below or above this additive expectation, respectively. Suppression is a hyper-antagonistic case in which drug A alleviates the effect of drug B. Insets: growth rates in the absence of drugs ('0'), and at fixed concentrations of drugs A and B individually and combined ('A+B'). The dashed horizontal line in insets indicates the additive expectation [4].

of compounds quickly becomes infeasible due to a combinatorial explosion. This limitation becomes even more severe if the drugs are administered in different temporal sequences which can considerably improve clearance compared to simultaneous administration [33]. In the long run, large-scale screens for potent drug combinations must be complemented by approaches that characterize the effects of drug combinations on cells in detail.

One important approach is to characterize the underlying mechanisms of drug interactions which are still largely unknown. The constituent drugs' modes of action and pharmacodynamics alone cannot explain drug interactions in an obvious way [34]. Drug interactions can be caused by relatively simple uptake effects, for example synergism results if one drug increases the permeability of the cell envelope to another drug [35]. Indeed, such an uptake effect likely causes the synergism between aminoglycoside

Figure 2



Example antibiotic interaction network in *E. coli*. The nodes of this network are key antibiotic classes labeled by their main target or mode of action and a representative drug. Edge colors indicate the drug interactions that are typically observed in *E. coli* between the different antibiotic classes: red, synergism; green, antagonism; blue, suppression. No line is shown for additive interactions. Data compiled from [10,28,42] and unpublished results. For clarity, relatively few antibiotics are shown; for a more comprehensive network, see [28].

and beta-lactam antibiotics [36]. Drug interactions can also be caused by direct physical interactions between the drugs at their target; this mechanism is at work for the antibiotics quinupristin and dalfopristin which both bind to the ribosome at different sites and reciprocally stabilize their binding [37]. For bactericidal antibiotics (which kill bacteria), the definition of drug interactions shown in Figure 1 is readily generalized to include negative growth rates (i.e. killing rates). Interactions between bactericidal and bacteriostatic antibiotics (which only inhibit growth) were long hypothesized to be predominantly antagonistic [35], as killing by bactericidal antibiotics often requires cell growth which is prevented by bacteriostatic drugs. This view was recently substantiated in research that found that antagonistic interactions are significantly enriched for bacteriostatic–bactericidal pairs among all pairwise combinations of 21 drugs [38].

Some drug interactions have more complex causes when the drugs perturb cell physiology and cause cellular responses which then affect the activity of the other drugs. Specifically, one study compared the growth of genome-wide *E. coli* deletion mutants [39] in the presence of trimethoprim and sulfonamides which target enzymes that catalyze sequential steps in the folic acid biosynthesis pathway (dihydrofolate reductase and dihydropteroate synthetase, respectively). Based on these data the authors provided evidence that the synergism between these drugs is partly caused by secondary effects on parallel branches of this pathway, downstream from the main targets [40••]. Such an effect can amplify the synergism that is expected from inhibiting the drugs' primary targets [41]. Another study elucidated the causes of suppressive drug interactions between DNA synthesis inhibitors and translation inhibitors: by genetically manipulating ribosome production, it was shown that non-optimal regulation of ribosome production under DNA stress results in a costly, excessively high global protein synthesis rate which is corrected by the translation inhibitor, causing suppression [42]. Such imbalances between major cellular synthesis processes could be a more general cause of other drug interactions [43]. In *Staphylococcus aureus*, an explanation for the suppressive interaction between vancomycin (which targets cell wall synthesis) and colistin (which targets the cell membrane) was suggested: colistin exposure triggers global gene expression changes that are similar to those in vancomycin resistant mutants, indicating that this response to colistin protects the cell from vancomycin [44]. Overall, there is an increasing number of drug interactions with partly characterized mechanism, but in many cases these mechanisms remain to be identified.

A long-term goal is the development of a systematic approach for unraveling the underlying causes of any given drug interaction. Identifying which genes effect drug interactions would be a major step forward [40••,42]. Mutant libraries enabling the study of genome-wide gene deletion and overexpression effects are available in *E. coli* [39,45] and an increasing number of other microbes [46]. Recently, the *E. coli* gene deletion library was used together with high-throughput techniques for measuring bacterial growth to identify cellular functions that control drug interactions; intriguingly, the same cellular functions, in particular LPS synthesis and ATP synthesis, were found to affect diverse drug interactions [47••]. Further, methods for quantifying the growth effects of genome-wide double-gene deletions have been developed in yeast and *E. coli* [21,48,49]. The genetic interactions between different cellular pathways that will be uncovered using these methods, together with existing chemical genomics data of single drug effects [40••,50], will suggest new hypotheses for drug interaction mechanisms which can then be tested in detail.

Combinations of antibiotics with other compounds

Drug interactions can also occur between antibiotics and compounds that have no antimicrobial activity alone but can, for instance, amplify the effect of an antibiotic [24]. Well-known examples are combinations of antibiotics with inhibitors of their resistance mechanisms which can 'revive' old antibiotics. This approach was successfully adopted in the famous 'augmentin' combination, in which a beta-lactam is combined with an inhibitor of the resistance enzyme beta-lactamase [51]. While, in the long run, bacteria also evolve resistance to such beta-lactamase inhibitors [52], they are a ray of hope in the otherwise dire situation in new antibiotic approvals. Motivated by the prior success of this approach, considerable efforts to develop new beta-lactamase inhibitors are underway and several such inhibitors are currently in clinical trials [53]. New compounds inhibiting enzymes conferring resistance to carbapenem beta-lactams and aminoglycosides were identified by screening natural products from environmental microorganisms [54••] and using a structure-guided approach [55], respectively. In both cases, the identified compounds were previously known for entirely different activities on plant leaves and as eukaryotic protein kinase inhibitors, respectively, highlighting the potential for repurposing drugs. Together, these results suggest that existing compound libraries offer an enormous, virtually untapped reservoir of candidate drugs that can synergize with antibiotics.

Exploring this idea, a recent screen tested over 1000 approved drugs for synergistic activity against bacteria when combined with the antibiotic minocycline (a translation inhibitor) and identified several new synergistic combinations with verified *in vivo* activity [56•]. In one case that was investigated in more detail, synergism was due to increased uptake of minocycline. A related screen of 30 000 compounds identified some that render the aminocoumarin antibiotic novobiocin, which usually only

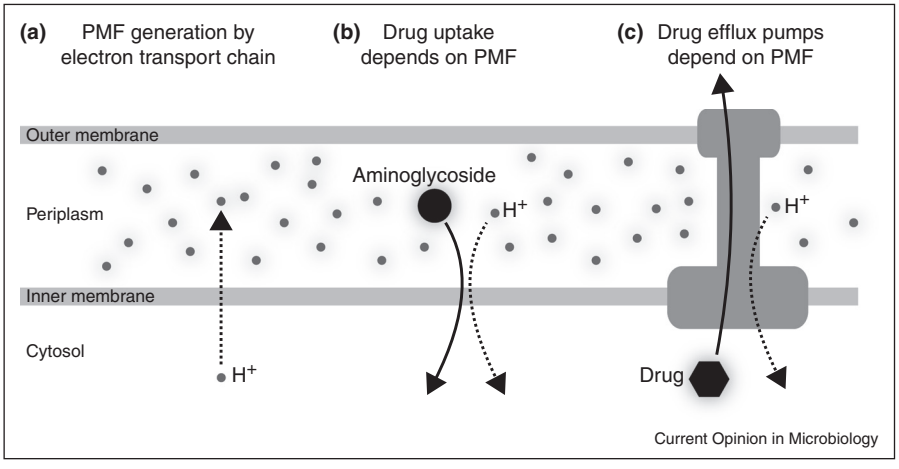
inhibits Gram-positive bacteria, effective against Gram-negatives [57]; again, synergism was likely due to effects on cell envelope permeability which facilitated novobiocin uptake. Furthermore, a study on methicillin-resistant *S. aureus* (MRSA) revealed that ticlopidine, an antiplatelet drug, synergizes with the beta-lactam cefuroxime by perturbing wall teichoic acid biosynthesis, a promising drug target in MRSA [58]. Synergism was also observed in *E. coli* between the antibiotic vancomycin and several established antibiotics — an unexpected effect as Gram-negative bacteria are usually resistant to vancomycin [59]. Similarly, silver, the antimicrobial activity of which is long known, was found to synergize with various antibiotics in Gram-negative bacteria by a mechanism that involves increased membrane permeability and other effects [60]. An analogous screen in yeast identified compounds that synergize with the antifungal fluconazole which inhibits ergosterol biosynthesis [61]. Finally, motivated by the idea to revive old drugs in new combinations, it was shown that bicyclomycin, an old inhibitor of the Rho transcription terminator with limited bactericidal activity [62], efficiently kills bacteria when combined with one of the bacteriostatic antibiotics tetracycline, chloramphenicol, or rifampicin [63]. These results highlight that the full potential of old drugs and other compounds with little apparent clinical value may be unlocked when deployed in strategic combinations with other drugs.

Signaling molecules and metabolites produced by microbes and plants provide an enormous reservoir of compounds that may show synergism or other interactions when combined with antibiotics. For instance, *E. coli* can use the signaling molecule indole to communicate the presence of antibiotics between cells in a population which then protect themselves by expressing drug efflux pumps and other mechanisms [64]. In *Salmonella typhimurium* indole triggers a similar protective mechanism against antibiotics involving the oxidative stress response [65]. The plant metabolite salicylate (the main active component of aspirin) causes a related phenomenon in that it antagonizes antibiotics by triggering the multiple-antibiotic resistance (*mar*) operon which leads to increased expression of the AcrAB-TolC multidrug efflux pump [66,67]. In contrast, specific metabolites, including several sugars and pyruvate, were shown to enhance the killing efficiency of aminoglycoside antibiotics against persister bacteria [68•] — a subpopulation of cells that has phenotypically switched into a dormant state and is notoriously hard to eradicate [69–71]. Similarly, the addition of metabolites like glucose and alanine restores the ability of the aminoglycoside kanamycin to kill otherwise antibiotic-resistant bacteria by increasing the proton motive force (PMF) which stimulates aminoglycoside uptake [72] (Figure 3). The interactions between antibiotics and metabolites identified so far are certainly only the tip of the iceberg, highlighting the need to explore such interactions more systematically.

Drug combinations that minimize resistance evolution

Slowing the evolution of drug resistance is a key motivation for using drug combinations. In some cases, this goal

Figure 3



The proton motive force (PMF) and drug efflux pumps play a central role in diverse drug combination effects. **(a)** Schematic illustrating how the PMF is generated by the electron transport chain in aerobic conditions: protons (H^+) are moved from the cytoplasm to the periplasm. Stimulating this process by adding glucose or other metabolites can restore aminoglycoside susceptibility in persisters [68•] and in aminoglycoside-resistant bacteria [72]. **(b)** The uptake of aminoglycosides depends on the PMF: the free energy released by moving protons from the periplasm to the cytoplasm is used for aminoglycoside uptake. Hence, bacteria can evolve aminoglycoside resistance by diminishing the PMF. **(c)** Many multidrug efflux pumps depend on the PMF: here, the free energy released by moving protons to the cytoplasm is used to expel drug molecules from the cell. In aminoglycoside-resistant strains with diminished PMF, the activity of drug efflux pumps is reduced, leading to collateral sensitivity with antibiotics expelled by these pumps [16••]. The expression of such multidrug efflux pumps (e.g. AcrAB-TolC or MdtEF-TolC) can further be stimulated by salicylate or indole [64,66,67], leading to suppressive interactions between these compounds and antibiotics.

can be achieved simply because several independent mutations are required to become resistant against a combination of drugs with different cellular targets. Microbes offer unique possibilities for studying resistance evolution in well-controlled experiments [73] because their generation times are short, population sizes in laboratory cultures are large, and the genomes of evolved strains can be re-sequenced affordably. Rates of resistance evolution vary considerably between antibiotics for reasons that are largely unknown [8,74]. Understanding the causes of differences in the propensity for evolving resistance to different drugs remains a challenge. Several studies investigated the effects of antibiotic combinations with different drug interactions on spontaneous resistance evolution and revealed a general trend that antagonistic drug combinations lead to slower resistance evolution than synergistic ones [11,12]. However, recent work on *S. aureus* suggested that this trend may not hold generally when bacteria evolve higher levels of resistance as the drug interactions themselves might change due to resistance mutations [75].

An alternative strategy for reducing spontaneous resistance evolution is to exploit evolutionary tradeoffs in which bacteria that evolved resistance to one drug become more sensitive to another [26]. Numerous studies explored such tradeoffs for large sets of antibiotics. Two effects can occur: resistance to drug A may confer cross-resistance to drug B; alternatively, the strain resistant to drug A may have become more sensitive to drug B — a phenomenon termed 'collateral sensitivity'. By evolving bacteria for resistance to a large set of drugs and quantifying the sensitivity of these evolved strains to the entire set of drugs, networks of cross-resistance and collateral sensitivity were mapped [15••,16••,17,18,76]. These studies consistently found that strains evolved for aminoglycoside resistance became more sensitive to various other antibiotics. Based on the genes that are mutated in the aminoglycoside-resistant strains, an intriguing mechanism for this effect was proposed: since aminoglycoside uptake requires the PMF, resistance typically evolves by diminishing the PMF; reduced PMF in turn impairs the function of multidrug efflux pumps that use the PMF to expel antibiotics from the cell, causing increased sensitivity to these antibiotics [16••] (Figure 3). It was further shown that differences in the extent of resistance development observed for different antibiotic combinations can be rationalized based on collateral sensitivity and cross-resistance of the constituent drugs [14,77]. Specifically, resistance mutations causing collateral sensitivity were suppressed under the corresponding drug combinations which slowed resistance evolution [14].

Collateral drug sensitivity networks may further serve as a basis for designing treatments in which multiple antibiotics are cycled over time. While harder to implement in practice, such drug cycling strategies may have advantages, such as lower toxicity, compared to combination treatments where drugs are applied simultaneously. Systematically exploring drug cycling strategies is challenging since, in addition to the choice of drugs and their concentrations, the frequency of drug switching can be varied. Nevertheless, recent work made progress in elucidating the effects of cycling pairs of antibiotics. For the aminoglycoside gentamicin and the beta-lactam cefuroxime, it was validated that strains evolved for resistance to each of these drugs individually were outcompeted by the wild type in the presence of the other drug, respectively, as predicted from their collateral sensitivity profile [15••]. A study investigating three antibiotic pairs in *S. aureus* found that daily switching between two drugs typically reduces the rate of resistance evolution and selects for different mutations than the corresponding single drug treatments [13•]. These effects could be rationalized from tradeoffs between the mutations conferring resistance to the individual drugs. In a related approach using targeted mutagenesis in multiple selection cycles, the adaptation of the resistance enzyme TEM-1 beta-lactamase to the beta-lactam antibiotics cefotaxime and ceftazidime was studied when the drugs were applied individually, in combination, or alternating over time; indications for evolutionary constraints were identified but, overall, the simultaneous or alternating application of the drugs had limited effects on resistance evolution [78].

A particularly promising approach for identifying drug combinations that minimize resistance evolution is to use competition experiments between drug-sensitive and resistant strains early in the drug-screening and combination design process. The potential of this approach is highlighted by the observation that suppressive drug interactions can invert selection: a drug-sensitive *E. coli* strain was shown to rapidly outcompete a doxycycline-resistant strain under the suppressive combination of doxycycline and ciprofloxacin — an effect that occurred for different resistance mechanisms and should hold more generally for suppressive drug interactions [10]. These observations motivated the development of an innovative screening technique, using neutral labeling of sensitive and resistant bacteria with different fluorophores to identify natural products that can select against drug resistance [79].

Perspectives

A fundamental problem in exploring the effects of drug combinations is the enormous number of experiments that are required to systematically explore all possible combinations of a set of drugs: testing all pairwise combinations of N drugs at one fixed concentration requires $\sim N^2$ experiments; investigating large numbers of drugs and combinations of more than two drugs rapidly becomes prohibitive due to a combinatorial explosion. Quantitative principles that can predict drug combination effects from fewer measurements could remedy this

situation. Interestingly, such general principles may indeed exist. Specifically, an entropy maximization approach was used to derive a formula that successfully predicts the growth response to combinations of more than two antibiotics from the responses to the constituent drugs and their pairwise combinations alone [80•]. Related work provided evidence that the global transcriptional response to antibiotic combinations follows general rules that enable its prediction from the responses to the individual drugs [81]; similar observations were made in cancer cells [82]. Such insights could be applied in advanced treatment strategies that use drug combinations to control the gene expression state of cells. While these principles need to be validated more broadly, they could play a key role in the future design of potent drug cocktails.

A limitation of current studies of drug combinations is that they typically focus on one or a few bacterial strains. However, even bacteria from the same species can show diverse responses to antibiotics; for instance, just a few mutations conferring resistance to one antibiotic can entirely change the cell's response to other drugs. This raises the question to what extent drug interactions are conserved in mutants and across microbial species. Several studies showed that drug interactions are often conserved in resistant mutants, but they might also change considerably [10,83]. The latter would be a challenge for the optimization of multidrug treatments [14,84]. Scaling laws that enable predicting the responses of resistant mutants or gene deletion mutants to two drugs based on few measurements were recently suggested [47••,83]. It remains to be seen how far drug interactions are robust to other genetic perturbations and to what extent they are conserved across species.

Conclusions

Drug combinations have great potential for improving antimicrobial chemotherapy. The field made considerable progress in developing high throughput techniques for identifying potent drug combinations, in understanding the underlying mechanisms of drug interactions and their potential to minimize resistance evolution, and in identifying general principles for the prediction of drug combination effects. Many future challenges remain. In particular, coarse-grained whole-cell models based on bacterial 'growth laws', which succeeded in quantitatively describing and predicting responses to individual antibiotics targeting the ribosome [85–89], should be extended to other drug classes. This approach could enable a more quantitative understanding of drug combination effects on cell physiology. On the experimental side, a key challenge is to develop techniques for the investigation of drug combination effects on bacterial communities rather than on individual strains in isolation. Specifically, community resistance mechanisms in which members of microbial communities protect each other from the presence of an antibiotic [90] can possibly be outsmarted by suitable drug combinations that remain to be identified. Another problem is that drug interactions and their consequences on resistance evolution can depend on environmental conditions. Thus, more expensive and time-consuming *in vivo* studies on the most worrisome pathogens need to be performed to validate central insights gained from basic research on non-pathogenic laboratory strains.

The rapidly emerging new concepts and experimental techniques for the investigation and optimization of drug combinations in microbes can become a driver of progress in other fields including cancer chemotherapy where drug combinations are extensively used [91]. Interdisciplinary research on drug combinations will lead to further exciting advances in microbiology, evolutionary biology, systems biology, and other fields.

Acknowledgements

I thank Andreas Angermayr, Michael Baym, Marta Dravecka, Csaba Pal, Laura Stone, Qi Qin, and Pamela Yeh for critically reading the manuscript and all members of the Bollenbach lab for fruitful discussions. This work was supported in part by Austrian Science Fund (FWF) Grant No. P 27201-B22, HFSP program Grant No. RGP0042/2013, and Marie Curie Career Integration Grant No. 303507.

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- •• of outstanding interest

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