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Systems-level antimicrobial drug and drug synergy discovery

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Here, we review the 'target-centric' genomic strategy to antimicrobial discovery and share our perspective on identification, validation and prioritization of potential antimicrobial drug targets in the context of emerging chemical biology, genomics and phenotypic screening strategies. We propose that coupling the dual processes of antimicrobial small-molecule screening and target identification in a whole-cell context is essential to empirically annotate 'druggable' targets and advance early stage antimicrobial discovery. We also advocate a systems-level approach to annotating synthetic-lethal genetic interactions comprehensively within yeast and bacteria models. The resulting genetic interaction networks provide a landscape to rationally predict and exploit drug synergy between cognate inhibitors. We posit that synergistic combination agents provide an important and largely unexploited strategy to 'repurpose' existing chemical space and simultaneously address issues of potency, spectrum, toxicity and drug resistance in early stages of antimicrobial drug discovery.

Antibacterial discovery is a challenging field. Since the golden era of antibiotics (~1940–1970), only two mechanistically and structurally new classes of antibiotics have reached the clinic: linezolid and daptomycin. This failure coincides with the emergence of serious life-threatening multidrug-resistant (MDR) bacteria[1] and, ironically, the blossoming of the 'omics' and post-omics era. The reasons we have tried and failed to identify new antibiotics are largely beyond the scope of this review[2–7]. However, even the relatively straightforward first operational step to addressing this challenge—namely, antimicrobial target validation and lead-finding strategies—deserve careful reassessment.

The relative failure of target-based antimicrobial discovery in the genomic era seems to be widely accepted and perhaps is best illustrated by Payne *et al.*[8] Despite a massive campaign focusing on 67 rigorously validated bacterial targets, each applied to industrial-scale *in vitro*–based ultra high-throughput (uHTS) screening and exhaustive follow-up experiments over a seven-year period, it was concluded that such efforts were unsustainable for the cost-effective identification of progressable antibiotic leads and that antimicrobial efforts would be best served by focusing on 'best-in-class' drug development (**Box 1**) rather than 'first-in-class' drugs (**Box 1**). Consistent with this notion, the majority (~70%) of new drugs approved between 1999 and 2008 were best-in-class agents[9]. However, it is interesting to note that the remaining drugs approved over this period, which comprise the first-in-class drugs (including daptomycin and linezolid), mostly originated from whole-cell phenotypic screens, driven initially by the bioactivity of the molecule[9]. Here, we examine genomic-era target-based antimicrobial discovery efforts to consider and develop innovative and successful chemical-genomic strategies (specifically, genomics-based technologies applied to small-molecule bioactivity studies) based upon whole-cell screening for the discovery of new classes of antimicrobials with new mechanisms of action (MOAs).

In particular, we discuss how new bioactive compounds are identified and can be linked to their specific target through chemical-genomic approaches. With defined target-compound antimicrobial pairs, a rational understanding of the biology pertaining to each target and the chemical tractability of each whole cell–active cognate inhibitor can be rigorously assessed. Perhaps counterintuitively, it is only after the compound-target connection has been made that the general operational code for genomic-based target validation strategies is best considered because knowledge of both the compound and target allows for a clearer understanding of the factors required for further development of a first-in-class small molecule. We also discuss the potential for exploiting fundamental aspects of synthetic-lethal genetic networks and compound synergy as a means to augment antimicrobial drug discovery and development.

Operational code for genomic and target-based discovery

The last 15 years of antimicrobial discovery have relied on an operational code consisting of three basic rules for identifying potential targets (**Fig. 1**): (i) a bioinformatics-driven approach, derived from whole-genome sequencing, is used to identify pathogen-specific genes with the desired conservation and spectrum and the absence of a human ortholog. (ii) Genetic analyses are performed to confirm that loss-of-function mutations result in a nonviable growth phenotype under standard laboratory conditions and/or a nonvirulent phenotype in a relevant animal model of infection. Loss-of-function mutations of this kind suggest that cognate small-molecule inhibitors of the target should recapitulate these growth phenotypes. (iii) There should be some evidence, derived from sequence, structure or biochemical information, that the target is druggable.

With these rules in mind, antimicrobial agents could be rationally developed to prevent or treat an established infection. Although such criteria are entirely logical, they are simplistic and may not predict 'high-value targets'. In fact, when considering only the first rule, many of the most successful antibiotics ever discovered, including macrolides, aminoglycosides, fluoroquinolones and sulfonamides, perturb processes that hardly satisfy the pathogen-specific ortholog criterion[10]. Similarly, prominent antifungal drugs including azoles and amphothericin show efficacy (albeit with significant toxicity in the case of amphothericin),

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Box 1 | 'New or used' antimicrobial targets

'Used' targets are those for which current or past antimicrobial agents act to elicit a clinically validated therapeutic effect. Drug discovery against such targets is described as best-in-class drug development. This approach aims to identify either an entirely new chemical series or analogs of existing chemical scaffolds to improve therapeutic efficacy or pathogen spectrum and simplify administration or to reduce drug dosing, cytotoxicity or resistance associated with the therapeutic class[5,109]. These efforts are largely directed toward improving current β-lactams, which target cell wall penicillin-binding proteins; fluoroquinolones, which inhibit DNA gyrase and topoisomerase enzymes; or macrolides, tetracyclines and aminoglycosides, which impair protein synthesis by targeting the ribosome[5,10]. Similarly, best-in-class antifungal efforts are largely focused on improving existing echinocandins and azoles, which inhibit cell wall β-(1-3)-glucan synthesis (Fks1) and ergosterol biosynthesis (Erg11), respectively[11,110].

Antimicrobials developed against new targets are described as first-in-class agents. New targets are not clinically validated; consequently, they come with risk from a drug development perspective. These risks include uncertainties regarding whether the target is susceptible to small-molecule inhibition (that is, druggable), its antimicrobial spectrum and its susceptibility to drug resistance. A myriad of possible drug resistance mechanisms may abrogate the effects of cognate inhibitors to new drug targets (**Box 3**). These include the intrinsic structural variation of a target between microbes, propensity for tolerating target-specific drug resistant mutations, susceptibility to compensatory bypass mutations or horizontal gene transfer[37,111,112]. First-in-class antibiotics are also vulnerable to indirect processes that reduce the effective drug concentration within cells, whether by induction of efflux pumps or hydrolytic or modifying enzymes or by altering cell permeability[37,111,112]. Despite these tremendous challenges, first-in-class agents provide the best long-term strategy to combat the dual threat of drug resistance and emerging microbial pathogens for which new therapeutics are sought[1,5].

despite the former selectively targeting fungal cytochrome p450 monooxygenases (Erg11) rather than human orthologs and the latter preferentially targeting lipid membranes containing fungal ergosterol versus cholesterol[11]. Thus, there is precedent and potential for deviating from the rules associated with the current operational code.

The second rule of target identification, attacking processes that are essential for pathogen growth and viability, has served as the foundation of antimicrobial drug discovery since the discovery of penicillin. Genetic strategies for assessing gene essentiality remain an important component to validating any drug target (**Box 2**). With a whole-genome sequence available along with robust orfeome predictions (that is, genome-wide annotation of protein-expressing open reading frames), a global loss-of-function mutation analysis is a logical and important next step. Starting with the first genome-wide gene deletion analysis completed in *Saccharomyces cerevisiae*[12,13], a variety of genetic strategies have been similarly applied to identify essential genes in a number of bacterial pathogens, including *Escherichia coli*[14,15], *Haemophilus influenza*[16,17], *Pseudomonas aeruginosa*[18,19], *Salmonella enterica* var. Typhimurium[20], *Staphylococcus aureus*[21,22], *Streptococcus pneumoniae*[16,23] and *Mycobacterium tuberculosis*[24]. Generally, these approaches involve transposon-insertion inactivation-based mutagenesis and provide a substantial catalog of essential genes, with the identification of indispensable genes only limited by the extent of the insertion bias of the transposon and genome coverage or saturation of the screen. Thus far, gene replacement strategies to comprehensively construct clean deletion mutations from start to stop codons have only been accomplished in *S. cerevisiae*[12,13], *Bacillus subtilis*[25], *E. coli*[15] and *Acinetobacter baylyi*[26].

Importantly, not all essential genes are equal as drug targets; their terminal phenotypes merit important considerations (**Box 2**). For example, essential genes with a cidal terminal phenotype (that is, cells die upon genetic inactivation) are generally preferred over

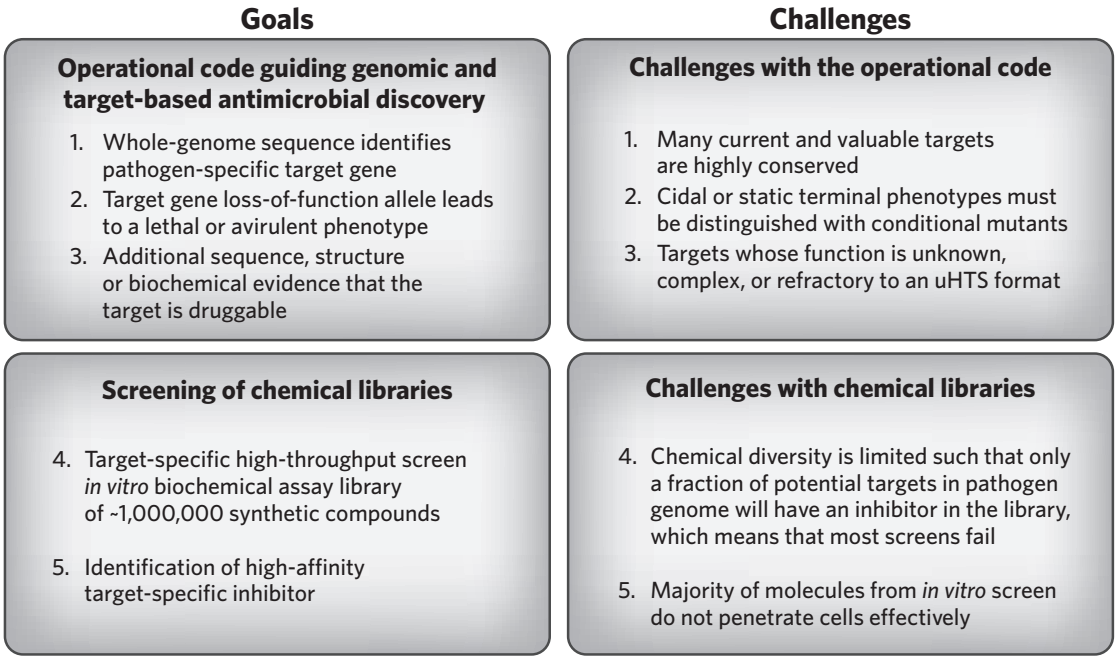

Figure 1 | The goals and challenges associated with the current operational code for antimicrobial target identification and target-based chemical library screening.

Box 2 | Targets

What do they look like; what do they tell us?

Antimicrobial drug targets are generally regarded as any protein, macromolecular structure or cellular process whose function is specifically required for growth, viability or virulence among a clinically relevant set of pathogens. Genetic demonstration that disrupting a target's function will abrogate the disease process is critical, with loss-of-function or gain-of-function mutations of the target predicting the effects of small-molecule antagonists or agonists, respectively. It is rare for a small-molecule inhibitor to completely phenocopy the effects of deleting the target. First, the inhibitor must be exquisitely selective (imagine the difficulty of achieving this if the target is a protein kinase or member of any other diverse protein family). It must also have effectively complete penetrance across diverse clinical isolates. Although this seems straightforward, different clinical isolates of a common bacterial species (for example, *S. aureus*) have extensive genetic diversity[113]; microbial pathogens of distinct species, orders and phyla are genetically more dissimilar to one another than yeast is to human[114]. Finally, unlike loss-of-function mutations, cognate inhibitors must penetrate and accumulate in cells. Therefore, although genetic validation of a drug target is necessary, whole cell–active target-specific inhibitors are invaluable chemical tools to survey the phenotypic consequences of interdicting a target across vast phylogenetic distances.

What are they good for?

In a reductionist approach, antimicrobial targets are interrogated in functional uHTS assays aimed to identify specific cognate inhibitor hits and lead compounds whose in-cell activity should mimic the genetic inactivation of the same target. Constructing a null mutation to establish the essentiality of a target is necessary, but conditional mutants of essential genes enable more sophisticated terminal phenotype studies both *in vitro* and in animal models of infection. Generally, conditional mutants are engineered by replacing the endogenous promoter with a heterologous promoter, which is either inducible or repressible by a specific metabolite or antibiotic supplement. Examples include IPTG[29], arabinose[27] and xylose[28], which are applicable to bacterial pathogens, and galactose[115], nitrogen[33] or tetracycline, which are used in yeast and fungal pathogens[30,31,116]. The tetracycline conditional promoter system is of particular interest because of its utility for examining *C. albicans* conditional mutants in mouse models of infection[34]. By infecting mice with such strains and administering tetracycline before or after establishment of an infection, the repressor could be used as a surrogate to a target-specific inhibitor. Terminal phenotypes could then be assessed on a large scale to discriminate between targets that would provide potential therapeutic utility to prevent infection or to treat an established infection. These studies are particularly relevant in cases where genetic inactivation of the target may lead to a phenotype potentially suppressed in a host environment, where a lack of efficacy would be predicted. For example, antifungal azoles inhibit ergosterol biosynthesis but specifically lack efficacy against *Candida glabrata*, which can scavenge sterol from the host[117,118]. Similarly, unlike *S. aureus*[119], *Streptococcus* spp. can use host fatty acids during infection, thereby potentially overcoming the antibiotic activity of inhibitors targeting this pathway[120]. *In vivo* validation studies therefore mitigate the risk of pursuing a new target whose function is either nonessential or redundant in a host environment.

essential genes that have a static terminal phenotype (that is, cell growth is arrested but cells remain viable). Inhibitors to essential gene products with a cidal terminal phenotype would provide greater efficacy to clear a microbial infection and/or be less likely to be limited by drug resistance than inhibitors with static terminal phenotypes. However, without specialized conditional mutations in essential genes, their terminal phenotypes cannot be induced and characterized in detail. For example, terminal phenotypes can be studied for specific essential gene targets in bacteria by constructing a merodiploid, whereby the deletion mutation is complemented with a second functional gene copy whose expression is controlled using an IPTG-, arabinose- or xylose-inducible promoter[27–29]. Similarly, in fungi, conditional mutants may be constructed directly by replacing the endogenous promoter with a heterologous promoter that is inducible or repressible, such as the tetracycline promoter[30–32]. Beyond assessing cidal or static terminal phenotypes, conditional mutants also facilitate scoring of other important target attributes, such as the extent of functional inactivation necessary to achieve growth impairment and definitive validation of gene essentiality and/or avirulence in a relevant animal model of infection[33,34].

The third rule of the target-based approach implies that the molecular function of potential targets is known and well characterized. Accordingly, enzymatic targets are highly valued in this paradigm simply because their functional activity can be easily reconstituted and assayed *in vitro*. Thus, potential targets

Box 3 | Targets: other considerations

Acquired drug resistance is an important consideration for any antimicrobial target and cognate inhibitor. However, without target-specific bioactive inhibitors, targets are typically evaluated in the context of general principles that affect drug resistance. For example, target-based resistance is substantially lower for antimicrobials that mechanistically act on multiple essential targets than for agents that inhibit a single target because multiple independently derived resistance mutations must be acquired in the former case to nullify the effect of the inhibitor[6]. Examples of this include the low frequency of resistance (10^{-9}–10^{-10}) associated with fluoroquinolones[10] and echinocandins[11], which each target multiple enzymes. Notwithstanding this general rule, important exceptions apply. For example, fosfomycin targets cell wall peptidoglycan synthesis via MurA inhibition and has a high frequency of resistance (10^{-7}) in the laboratory[6]. Despite this, fosfomycin is clinically efficacious in treating urinary tract infections as the bacterial burden associated with such infections is below the frequency of spontaneous drug-resistant mutations and high clinical exposure levels of the drug are achieved[6]. Similarly, drug resistance mechanisms may carry a substantial fitness cost and attenuated virulence, as is the case with fosfomycin-resistant isolates[6] and/or restored antibiotic susceptibility to other agents, thereby mitigating their importance in a therapeutic context (**Box 4**).

Drug resistance bypass mechanisms are also commonly encountered, especially those affecting drug susceptibility by virtue of cell permeability or efflux mechanisms. These include loss-of-function mutations in cell surface porins, such as those normally used by β-lactams to transit through the outer membrane of Gram-negative bacteria (for example, OprD[37]), as well as the induction of MDR efflux pumps common to all microbes (for example, the AcrAB-TolC efflux family in bacteria[37] and the pleiotropic drug resistance family in fungi, which effectively efflux azoles[121]). Accordingly, essential targets localized at the cell surface circumvent such issues. Similarly, efflux-specific resistance to β-lactams is rare among Gram-positive bacteria because their targets (penicillin-binding proteins) are localized within the cell wall. Conversely, β-lactam resistance within Gram-negative bacteria is problematic as penicillin-binding proteins are localized within the periplasmic space and are thus vulnerable to efflux pumps[37]. Despite intensive genomic efforts to identify essential cell surface targets, few have been identified, in part, owing to genetic redundancy.

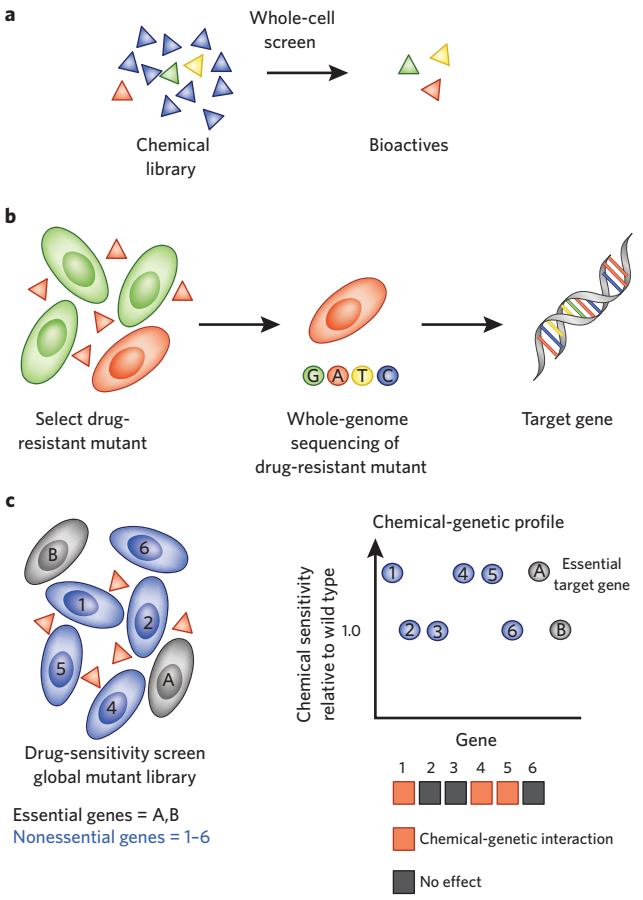

Figure 2 | Identifying bioactive antimicrobial compounds and linking them to their target with forward and reverse chemical-genetic approaches. (**a**) Whole-cell screening strategies for bioactive compounds identify a cell-permeable and antimicrobial subset. (**b**) In a forward genetic approach, selection for a drug-resistant mutant, followed by whole-genome sequencing, identifies mutations that lead to the drug-resistant phenotype, which often occur within the gene encoding the drug target. (**c**) Chemical-genetic screens involve screening genome-wide sets of mutants, including mutations that partially inactivate essential genes and deletion or null alleles of nonessential genes for hypersensitivity to a bioactive compound (left). The most hypersensitive essential gene mutant often identifies a candidate target gene, whereas the set of hypersensitive nonessential gene deletion mutants forms a chemical-genetic profile that identifies functionally related genes. Upon exposing a mutant collection to a specific bioactive compound (red triangle), mutations in the essential gene A lead to chemical hypersensitivity, which identifies a candidate target gene, and mutations in the set of nonessential genes 1, 4 and 5 lead to chemical hypersensitivity and generate a chemical-genetic profile (right).

that satisfy all other criteria, which is a high bar, but whose function is unknown, complex or refractory to reconstitution in an uHTS format are ignored at this early stage of target triaging. Conversely, targets that satisfy this rule are highly susceptible to developing target-based drug resistance (**Box 3**).

Perhaps an even greater unintended consequence of the operational code of target-centric genomics approaches to antimicrobial discovery is its reliance on *in vitro* biochemical screening strategies, thereby ignoring the important issue that target-specific inhibitors must also penetrate cells and thwart diverse efflux systems, hydrolytic and modifying enzymes to achieve whole-cell bioactivity (**Box 3**). This is particularly problematic as several of the most medically important pathogens for which new antibacterials are urgently sought are MDR Gram-negative bacteria[1], which are naturally protected from chemical stress by a second (outer) membrane[35] and diverse and highly efficient drug efflux systems[36,37]. Consequently, little success has been achieved in converting highly potent target-specific inhibitors discovered through cell-free screening efforts into whole-cell bioactive leads with a potency and spectrum sufficient for drug development.

Understanding the limitations of chemical space is paramount. Take, for example, a library of 1,000,000 synthetic compounds—a standard library size in most industry programs—and the objective of identifying bioactive anti-*Pseudomonas* antibiotic leads. Less than ~1,000 compounds in such a library (given our experience of a likely hit rate of <0.1%) will show whole-cell growth-inhibitory activity. Moreover, not all bioactive compounds act through a target-specific MOA; most act nonselectively as alkylating agents, DNA intercalators, detergents and so forth. Thus, perhaps less than 5% of bioactive compounds would act through target-specific mechanisms[38]. At this rate of attrition, fewer than 50 compounds in the starting library are expected to show whole-cell activity and act through a target-specific MOA. Given that pathogenic bacteria, such as *P. aeruginosa*, have ~300–400 essential genes[18,19], we would expect >85% failure rate in screening any individual target in an uHTS campaign for a target-selective whole cell–active agent. This failure rate is conservative because we ignore the possibility that the bioactivity of a compound may depend on its ability to inhibit multiple members of a protein family or that sets of structurally related compounds may be biased toward a particular target or pathway. Success may also vary with the target organism; for example, whole-cell bioactivity is generally easier to obtain when screening Gram-positive bacteria or yeast[38,39], and hit rates will generally improve by screening natural product libraries, albeit with a whole new set of challenges[4,5,38]. Profound attrition rates 'stack the deck' against success in a target-based screening paradigm. Therefore, although the rules of the operational code driving genomic and target-based antimicrobial discovery seem logical, perhaps it is not surprising that the systematic and ordered application of these rules has not yielded success in developing first-in-class agents[8]. We suggest that these rules should only be considered once a target and its small-molecule lead have been identified, and, perhaps most importantly, they should be considered as guidelines rather than rules.

Bioactive compound libraries

Considering that the principal limitations of target-based screening are that few (if any) cognate inhibitors to an individual target exist in a chemical library and that our knowledge of how to optimize whole-cell activity through medicinal chemistry is limited, a pragmatic solution to this problem most likely involves greater initial focus on compounds with intrinsic bioactivity against microbes of interest (**Fig. 2a**). Annotating those compounds in a chemical library with inherent antimicrobial activity against medically important pathogens and/or model organisms is technically straightforward[39]. Such a first step was routinely used in empirical antimicrobial screening long before enormous chemical libraries and automated uHTS methods were embraced by industry. Moreover, a trend toward sharing publicly available chemical libraries annotated for antimicrobial activity is emerging[39–41]. Addressing the bioactivity problem in this way, however, presents a new challenge: how to efficiently link drug-like bioactive small molecules to their molecular target. Fortunately, substantial progress has been achieved in this area, warranting a return to the historically successful compound-centric approach to antimicrobial lead finding.

Target identification and antimicrobial lead discovery

Forward and reverse chemical-genetic strategies are equally applicable to interrogating MOA and target identification of

Figure 3 | Interpreting chemical-genetic and genetic interaction profiles. Sets of compounds with similar chemical-genetic interaction profiles (for example, genes 1, 4 and 5 are all sensitive to both compounds represented by red and blue triangles) often have similar modes of action (left). Sets of genes with similar genetic interaction profiles (for example, genes A and B both show genetic interactions with genes 1, 4 and 5) have similar functional roles (right). If the chemical-genetic interaction profile of a compound resembles the genetic interaction profile of a gene, then a target pathway is predicted (for example, the compounds represented by red and blue triangles may target a pathway containing genes A and B).

drug-like bioactive compounds. Briefly, forward chemical genetics is simply 'old school' microbiology—selecting for mutants able to grow in the presence of an otherwise lethal concentration of an antimicrobial agent. Resistant mutants are powerful because they often identify the target of the bioactive compound[42]. Moreover, with next-generation sequencing (NGS), microbial genomes can be sequenced, assembled and annotated on a timescale of days, such that mapping of drug-resistant mutations can be largely automated[43,44] (**Fig. 2b**). The approach is powerful because it (i) is comprehensive in nature without assay development or target bias, (ii) is rapid and applicable to any microbe with a haploid genome, (iii) does not rely on pathogen-specific tool building and (iv) recapitulates the classical era of antibiotic discovery but leverages post-genomic technology.

There are multiple examples of successfully linking bioactive small molecules to their target by combining drug-resistant mutant selection (DRMS) and whole-genome sequencing. An elegant early example of this forward chemical-genetic strategy is Bedaquiline, an electron transport chain inhibitor shown to target the atpE subunit of the ATP synthase, which is now in phase 2 clinical trials for the treatment of tuberculosis[45]. Similar approaches have yielded multiple new early stage antituberculosis leads targeting distinct aspects of cell wall synthesis[46,47] or mycolic acid transport[46,48]. Coupling DRMS with NGS has also led to the identification of decoquinate, a potent cytochrome B1–specific inhibitor effective against *Plasmodium falciparum*, the causative agent of malaria[49], and of new small molecules belonging to the pyrazolopyrimidinone class targeting *S. aureus* and *E. coli* tRNA synthases (Smith, J.S., personal communication).

Reverse chemical-genetic screens exploit genome-wide collections of mutants that are available, for example, in *S. cerevisiae*[12,50–53], *C. albicans*[38,54,55] and *S. aureus*[56,57], where drug susceptibility phenotypes can be rapidly mapped to specific genes by systematically screening antimicrobial agents against a defined and (ideally) comprehensive mutant collection (**Fig. 2c**). In principle, provided the corresponding drug target to a bioactive (growth inhibitory) agent is partially depleted from the cell, the mutant to the target will show chemical hypersensitivity (that is, reduced fitness) to its cognate inhibitor over the wild-type strain or other mutants[50,58]. Because our bioactive compounds are growth inhibitory by definition, the target should be an essential gene. Technically, mutant strain collections for essential genes may be constructed as heterozygote deletion mutants in diploid microbes[50,54] or by transposon insertion[59], disruption of 3′ noncoding sequence[60] or antisense interference[56,57] to partially inactivate the target gene in a haploid organism.

In addition to mutations to the essential target gene, those in nonessential genes may also result in hypersensitivity to a bioactive compound, especially nonessential genes that operate within functionally related pathways (see synthetic lethality below; **Fig. 2c**). Thus, testing a genome-wide set of mutants, covering both essential and nonessential genes, for sensitivity to a bioactive compound generates a chemical-genetic profile that links the compound to a specific set of functionally related genes[53]. The most hypersensitive essential gene mutant often identifies the compound's target, whereas the set of nonessential gene mutants generates diagnostic information about the related buffering pathways. In particular, bioactive compounds that share highly similar chemical-genetic profiles often share a similar function and target the same pathway (**Fig. 3**).

Elegant 'bar coding' strategies to uniquely identify individual mutant strains also exist and enable the pooling of defined mutant strain collections and screening for chemical hypersensitivity phenotypes in a highly parallel coculture format. Such assays are commonly referred to as a fitness test, haploinsufficiency profiling (for heterozygous diploid deletion mutants of essential genes) or homozygote profiling (for homozygous diploid deletion mutants of nonessential genes or for haploid profiling of nonessential deletion mutants). The relative abundance of mutant strains following drug treatment and competitive growth in pools can be determined either by microarray analysis or genome sequencing[38,54,61–63]. Hybrid strategies to the traditional forward and reverse chemical-genetic screens have also been developed[42,64,65].

Forward and reverse chemical-genetic strategies need not be mutually exclusive. For example, reverse chemical-genetic

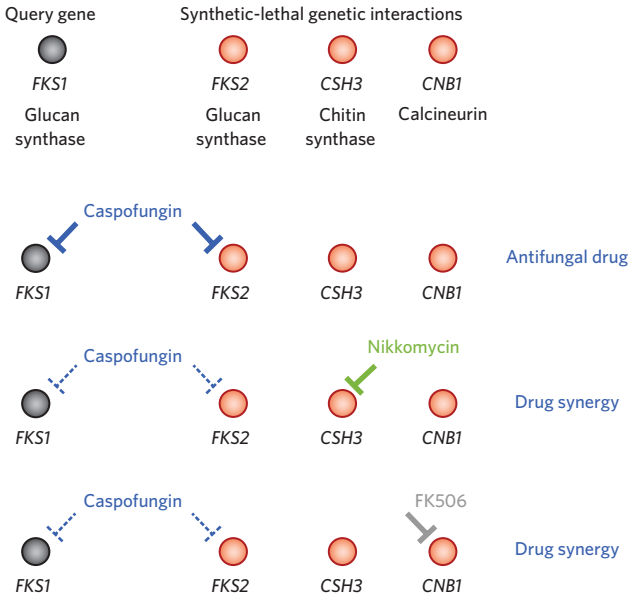

Figure 4 | Genetic interactions are predictive of chemical-genetic and chemical-synergy interactions. A deletion mutation in *FKS1*, which encodes a biosynthetic enzyme for yeast cell wall (1,3)-β-glucan biosynthesis, is synthetically lethal with deletion mutations in *FKS2*; an *FKS1* paralog, *CHS3*, which encodes cell wall chitin synthase; and *CNB1*, which encodes the regulatory subunit of calcineurin. The antifungal compound caspofungin inhibits (1,3)-β-glucan synthase activity to kill cells. However, substantially reduced amounts of caspofungin, as indicated by the dashed inhibitory bar, are required to similarly kill cells in combination with the chitin synthase inhibitor nikkomycin or the calcineurin inhibitor FK506. Such combinations have synergistic activity, reflecting the synthetic lethality between *FKS1* and *CHS3* or *FKS1* and *CNB1*, respectively.

Box 4 | Synthetic-lethal targets and the design of synergistic combination agents

β-lactamase inhibitors combined with β-lactam antibiotics represent the 'gold standard' of antibiotic combination agents. These combinations include sulbactam plus ampicillin, clavulanic acid plus ampicillin and tazobactam plus piperacillin[109]. They are paired because β-lactamase inhibitors restore efficacy against drug-resistant bacteria that have acquired β-lactamase enzymes and effectively hydrolyze the β-lactam antibiotic[10,109]. Similar, albeit less successful, strategies have aimed to identify small-molecule inhibitors of drug efflux pumps that cause antibiotic resistance[36,37]. In both cases, combining these agents is highly synergistic, meaning that restored bioactivity is achieved using a fractional concentration of each agent exceedingly below their effective concentration used when administered individually[122,123]. These strategies are designed to neutralize the underlying drug resistance mechanism; the targets themselves, however, are not synthetically lethal. Conversely, a systems-level approach considers the interdependence of functional networks within the cell that, if interdicted genetically or pharmacologically, achieves the same synergistic effect. Synthetic-lethal targets need not be essential on their own, although such targets are generally preferred. Combining synergistic target-specific inhibitors offers important advantages. Provided both inhibitors are potent bioactive agents targeting distinct essential proteins, their combination effectively reduces the frequency of resistance associated with each individual agent[96,97]. Further, substantially lower concentrations of each inhibitor are required to elicit efficacy compared to the amount required for single agents. Therefore, reduced exposure may mitigate cytotoxic effects[124]. Finally, as the two targets produce a synthetic-lethal phenotype, target-based drug resistance mutations to one member of the pair may resensitize the pathogen to the companion antibiotic[97]. Notwithstanding the unique issues to such a strategy (for example, codosing or coadministration requirements and clinical and regulatory complications), the opportunity to rationally develop combination agents holds tremendous promise.

strategies are an effective approach to discriminate between target-specific and nonspecific bioactive agents, as the former uniquely shows pathway-specific profiles using a fitness test–based screening paradigm[38,56]. Accordingly, such compounds may be further evaluated by DRMS and NGS to identify their cognate target, as we have shown with *S. aureus* inhibitors targeting SAV1754, a component of cell wall peptidoglycan biosynthesis[66], and antifungal natural product-derived parafungins, which target the *C. albicans* polyA polymerase[55,67]. In each case, sufficient pathway-specific profiles suggestive of target-specific inhibitory activity were identified in the fitness test assay to warrant DRMS and ultimately target identification. Converse to individual target-based screening, diverse postgenomic strategies now exist to rapidly identify drug targets to bioactive agents in the context of the pathogen's global compendium of possible targets. Accordingly, a compound-centric view emerges, and for the first time we have the opportunity to empirically address a fundamental consideration of any high-value target, namely whether it is druggable as an antimicrobial target.

Genetic interactions enlighten target discovery

Genetic interaction studies in baker's yeast highlight important and underexploited opportunities to expanding target sets and tackling core issues in antimicrobial drug development. A genetic interaction is observed when a double mutant shows a more extreme phenotype than the expected effect associated with the combination of the two corresponding single mutations. Genetic interactions can be negative (more exaggerated) or positive (less exaggerated) in terms of phenotype with respect to the expected double mutant phenotype[68]. Synthetic lethality represents an extreme example of a negative interaction where two mutations, each causing little to no fitness defect on their own, result in a lethal double mutant phenotype[69]. The potential for therapeutically harnessing synthetic lethality provides a new avenue for antimicrobial drug discovery.

The yeast gene deletion project identified ~1,000 essential genes and ~5,000 nonessential genes, generating ~5,000 viable deletion mutants[13], which represent an ordered set of strains that can be used for mapping genetic interactions systematically. The yeast synthetic genetic array (SGA) methodology automates yeast genetics and enables the mapping of genetic interactions on a large scale. SGA combines arrays of either nonessential gene deletion mutants or conditional alleles of essential genes with robotic manipulations for high-throughput construction of haploid yeast double mutants and identification of genetic interactions[70,71]. A genome-scale colony size scoring methodology[72] enables quantitative analysis of negative and positive genetic interactions. In a recent large-scale endeavor, ~5.4 million gene pairs, covering ~30% of the *S. cerevisiae* genome, were examined to reveal ~100,000 negative genetic interactions and provide the first view of the genetic landscape of a cell[73]. This global set of quantitative negative genetic interactions includes both synthetic-lethal and synthetic-sick (slow-growing) phenotypes. However, only the most extreme synthetic-lethal interactions (~10,000 interactions) are likely to be relevant to antimicrobial target discovery. Nevertheless, this means that the number of synthetic-lethal gene pairs is on the order of approximately ten-fold higher than the number of essential yeast genes, offering an astounding potential for antimicrobial therapy through combinatorial targets.

A detailed analysis of the topology of the *S. cerevisiae* genetic network has revealed several general principles. For nonessential genes, most synthetic-lethal genetic interactions occur between pathways and complexes, mapping those that function together to control essential functions. In the case of essential genes, in addition to these between-pathway interactions, the combination of two hypomorphic (partially functional) alleles often results in a synthetic-lethal interaction, and thus genetic interactions also occur within pathways. The average gene may show on the order of ~30 negative genetic interactions, and thus the genetic network is complex, yielding a functional wiring diagram in which numerous genes and their corresponding pathways buffer each other. On a global scale, SGA has the potential to map synthetic-lethal interactions among the majority of yeast genes and their corresponding pathways.

The genetic interaction profile for a given query gene is diagnostic of its role because genes with similar patterns of genetic interactions often function within the same pathway or complex[71,73] (**Fig. 3**). A global network in which genes are linked to one another based upon the similarity of their genetic interaction profiles generates a functional wiring diagram of a cell[73,74]. Moreover, because a loss-of-function mutation in a gene provides a model for the physiological effect of an inhibitory molecule that targets the gene product[74], the global network of genetic interaction profiles provides a key for interpreting chemical-genetic interaction profiles and thus can link compounds to their target pathways[73] (**Fig. 3**).

Specific genetic interactions may also be relevant to antimicrobial drug discovery. The genetic interaction profile of the nonessential gene *FKS1* (**Fig. 4**), which encodes a biosynthetic enzyme for yeast (1,3)-β-glucan biosynthesis, provides an interesting

example. *FKS1* has a paralog encoded by another nonessential gene, *GSC2* (henceforth referred to as *FKS2*), and the antifungal drug caspofungin blocks (1,3)-β-glucan biosynthesis by inhibiting both Fks1 and Fks2 (ref. 75). Accordingly, the *fks1Δ fks2Δ* double deletion mutant is synthetically lethal[71]. Because *FKS1* is nonessential, an *fks1Δ* query mutation can be screened for additional synthetic-lethal partners, revealing negative genetic interactions with a set of ~100 genes and extreme synthetic-lethal interactions with ~20 genes[73]. In particular, *FKS1* is synthetic lethal with *CHS3*, a chitin synthase required for synthesis of the majority of cell wall chitin[76], and with *CNB1*, which encodes the regulatory subunit of calcineurin, a calcium- and calmodulin-regulated protein phosphatase that activates the Crz1 stress-responsive transcription factor[77].

The *FKS1-CHS3* genetic interaction predicts that a *CHS3* deletion mutant should be hypersensitive to caspofungin, which is indeed the case[75]. Furthermore, because *FKS1* and *CHS3* loss-of-function mutations have a synergistic genetic interaction[78,79], then a specific inhibitor of chitin synthase, such as nikkomycin[80], should be synergistic with caspofungin. Indeed, this seems to be true in a number of different fungi, including the pathogens *C. albicans*[81] and *Aspergillus fumigatus*[82]. Analogously, the *FKS1-CNB1* genetic interaction predicts that *cnb1Δ* and *fks1Δ* deletion mutants should show sensitivity to both caspofungin and the calcineurin inhibitor, FK506, respectively, and that caspofungin and FK506 should also act synergistically. Indeed, a number of different studies provide evidence to support these predictions both in *S. cerevisiae* and major fungal pathogens[83–85].

One of the over-riding benefits of SGA applied to antimicrobial target discovery is that it provides a productive avenue for identifying new drug targets. Global synthetic-lethal genetic networks would identify numerous pairs of nonessential genes that can be targeted in combination to kill the pathogen, and thus the antimicrobial target space is expanded well beyond that of the subset of essential genes. Synthetic-lethal interactions with conditional alleles of essential genes also enable the identification of genes and pathways that buffer the activity of an essential cellular function and thereby predict targets whose cognate inhibitors would show strong synergistic activity with existing antimicrobials. As such, clinically used antimicrobials may be improved in terms of their efficacy, spectrum, resistance and safety profiles if paired with such cognate inhibitors (**Box 4**). As illustrated above, SGA synthetic-lethal interactions predicted that FK506 would potentiate the activity of caspofungin, enabling combination therapy involving a lower dose of caspofungin. Synergistic effects causing host cytotoxicity might not be expected, provided that one (or both) of the targets is absent in humans and that the synergy of the drugs enables a lower dose of each therapeutic.

Applied synthetic lethality and chemical synergy

A comprehensive set of deletion mutations have also been made for *E. coli*, identifying over 300 essential genes and generating ~4,000 viable deletion mutants[15]. This Keio collection has been extensively used in chemical-genetic screens to identify genes required for tolerance and susceptibility, to most of the common antibiotic drug classes[86–89]. Screening the Keio collection for chemical hypersensitivity to a specific antibiotic is analogous to systematically scoring double mutants for genetic interactions with the antibiotic target gene. Similarly, chemical-genetic screens have also been performed using transposon insertional libraries of other important bacterial pathogens, including *M. tuberculosis*[90], *P. aeruginosa*[91] and *A. baylyi*[92], revealing large numbers of genes that influence drug susceptibility and are therefore candidate antibiotic potentiation targets. Perhaps surprisingly, the hypersensitivity observed is typically rather modest, ranging from a two-fold to eight-fold change in minimal inhibitory concentration of the antibiotic between wild-type and mutant strains. This modest effect may reflect, at least in part, that chemical-genetic screens for antibiotic potentiation targets are often restricted to nonessential genes. Analogous examples that focus on essential genes are rare; however, we have recently reported one such study[93]. To identify targets that, if partially depleted from a cell, restore the susceptibility of methicillin-resistant *S. aureus* (MRSA) to β-lactam antibiotics, a phenotypic screen was performed using an inducible RNAi methodology to knock down expression of ~250 different essential genes. Remarkably, over 10% of the genes surveyed provided specific tolerance to β-lactam antibiotics versus other antibiotic classes, highlighting aspects of cell wall biogenesis and cell division that buffer MRSA from the full effects of β-lactams[66,93–97]. We have used these findings to predict new combinations of synergistic drugs against MRSA[66,93,96,97]. Consistent with FtsZ as a member of the MRSA β-lactam potentiation network, the potent FtsZ inhibitor PC190723 (ref. 98) is uniquely synergistic with β-lactam antibiotics, including when tested in a mouse skin infection model of MRSA[97]. Further, multiple PC190723-resistant strains containing FtsZ amino acid substitution mutations have substantial and specific hypersensitivity to β-lactams, recapitulating the phenotype of the FtsZ antisense strain[97]. These results highlight an important additional potential benefit of combination agents; namely, each member of the combination agent pair has the propensity to counteract the resistance associated with its companion antibiotic (**Box 4**).

Empiric screening for synergistic agents

Previous examples demonstrate how synthetic-lethal interaction or chemical-genetic interaction maps may be used to predict chemical synergy. Alternatively, synergy may also be identified by directly screening compound libraries; for example, recent screens against MRSA identified β-lactam synergists[66,96,99,100]. Interestingly, drug resistance analysis of two prioritized bioactive agents resulting from the screen led to the identification of their respective targets, the essential cell wall protein SAV1754 (ref. 66) and the type I signal peptidase, SpsB[96]. As these targets are in the MRSA β-lactam potentiation network and cognate inhibitors have highly selective synergy with β-lactams, these studies reinforce the predictive power of the chemical-genetic interaction network.

Clinically used drugs that were developed for other therapeutic indications but may potentiate the effects of existing antibiotics provide another important chemical source for synergistic agents[100–103]. Ejim *et al.*[103] identified loperamide as a strong and specific potentiator of tetracycline antibiotics across multiple Gram-negative bacteria, including drug-resistant clinical isolates of *P. aeruginosa*. Further, administering loperamide and minocycline (a tetracycline analog) together demonstrated therapeutic synergy in a mouse model of *S.* Typhimurium[103]. Repurposing existing therapeutics as possible antibiotic synergists comes with the benefits of the extensive toxicological and pharmacological information associated with any approved drug[40,104].

Finally, target-specific inhibitors may be screened *ad hoc* for synergistic activity against specific antimicrobial agents, particularly when motivated by a well-characterized molecular function of the drug target. Take, for example, HSP90, a molecular chaperone that indirectly mediates susceptibility to azole and echinocandin-based drug resistance[105,106]. As Cowen *et al.*[105] correctly predicted, the Hsp90 inhibitor geldanomycin has marked synergy with both antifungal drugs, both *in vitro* and in a non-vertebrate model of candidiasis, and such synergy extends across *C. albicans* and *A. fumigatus*. Moreover, though synthetic-lethal genetic interactions highlight possible synergistic agents that should target the interaction, the potential for synergy among drugs may be broader than the genetic interaction profiles of their major targets[107].

Conclusions

We describe an integrated approach to antimicrobial lead discovery that is rooted in empirical whole-cell screening for small molecules with intrinsic bioactivity whose MOA may be rapidly determined using a variety of forward or reverse genomic platforms to identify and subsequently validate their target. Such a compound-centric approach provides a highly efficient genome-wide strategy to identify druggable targets with companion target-specific inhibitors. Rather than up-front genetic, bioinformatic and biochemical target prioritization and subsequent *in vitro*–based screening of individually 'anointed' targets (with the exceedingly high rate of failure associated with this strategy[8]), target-inhibitor pairs identified in our strategy can be ranked according to both the potential therapeutic relevance of each target and the chemical tractability, potency and bioactive spectrum of each cognate inhibitor series. Precise genetic strategies to evaluate the therapeutic effect of inactivating the target (both *in vitro* and *in vivo*) exist and provide an absolute benchmark for the achievable efficacy of the lead, which may itself still require substantial medicinal chemistry improvements before animal efficacy studies are possible. Stated simply, few bioactive target-specific inhibitors exist in a given pharma-scale screening library, and they are not readily identified by target-based screening or easily optimized to have the necessary antimicrobial activity to progress as drug leads. Consequently, it is imperative to identify all of the target-specific bioactive antimicrobial drug leads in a library. Doing so involves returning to the historically successful approach of empiric screening, applying innovative uHTS methods[101,108] and leveraging post-genomic methodologies including DRMS and NGS to facilitate small-molecule MOA determination. Because the necessary potency, spectrum and resistance and other issues inflict enormous attrition on the development of antimicrobial leads as single agents, we recommend addressing these impediments through combination agents (**Box 4**). SGA analysis underscores the immense synthetic-lethal interaction networks that comprise the yeast cell[73], and similar studies performed in microbial pathogens should likewise identify a bounty of previously unexploited drug targets. Importantly, understanding the genetic interaction networks that sustain cell viability provides an exciting opportunity to rationally predict those compound combinations expected to pharmacologically recapitulate the synthetic lethality observed between their targets. Finally, the immense network of genetic interactions revealed in yeast underscores the vast number of synergistic relationships possible among compounds in any chemical library. Indeed, contemplating the use of chemical libraries in these ways resets the suitable chemical space available within pharmaceutical synthetic libraries to discover new antimicrobial agents.

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Competing financial interests

The authors declare competing financial interests: details accompany the online version of the paper.

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