Genetic Strategies for Identifying New Drug Targets

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ABSTRACT Genetic strategies have yet to come into their own as tools for antibiotic development. While holding a lot of initial promise, they have only recently started to bear fruit in the quest for new drug targets. An ever-increasing body of knowledge is showing that genetics can lead to significant improvements in the success and efficiency of drug discovery. Techniques such as high-frequency transposon mutagenesis and expression modulation have matured and have been applied successfully not only to the identification and characterization of new targets, but also to their validation as tractable weaknesses of bacteria. Past experience shows that choosing targets must not rely on gene essentiality alone, but rather needs to incorporate knowledge of the system as a whole. The ability to manipulate genes and their expression is key to ensuring that we understand the entire set of processes that are affected by drug treatment. Focusing on exacerbating these perturbations, together with the identification of new targets to which resistance has not yet occurred—both enabled by genetic approaches—may point us toward the successful development of new combination therapies engineered based on underlying biology.

Bedaquiline was the first novel antitubercular to be approved by the FDA in over 4 decades (1); delamanid is likely to follow soon (2), and there are a number of others in the pipeline (SQ109, linezolid analogues [3–5]). In fact, tuberculosis drug development is one of the few dynamic branches of an otherwise stagnating field. Although there is some basic research into antibiotics in general, little progress is being made in bringing suitable leads into the clinic (6). Only two systemic antibiotics were approved by the FDA between 2008 and 2012, compared to six from 1998 to 2002 and 13 from 1988 to 1992, a number that must increase if we are to retain the upper hand over infectious diseases (7). While this is, in part, due to stringent FDA regulations and the withdrawal of large pharmaceutical companies from antibiotic research (8, 9), it is also due to the high attrition rate in antibiotic discovery, which is further exacerbated by the so-called discovery void (10).

While rational drug design has led to the successful identification of many potent antivirals (maraviroc, raltegravir, zanamivir), antitumor drugs (imatinib, gefitinib), and anti-inflammatory drugs (celecoxib, rofecoxib), target-based approaches played no role in the development of any of the antibacterials that are currently in trials. Our understanding of medicinal chemistry, structural biology, and genomics has delivered unprecedented insights into structure-activity relationships (SARs), detailed knowledge of the three-dimensional structure of proteins, and extensive catalogues of genetic information, but the success of target-based drug development remains elusive. The extent of the problem is illustrated by the failure of a large and intensive target-based campaign to develop novel antibiotics. Seventy high-throughput screens designed to identify inhibitors of essential enzymes were able to identify leads against only five targets: phenylanalyl-, methioninyl-tRNA synthetase (PheRS, MetRS); peptide deformylase (Pdf); peptide deformylase (Pdf); enoyl-acyl
carrier protein reductase (FabI); and 3-ketoacyl-acyl carrier protein III (FabH) (11). As a result, rational drug design is still confined to highly specialized applications such as optimization and development of next-generation versions of existing drugs (12, 13).

In this article we will focus on recent advances in the field that point to ways in which genetics could be used to improve the success and efficiency of drug development. It is important that we recognize lessons from past gene-oriented efforts, particularly the need for making sure that all approaches be based on a whole-cell system (to incorporate in vivo activity and cell permeability from the start), and that we focus on in vivo–validated targets. By doing so there are significant advantages to be had when combining genetic approaches and more traditional screening methods. Key among these are:

1. **Targeting biology that is relevant to infection.** We will describe how unbiased genetic approaches can be used to identify key mediators of virulence and identify novel targets that are necessary for survival in the host.

2. **Strategies to identify drug synergy from the start.** Synergy, while difficult to attain, is highly desirable, because it allows treatment to be more efficient at lower drug concentrations, therefore improving the safety profile of any antibiotic. We propose an approach that may lead to the identification of drug synergy from first principles, which could result in shorter drug treatments and possibly antibiotic combinations with longer “shelf-lives” in the clinic.

3. **Compound-driven selection of targets.** We will discuss advances in methodologies that would allow researchers to identify compound mechanisms of action (MOAs) concurrently with performing screens for inhibitors. This should lead to the identification of novel validated targets in conjunction with new drug scaffolds.

4. **Exploring chemical space populated by weak inhibitors.** Screening hits need to undergo several rounds of chemical iteration to become optimized leads; we will show how a biology-driven identification and selection of hits can allow us to identify compounds with novel MOAs that would be missed by standard screening strategies.

While the current pace of antibiotic development has been disappointing, utilizing these strategies could expand the number of potential compound candidates and reveal new mechanisms that could be exploited to enhance drug efficacy.

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**CURRENT TARGET-BASED DRUG DISCOVERY IN MYCOBACTERIUM TUBERCULOSIS**

The tuberculosis field has long evaluated basic research with an eye on the identification of novel antibiotic targets. Genes are regularly assessed for their contribution to virulence in the mouse model of infection, and multiple systematic assessments of gene essentiality both in vitro and in vivo have already been used to gain a global perspective (14–19). As a result, there is already a growing body of knowledge about potential targets and metabolic pathways whose disruption should have a profound effect on Mycobacterium tuberculosis viability in vivo. The glyoxylate shunt enzymes isocitrate lyase (20, 21) and malate synthase (22) are good examples and are currently being explored as drug targets. Similarly, Beste et al. recently showed that anaplerotic reactions are likely to be important during infection (23). This is corroborated by the fact that inhibitors of carbonic anhydrase were shown to have an antibiotic effect on M. tuberculosis (24). Peptide deformylase, mentioned in the introduction as one of the few chemically validated drug targets (25), seems to be tractable in M. tuberculosis as well (26). Similarly, methionine aminopeptidase is showing promise as a target for antimycobacterial intervention (27).

In addition to these novel enzymes, there are also a number of known targets whose inhibition constitutes the backbone of current chemotherapeutic interventions, e.g., RNA polymerase and enoyl-acyl carrier protein reductase (InhA). By analogy to what is common practice in other parts of the antibiotic discovery field, targeting these with novel chemical entities is another possible strategy to overcome drug resistance. An example was recently reported in which Hartkoorn et al. showed that pyridomycin—a compound of known antitubercular activity—inhibits InhA (28). Unfortunately pre-existing resistance to isoniazid interfered with the compound’s efficacy, particularly in strains where InhA overexpression was the mechanism of resistance. Encouragingly, though, mutations in InhA leading to pyridomycin resistance did not influence isoniazid activity, and perhaps more importantly, catalase (KatG), which is required for the activation of isoniazid and whose mutation is the most common mechanism of resistance to isoniazid, did not affect pyridomycin MIC. These findings therefore provide an encouraging example of targeting known targets in the future.
Augmenting Existing Drugs through Target-Based Approaches

Due to extensive research into antibiotic resistance in *M. tuberculosis*, we currently understand many of the molecular mechanisms that underpin it. Loss of prodrug activation is a common theme among *M. tuberculosis* mechanisms of resistance, because many antituberculars are prodrugs (isoniazid, ethionamide, pyrazinamide, delamanid). As a result, ensuring prodrug activation is an interesting avenue to pursue to augment existing therapies. An example of where this was done successfully in *M. tuberculosis* is the EthR inhibitor—BDM31343 (29). EthR is a negative regulator of EthA, whose activity is necessary for the activation of ethionamide. The authors took advantage of the knowledge of the structure of EthR and the extensive understanding of the biology of transcriptional regulators belonging to the TetR-family of proteins. Starting from 131 different inhibitor candidates identified based on crystallographic data, the authors were able to optimize the molecules and finally identify a BD31343, which had good pharmacological properties and was able to synergize strongly with ethionamide in the mouse model of infection. BD31343 is still in preclinical development, but it is likely to be the first “designer” antimycobacterial compound to enter clinical trials (30).

**TARGETING BIOLOGY THAT IS RELEVANT FOR INFECTION**

The key determinant of the success of any target-based approach is the “quality” of the selected targets. Ideally, we would like to have a long list of protein targets whose inhibition is specific (not affecting the host), physiologically relevant, and chemically tractable so we can readily bypass resistance if and when it occurs. However, this is not an easy task. As mentioned at the beginning of this article, gene essentiality and conservation across microbial species are not sufficient attributes for the successful identification of suitable antibiotic targets. Additional parameters that should be considered are protein “druggability” (31, 32) and vulnerability (33). Can a small molecule interfere with the process we want to target? Does inhibiting that pathway sufficiently perturb the normal activity of a cell to result in stasis or preferably death? Each drug development program defines its own criteria through which it selects suitable targets (34), but the general theme is the same: while we can use structural biology to try to determine whether we can develop an inhibitor to an enzyme (35), we cannot predict whether inhibition will have the desired biological effect in vivo. The only way to identify optimal targets is to systematically perturb relevant pathways experimentally. One way to do this is to use random mutagenesis with transposons as described below.

**Essentiality Screens as Tools for Drug Development**

An essential gene can be broadly defined as a gene whose product is required for survival in a specific environment. This is an operational definition; in fact, different mutations produce different extents of growth attenuation. Nevertheless, “essential” is a good shorthand term for those genes that encode cellular processes that are virtually indispensable for cell growth under any condition. Many of these are known sites of antibiotic action, e.g., translation, transcription, and cell wall biosynthesis. In addition to these genes that produce attenuation under the most basic conditions, there are also conditionally essential genes, whose function is dispensable under some conditions but essential in others; virulence determinants fall into this category. Genetic tests of essentiality rely on the inability to disrupt a given gene using either targeted or random approaches. Phage-mediated transposon mutagenesis has become the gold standard for random mutagenesis and has been successfully applied to all domains of life (36–38) including mycobacteria (14, 16, 19, 39). The distinct benefit of using transposon mutants rather than other approaches is the ability to interrogate the genomes of a highly complex library in a single experiment, particularly when using next-generation sequencing technologies to acquire data. At the simplest level, the frequency of gene disruption correlates with its essentiality: nonessential genes tolerate many insertions, while none are found in essential genes (see Fig. 1).

However, as pointed out earlier, gene essentiality is not a sufficiently powerful discriminator for target selection. One way to populate the list of genes of interest in a meaningful way is to focus on conditionally essential genes whose function is interwoven with pathogenesis. For example, transposon mutagenesis techniques were used to study the animal models of infection (15–18) and for the identification of genes required for survival in the macrophage (40). The results of these studies afford an eagle-eye view of the biological processes that are likely to be important during infection and provide important clues on the aspects of metabolism that should be investigated further as potential targets for intervention. Unlike screens focusing on core processes, where gene essentiality is statistically defined through the analysis of negative data (absence of specific transposon mutants in the population), conditional essentiality screens provide a direct measurement of the mutant fitness during
infection, which allows target prioritization based on the degree of the mutant’s growth or survival defect. While no specific gene identified in these studies has been selected for a concerted drug development effort to date, we already have a wealth of information to help choose possible targets and enhance the pace of drug development.

This point is illustrated very clearly by cholesterol metabolism. The Mce4 gene cluster is homologous to genes first identified during studies of mycobacterial cell entry (41) and is essential for M. tuberculosis survival in mice (15), probably due to its role in cholesterol uptake by the bacterium (42). It was later shown to be required for survival of M. tuberculosis in the macaque model of tuberculosis (18). Cholesterol is therefore an important source of carbon for M. tuberculosis during infection (43), but it is also a source of a number of intermediates, such as catechols and propionyl-coenzyme A, which are toxic if not cleared by the cell (43–46). Griffin et al. (45) successfully used transposon mutagenesis to single out genes involved in cholesterol uptake, catabolism, and central metabolism adaptations required to sustain growth on this unusual carbon source. Pathways such as this, which perform an essential function and generate toxic intermediates, could be targeted in two ways. One could simply inhibit the essential function. Alternatively, knowledge of the pathway gained from genetic studies can be exploited to inhibit steps that create toxic metabolites, leading to more dramatic drug effects.

The validity of combining transposon mutagenesis with growth conditions that mimic infection for the identification of new drug targets is further illustrated by investigations into key mediators of pH homeostasis in M. tuberculosis. Vandal et al. used this approach to single out Rv3671c, a transmembrane serine protease, as crucial for the maintenance of intracellular pH (47). Furthermore, they were also able to confirm that loss of Rv3671c compromises the ability of M. tuberculosis to establish chronic infection in mice. They proposed that Rv3671c would be a good target, despite not being essential for growth in vivo (48). Protease inhibition is one of the best-understood mechanisms of drug action, and it is therefore likely that Rv3671c may well prove to be druggable.

Engineering Drug Synergy and Limiting Drug Resistance

Another valuable use of transposon mutagenesis is to screen for synthetic lethality. Drug perturbations of a cell do not always have a unique effect on the system (49, 50). Therefore, using transposon mutagenesis in the context of drug treatment may give rise to the identification of companion targets. While not the same as synthetic lethality in the classical sense of the word, such targets could lead to potent synergistic compounds. At the simplest level, transposon mutagenesis in this context can identify prodrug activators, e.g., KatG for

**FIGURE 1** High-frequency transposon mutagenesis. (A) Any gene can have multiple potential transposon insertion sites (marked with dark gray bars). Transposon insertion is usually selected for by using antibiotic markers encoded within it and on a basic level results in the disruption of gene function. Identifying the site of insertion relies on the same principle as genome sequencing. Genomic DNA is sheared and an adaptor of known sequence is ligated to the fragments. In the case of transposon insertion site scoring, the resulting pool is amplified using primers specific for the transposon (in the simplest case these are the same for both flanks of the transposon; P1) and a primer specific for the adapter (P2). The number of reads mapping to each genomic locus is proportional to the abundance of the strain carrying this insertion. (B) The frequency of transposon insertion reflects gene essentiality. Genes that can tolerate insertions in multiple sites throughout their coding sequence are deemed nonessential. An organism cannot tolerate the disruption of an essential gene; therefore, no insertions can be detected. Genes that are not essential in a wild-type background under “normal” conditions but become essential once the system is suitably perturbed (e.g., low pH, presence of another mutation, drug treatment) provide a special case and are considered conditionally essential. Statistical methods should be used to determine whether a gene has a significantly low number of insertions. (C) More elaborate transposon architectures may include the presence of transcriptional terminators (Ω) or outward-facing inducible promoters (adapted from reference 69). Using such systems provides greater information because transposon insertion gains additional modalities that go beyond simple gene disruption. Since the directionality of insertion carries information, it is important to be able to use different primers for each transposon flank (P1L, P1R) during insertion scoring. doi:10.1128/microbiolspec.MGM2-0030-2013.f1
isoniazid and EthA for ethionamide (14). An example of a companion drug that interferes with prodrug activation is BDM31343 (29), discussed above. A systematic drug-induced conditional essentiality screen should result in the identification of multiple mediators of drug toxicity (50, 51) as well as shed light on common death pathways in the cell, provided, of course, that these enzymes do not form part of the core essential genes. Genes identified in this way would be unlikely to represent valid drug targets in their own right because they are, by definition, not essential under the screening conditions. Nonetheless they should provide compelling candidates for the identification of synergistic interactions, which in turn would have obvious benefits when devising next-generation combinations for treatment of tuberculosis.

In a similar vein, and perhaps more ambitiously, it is possible to perform transposon mutagenesis screens for true synthetic lethality in a mutant background. In this case one would first identify a novel target candidate and then perform a screen for genes whose activity becomes indispensable under conditions of diminished activity (or absence) of the primary target. Such approaches have been used successfully in the study of nutrient uptake (52) and propionyl-coenzyme A toxicity (53, 54). A systematic search may identify further interactions of a similar kind. In the first instance, one could focus on gene pairs (or networks) that can be inhibited using a single inhibitor for multiple targets—a feature that appears to be singly paramount for stemming antibiotic resistance (10, 55). Even if a single molecular entity would be impossible to generate, genetic screens of this nature could prove invaluable for devising future combination treatments. An important advantage of such a strategy is the fact that drug synergy would be built in at the developmental stages, much like β-lactamase inhibitors augmenting β-lactam antibiotic action, and not through trial and error, as was the case with trimethoprim and sulfamethoxazole. A powerful set of examples illustrating the appeal of this approach is provided by research aiming to resensitize methicillin-resistant Staphylococcus aureus (MRSA) to β-lactam antibiotics (reviewed in reference 56). Of particular interest is the identification (57) and further elaboration (58–60) of the synergistic action of β-lactams and teichoic acid inhibitors. Furthermore, in a comprehensive screen based on antisense-mediated gene silencing (61), Roemer and coworkers identified a number of auxiliary factors mediating β-lactam resistance (62) including SpsB (a signal peptidase) and the Z-ring proteins. Crucially, both of these were recently shown to be druggable, and the compounds that interfere with their function were strongly synergistic with β-lactams (63, 64).

So far we have focused entirely on the identification of targets based on genetic manipulation of cells. An alternative approach would be to use the well-established approach of whole-cell chemical screens to identify active compounds and to use genetic approaches to systematically identify their targets, ideally, concurrently with the screen for inhibitors. The key benefit of this compound-centric approach is that we are able to generate targets whose role in viability has already been validated, and we already have a chemical entity on which to build our search for inhibitors.

**USING DRUG SCREENS TO PROSPECT FOR NEW TARGETS**

One of the major advantages of whole-cell screening programs over target-based approaches is the immediate validation inherent in the experimental design. Compounds are selected based on the very clear endpoint readout of “Does it inhibit bacterial growth?” In a recent screen for antimycobacterial compounds (65), testing in excess of 100,000 compounds led to the identification of 1,549 hits. The prioritization of these for further development is currently based almost entirely on chemoinformatic approaches (66, 67). However, knowledge of a compound’s specific target accelerates the medicinal chemistry required to improve lead compounds. Experience from the pharmaceutical industry shows that approximately 5% of screening hits inhibit a specific protein (13), suggesting that a screen like the one mentioned above could identify as many as 77 targets. Identifying compounds with a specific MOA remains a resource-intensive undertaking, but a number of approaches have been developed in a multitude of systems that strive to enrich the quality and depth of information we get from a drug screen by adding a biological component beyond the simple growth inhibition endpoint. The importance of knowing the biology of a compound and its relevance for bacterial survival during infection as soon as possible cannot be overstated and is discussed later in this article.

In order to identify targets through the characterization of their inhibitors, it is essential that a methodology is available that allows the systematic, rapid, and scalable interrogation of the entire genome. The simplest approach is the established identification of spontaneous
resistance mutants. With the advent of powerful (and relatively cheap) next-generation sequencing platforms, such as Illumina, it has become feasible to rapidly identify resistance-mediating point mutations through whole-genome sequencing. An early example of the power of such an approach is the discovery of ATP synthase as the target for bedaquiline (68). When resistant mutants can be isolated, this approach has proven generally useful. However, not all compounds have a single target, and isolation of resistance mutants in cases where a single molecule affects multiple targets has very limited success, even if the constellation of mutations present within the tested bacterial population is enhanced by exposing it to ethyl methanesulfonate (EMS) prior to selection. More importantly, it is unfeasible to adapt this approach to high-throughput target identification.

More suitable approaches to fulfill the aforementioned criteria are those based on specialized libraries where either each gene in the genome is perturbed or its expression is controlled. Broadly speaking, such methodologies rely either on gene disruption through transposon mutagenesis (51, 69) and knockout libraries (13, 50, 70), or modulating expression using knockdown (71–73) and overexpression libraries (74, 75). Different approaches lead to different types of information. For example, gene disruption libraries are not compatible with the identification of essential genes (which, by definition, cannot be disrupted) and are therefore unlikely to lead to the identification of the target as such, but they have been used to describe the phenotypic landscape of a cell whose integrity has been perturbed by a drug (50) and can indirectly identify the inhibited pathway (76). As mentioned earlier, such approaches would be very well suited to identify conditionally essential targets, e.g., targets that are required specifically during infection. Similarly, they could also lead to the identification of companion drugs and consequently companion drug targets. An exception to this rule is the specific application of transposon mutagenesis to the identification of S. aureus growth inhibitors described by Wang et al. (69) and the identification of essential genes through promoter replacement in Vibrio cholerae (77). Normally, the transposon-based approaches used in such screens usually only carry an antibiotic resistance marker. Wang and colleagues modified their transposon to carry an outward-facing promoter as well as a transcriptional terminator. By doing so they can simultaneously screen for resistance mechanisms that arise through gene disruption and early termination (polar effects), as well as gene overexpression. Given that their transposon has a clearly defined directionality, they can correlate the site of transposon insertion as well as the orientation of the transposon to deduce which mechanism is driving resistance when the library is exposed to a specific drug, thus generating a multilayered biological output. However, modifying transposon sequences is not trivial, and the adaptation of their technique to mycobacteria can provide a significant challenge.

Target overexpression leads to an increase in the effective MIC for a drug, while target knockdown leads to the reverse effect. Measuring the relative enrichment or depletion of a particular strain within a highly complex population can therefore lead to the identification of the actual target for a compound—provided a single target exists. A key difference between the two is that overexpression is easily achieved by introducing an inducible copy of the gene on a plasmid that is transformed en masse into the target population (70). The generation of a knockdown library, on the other hand, requires a significantly larger resource investment because it relies on the generation of promoter replacement strains and has therefore been done in a systematic manner only in a handful of microorganisms (13, 70, 73, 78). Similarly to gene disruption libraries, expression modulation libraries could also be used to identify the broader impact of a compound on cell physiology, possibly paving the way for the identification of novel synergistic compounds. Importantly, modulating the expression of transcription and sigma factors may lead to more pleiotropic effects and could potentially give rise to the identification of multiple targets simultaneously. Another important technical distinction between overexpression and knockdown libraries is the differential ability to identify protein complexes as targets. Overexpressing a single component of a multiprotein complex is unlikely to lead to a significant change in MIC. Depleting a component, however, should lead to a shift in MIC consistent with the deletion of the target. It is important, therefore, to think of these approaches as complementary, and the choice of system should be influenced largely by the availability of the tools.

This approach has not been extensively developed in mycobacterial species. There is a wealth of proof-of-concept evidence in the literature showing that all of the above approaches could be implemented in mycobacteria (28, 33, 79–81), but there has been no real effort to date to perform such screens in a systematic manner. One of the few attempts at generating a high-throughput system to use for gene overexpression is random inducible controlled expression (RICE), but it has not been applied to the identification of potential antibiotic targets (82).
Forward Genetic Approaches (e.g. Tn, EMS, γ-radiation)

Drug target  \[ \rightarrow P \]  Select resistance  \[ \rightarrow P^* \]  Target mutation  \[ * \]  Prodrug Activator  \[ \rightarrow \]  Auxiliary factor (TF, efflux, bypass)

**Advantages:**
Minimal background knowledge required, robust and versatile, can lead to MOA and mapping of the whole drug landscape.

**Limitations:**
Hard to miniaturize, MOA requires significant amount of compound, limited information on essential genes, no information on target vulnerability.

Reverse Genetic Approaches (e.g. expression modulation)

Gene of interest  \[ \rightarrow P_{\text{inducible}} \]  Tag\(_{\text{degradation}}\)  Depletion  \[ \rightarrow \]  Target validation & vulnerability (Drug hypersusceptibility, depletion toxicity)

Depletion & Over-expression  \[ \rightarrow \]  Compound screening (Target identification)

Phenotypic drug landscape (Identify auxiliary factors)

**Advantages:**
Complete target validation (including essential genes), compatible with high-throughput screening, provides gene-specific as well as whole genome information.

**Limitations:**
Very resource- and knowledge-intensive to set up, limited by existing knowledge of genome organisation.
A different modality of this approach based on using substrate analogues, rather than growth inhibitors, could be used to identify families of targets based on the presence of a specific enzymatic activity. The benefit of performing such a screen would be to pinpoint groups of targets that could be inhibited using a single molecule, therefore potentially leading to the identification of compounds to which resistance would occur more slowly. Activity-based protein profiling approaches (83) are based on the ability to derivatize molecules such that they form a covalent bond with their target when the latter exhibits enzymatic activity against the bait. In this manner it may be possible to identify M. tuberculosis-specific homologues of targets that have been validated in other species. An example of such a study has been reported recently for the identification of ATPases in M. tuberculosis (84).

**BIOLOGICALLY RELEVANT TARGET VALIDATION**

Biological validation increases the confidence in a target and can decrease the risk associated with investing in further chemical development of compounds. The exact approach to validation varies but should take into consideration multiple parameters such as gene essentiality in vivo, target druggability, and selectivity as well as vulnerability. A cautionary tale comes from a screen that led to the identification and extensive optimization of a very promising antimycobacterial lead by Novartis—a specific glycerol kinase inhibitor. The company committed a full team to the project, achieving excellent in vitro and pharmacological results, although the compound was found to be ineffective when tested in the mouse model of infection. Its activity was shown to be specific to growth on glycerol—a carbon source regularly used for culture but not encountered by the bacterium in vivo (85). While our knowledge of biology at the time would probably not have prevented the company from pursuing the lead, one could argue that knowing the target immediately and validating it in vivo either by creating a knockout or a conditional knockout strain would have allowed the researchers to terminate the program earlier and incur fewer losses.

Another important determinant of target viability is vulnerability. This concept can be defined as the proportion of an enzymatic activity that needs to be inhibited in order to achieve growth arrest and death. In principle, more vulnerable targets, i.e., those requiring less inhibition, are more attractive targets. This cannot be known a priori, and not all essential targets are equally as vulnerable. For example, mycobacteria are exquisitely sensitive to RNA polymerase and InhA depletion, but they can withstand great levels of dihydrofolate reductase (DHFR) and alanine racemase (Alr) depletion. Alr and dihydrofolate reductase are present in great excess within cells, and mycobacteria can easily withstand almost complete depletion of the protein in the cell without significant growth attenuation (33). The targets of isoniazid and rifampin are very vulnerable, which could be one reason why these compounds form the backbone of current tuberculosis chemotherapy. Cycloserine, which targets Alr, is a less effective antibiotic (though an antibiotic nonetheless, suggesting that vulnerability is only one component in prioritizing targets).

Loss-of-function mutants are not suitable tools to assess the vulnerability of potential targets. Thus, we must use dose-dependent target depletion to quantify the extent to which a gene product is required for normal cellular function. Strains in which the levels of a gene product are controlled, through either direct promoter replacement or inducible protein degradation, are key for validating drug targets both in vitro and in vivo, and they have been used successfully in the case of, among others, isocitrate lyase, Rv3671c (81), Clp protease (86–88), and DNA gyrase B (89). In addition, glutamine synthetase (GlnA1) was shown to be essential in vitro and in vivo (90), although depletion of GlnA1 was not sufficient to arrest bacterial growth (91), therefore failing to validate GlnA1 as a target. Currently, the main obstacle to overcome for a systematic study and validation of knockdown mutants is the lengthy and technically difficult strategy to generate promoter replacement and inducible degradation strains (see Fig. 2).

Identifying potential antibiotic targets is a useful but adjunctive approach to developing new antibiotics. Ultimately, however, we need compounds that inhibit these targets. How do we proceed to getting new drugs?

**FIGURE 2** Summary of genetic approaches to studying drug targets. Both forward- and reverse-genetic approaches can be used to provide overlapping and complementary tools for the investigation of antibiotic targets. The limitations and advantages are summarized in the boxes. The asterisk refers to a mutation within the promoter or coding region resulting in drug resistance. P, promoter; Tn, transposon; EMS, ethyl methanesulfonate; TF, transcription factor; MOA, mechanism of action. doi:10.1128/Microbiolspec.MGM2-0030-2013.f2
FINDING MORE AND BETTER COMPOUNDS

As stated earlier, genome-driven large-scale screening campaigns for potent bacterial growth inhibitors to be developed into new antibiotics have yielded a disappointing number of hits and only a handful of antibiotic targets. One important drawback is that until recently, existing chemical libraries were derived from existing drug development programs. Unfortunately, because pharmaceutical development is strongly oriented toward chronic diseases, antibiotics have not been a big part of chemical synthesis campaigns. Thus, many of the compounds available in current libraries are minor modifications of compounds effective in cancer or diabetes. And the rules that govern most synthetic efforts (92) do not apply to many of the most effective antibiotics. The vast majority of potential mycobacterial targets bear little resemblance to those that are of current pharmaceutical interest, and it is likely that there will be only a limited number of inhibitors among them. Moreover, compounds must both enter the bacterial cell and avoid inactivation and efflux, properties that seem restricted to a rather small set of chemicals. Thus, increasing the chemical space among potential inhibitors could have a considerable effect on the success rate of screens in general and perhaps even target-based strategies in particular.

Significant steps have been taken to improve the quality of compound libraries used for screening (65, 93, 94). While there have been a number of hits identified during these campaigns, the problem of compound prioritization remains, delaying lead optimization in resource-limited settings. The logical progression of a screening campaign is toward a more focused approach based on specific chemical scaffolds. Picking diverse, previously underexplored chemistries is producing good results, and many of the clinically most advanced drug candidates have been initially identified starting from a select set of chemical scaffolds such as diarylquinolines (68), nitroimidazoles (2, 95), and ethylenediamine analogues (96). In addition, others, including benzo-thiazinones (97) and diphenyl-pyrroles (98), are currently going through preclinical development with very promising results.

It is important to keep in mind that focusing solely on inhibitors of exponential growth may not produce the most effective tuberculosis therapies. The clinical significance of bacterial populations recalcitrant to treatment during active tuberculosis has been appreciated for a long time, and historically, treatments were developed and evaluated with the aim to limit the rate of relapse thought to occur due to failure to clear nongrowing bacteria (99, 100). Two drugs were shown to be particularly successful in reducing the rate of relapse following isoniazid treatment: rifampin and pyrazinamide (101). The latter was also shown to greatly improve the early bactericidal activity of multiple drug combinations (102), leading to a higher proportion of culture-negative patients 2 months into treatment when compared to combination therapies without it (103). In fact, pyrazinamide is one of the four drugs currently administered during the directly observed treatment for tuberculosis endorsed by the World Health Organization (WHO) (104). It is also likely to remain relevant for future combination therapies because it greatly improves the efficacy of both bedaquiline and PA-824 (105). However, pyrazinamide itself is virtually inactive against actively growing M. tuberculosis under routine culture (and screening) conditions and would therefore not be detected under current screening approaches. Unfortunately, the mechanism of pyrazinamide action remains elusive (106, 107), limiting its use as a tool for identifying new drug targets. Perhaps the most important aspect of pyrazinamide as a drug is its ability to kill M. tuberculosis that is not actively growing (108). This is an area of tuberculosis drug discovery that has received increasing attention (e.g., bedaquiline and PA-824 are both active against nongrowing bacilli), and exploring it further is essential to guarantee the success of future treatment regimens that rapidly eradicate reservoirs of persistent bacteria.

Expanding Screening Conditions

As discussed above, the in vitro conditions that produce maximal bacterial growth rates do not necessarily reflect those found during infection (109). It is clear that nutrient sources, pH, and oxygen availability might all differ. Moreover, treatment occurs in the presence of host immunity, which might itself alter responsiveness to antibiotics; e.g., vitamin D synergizes with pyrazinamide to improve macrophage killing of M. tuberculosis (110).

Several groups have tried to replicate facets of the in vivo growth milieu in vitro (111). For example, nutrient limitation can be modeled with starvation, incubating cells in the absence of any carbon source (112), gradually depleting nutrients (113), or combining carbon limitation with other stresses such as low oxygen tension and low pH (114). Bacteria can be grown with alternative carbon sources, such as lipids (115), at low oxygen tension (116–118), under acidic conditions...
Conditions mimicking the in vivo environment produce different physiologic responses, but one common characteristic they share is slowing or stopping bacterial replication. An alternative to many different experimental conditions would be to prevent or limit replication using genetic rather than physiologic manipulation, as has been done using a streptomycin-dependent strain of M. tuberculosis (124, 125). Bacteria in this model show a modified drug susceptibility profile, suggesting that they could provide an important tool in identifying the next generation of antibiotics whose activity would be exerted through the disruption of growth processes. Therefore, using attenuation of gene expression would achieve two goals. In the first instance, compounds that specifically inhibit strains under-expressing a gene of interest, but not a nonrelated control, would already demonstrate a potential for target specificity. Second, by using hypersusceptible strains, we could identify inhibitors whose initial activity is too weak to be identified in screens where wild-type cells are used, therefore allowing the screeners to explore the part of chemical space populated by weak but specific inhibitors. At the end of such a campaign we should have a number of hits that appear to have a specific activity against biologically validated targets—thus combining the advantages of target-based approaches, namely target specificity with whole-cell activity inherent in empirical approaches.

The viability of such approaches to identify novel inhibitors has already been extensively demonstrated in S. aureus (71, 72, 128) and Candida albicans (73). In these settings researchers were able to screen entire libraries of under-expressing strains against whole chemical libraries and successfully identify inhibitors of growth and their MOA in a single screen, greatly reducing both the time and resources necessary for selecting interesting hits. The first steps providing proof-of-concept studies toward applying this approach to mycobacteria have been made already: Abrahams and coworkers used promoter replacement to decrease the expression of the dfrA gene in M. tuberculosis (130). They then used the panC knockdown strain in a target-based whole-cell screen (TB-WCS) of a small compound library (600 compounds) to identify 37 hits. The authors also used specific culture conditions to maximize the susceptibility of the panC mutant strain. Kumar et al. took a similar approach focused on dfrA (130). They first screened a library of 32,000 compounds in an in vitro system and then used the hits to screen against a DfrA-depleted strain. They were able to identify 52 primary hits and only 3 confirmed hits (showing suitable dose-response kinetics), with a single confirmed lead with specific DfrA activity in M. tuberculosis. This hit rate is consistent with those previously reported for strategies using biochemical screens as starting points (11). The choice of target may have been unfortunate since DfrA is not a very vulnerable target in mycobacteria (see above).
CONCLUDING REMARKS

Every successful drug development program has a specific target product profile that serves as a roadmap to guide the developmental effort toward the ultimate goal. A similar list of desirable properties could be drawn up for the ideal drug target. A target should be vulnerable, as defined in this article, and druggable, with the potential for good structure-activity relationship analysis to facilitate the development of next-generation drugs in the same vein as β-lactams. We should aim to identify groups of interconnected drug targets whose inhibition has a synergistic effect. The goal would be to generate tailored combinations of drugs that would limit the evolution of drug resistance as well as decrease the necessity for high doses of drug to achieve inhibition. The former would improve the shelf life of new treatments, while the latter would contribute significantly toward their safety profile. It may even be possible to assemble multiple such combinations. We should aim to find targets whose mutation carries a great fitness cost in the presence of its companion drug, either by penalizing the resistance mechanism itself, by blocking metabolic bypass routes, or by any other mechanism.

The starting point could be a validated drug target or a compound with a highly desirable pharmacological profile. By harnessing the combination of genetic approaches described here and using whole-cell-based screens mimicking the native environment found during infection, we should be able to identify companion drugs and drug targets that fit the above criteria.

It could be argued that detailed biological knowledge is not essential for the development of novel antibiotics. A whole suite of antimycobacterials was developed in the absence of such knowledge. While this is true for current drugs, the incorporation of biological goals can provide the right conceptual framework to facilitate the progression of future drug development programs. Furthermore, if biological considerations form the backbone of a project from the earliest stages of drug development, it may be possible to identify a constellation of targets whose vulnerability could be renewed in the face of drug resistance.

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REFERENCES

Genetic Strategies for Identifying New Drug Targets


