REVIEWS

Drugs for bad bugs: confronting the challenges of antibacterial discovery

David J. Payne, Michael N. Gwynn, David J. Holmes and David L. Pompliano

Abstract | The sequencing of the first complete bacterial genome in 1995 heralded a new era of hope for antibacterial drug discoverers, who now had the tools to search entire genomes for new antibacterial targets. Several companies, including GlaxoSmithKline, moved back into the antibacterials area and embraced a genomics-derived, target-based approach to screen for new classes of drugs with novel modes of action. Here, we share our experience of evaluating more than 300 genes and 70 high-throughput screening campaigns over a period of 7 years, and look at what we learned and how that has influenced GlaxoSmithKline's antibacterials strategy going forward.

Infectious Diseases Centre of Excellence for Drug Discovery, GlaxoSmithKline, 1250 South Collegeville Road, Collegeville, Pennsylvania 19426, USA. Correspondence to D.L.P.: e-mail: David.L.Pompliano@gsk.com
doi:10.1038/nrd2201
Published online
8 December 2006

Antibiotic discovery is not very fashionable these days, and yet resistance has evolved to every antibiotic ever placed into clinical practice, irrespective of the chemical class or molecular target of the drug. Despite various bacterial threats to public health (multiply drug-resistant strains, emerging pathogens and biothreat organisms), most large pharmaceutical companies and many biotechnology companies have left the area. Many factors contributed to this exodus, but the fact remains that a better return on investment can be made in other disease areas (at least based on commercial analysis and forecasting). Doubtless the strict regulatory requirements and the competitive commercial environment figures prominently in the calculus, especially for public companies that have responsibilities to shareholders[1–8]. What might be less well appreciated is just how difficult it is technically, and how much time it takes (according to statistics from the Centers for Medicines Research; see Further information), to make a novel antibiotic (FIG. 1). Converting an early chemical prospect into a medicine that can be used in people is a profound scientific challenge, the difficulties of which are not going to be mitigated by a change in the commercial landscape or public policy. The corporate withdrawal has not only forsaken the antibacterial pipeline but has also greatly diminished the overall capability to generate novel antibacterials. The current portfolio of compounds in clinical trials consists largely of derivatives of chemical classes for which there are already underlying resistance mechanisms — hardly the pharmaceutical firepower needed to face bacteria that are evolving on a timescale of hours. Although the emergence of resistant strains is unpredictable, it is inevitable, and we must be prepared. Excluding the resistance-mediated decline in efficacy, current antibiotics have side effects, difficulties with dosing regimens and restrictions on use, particularly for children, that constrain their utility. There is still a great need[5,8], and commercial opportunity, for novel antibacterials.

By the mid-1990s, there was little enthusiasm for making yet another incremental improvement to a β-lactam, macrolide or quinolone. Then, in 1995, the determination of the complete DNA sequence of a bacterial genome from *Haemophilus influenzae* changed everything. The prospect of hundreds of new genes to explore as possible targets sparked new interest in antibacterial discovery and fired the imagination. Embracing the genomics approach, GlaxoSmithKline (GSK) spent 7 years (1995–2001) evaluating more than 300 genes for their potential as targets for novel antibacterials and showing genetically that more than 160 of them are essential. In total, 70 high-throughput screening (HTS) campaigns of individual targets, complete macromolecular biosynthetic pathways and whole-cell screens were run against our synthetic chemical collection at that time. Our aim was to find a novel antibacterial compound that had either Gram-positive or broad-spectrum activity. Now it is time to take stock of what was achieved, to understand what our experience taught us about the antibacterial discovery process and to explain how those lessons influenced our strategy to develop novel antibiotics.

Discovery approaches

Exploiting genomics. The most clinically relevant antibacterials inhibit a very short list of cellular targets. The quinolones block DNA gyrase (and topoisomerase IV); the macrolides, tetracyclines and aminoglycosides inhibit ribosomal function; and the β-lactams shut down cell-wall biosynthesis. These antibiotics are classified by their chemical structure, as opposed to the target they

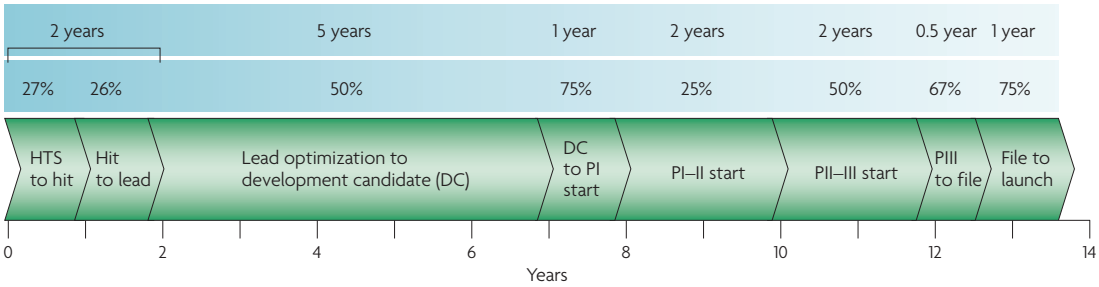


Figure 1 | **Estimated success metrics and timelines for the development of a systemic broad-spectrum antibacterial.** The figure shows timelines and risks associated with antibacterial drug discovery. The probability of success (percentages) up to the beginning of Phase I trials are based on GlaxoSmithKline (GSK) metrics and the clinical success rates are based on industry averages (data taken from reports by the Centers for Medicines Research (CMR); see Further information). Lead optimization is the most challenging and lengthy phase in antibacterial drug discovery. Probability of success during lead optimization is strongly linked to the size of the medicinal chemistry effort devoted to the project. GSK committed large teams of chemists for an uninterrupted period of 5 years to achieve a 50% success rate. Lead optimization efforts with less resources could easily take considerably longer.

inhibit. Incremental structural modification of these agents, although historically the method of choice, does not fundamentally change their interaction with the target. Although new derivatives can improve efficacy temporarily, the underlying resistance mechanisms, whether they are target-based, efflux or enzyme-mediated, are still present in the environment.

Besides the promise to unveil a treasure trove of new targets, genomic information built the case for pursuing known, but underexploited, targets. DNA sequence comparison of the genomes of different (but relevant) Gram-negative (*H. influenzae* and *Moraxella catarrhalis)* and Gram-positive (*Streptococcus pneumoniae*, *Staphylococcus aureus* and *Enterococcus faecalis)* pathogenic bacteria revealed genes that were highly conserved. Their commonality across such phylogenetically diverse species suggested canonical functions required for survival of bacteria in general rather than just species-specific roles. Using the *S. pneumoniae* genome as the primary comparator, genes that were in common across five pathogens with highly conserved amino-acid sequences and that also only occurred as single copies (to avoid potential resistance mechanisms) were designated as potential broad-spectrum targets[9]. To favour uniquely bacterial targets, those genes with a close human homologue were eliminated from further consideration.

The sequence analysis provided a hypothesis of essentiality that was tested genetically. Using allelic-replacement mutagenesis, we swapped the target gene with an antibiotic resistance marker (FIG. 2a), being careful not to disrupt the expression of downstream genes[10]. The absence of growth of organisms lacking a target gene strongly suggests, but does not guarantee, that the gene is required for viability. Growth in the absence of the target gene, however, marked the gene as being dispensible for survival, and therefore not very attractive as a target. As some genes were essential in *S. aureus* but not so in other pathogens and vice versa, the gene knockout must have failed to grow in at least two Gram-positive organisms before we advanced the target. Observing how growth varied with the level of gene expression was the second level of analysis used to assess the relative importance of the target gene to viability[11–13]. We modulated the target gene expression using inducible promoters in two different ways: directly, by placing the target gene under control of the promoter (FIG. 2b); and indirectly, by controlling the expression of antisense RNA that hybridized to and blocked transcription of the target gene (FIG. 2c). Decreased expression that led to impaired growth further supported the importance of the target gene to survival. These constructs also provided tools to confirm the mode of action (MOA) of hits from HTS: decreased expression of the putative target led to increased sensitivity to growth inhibition by the compound and vice versa.

More than 350 *S. pneumoniae*, *S. aureus* and *H. influenzae* candidate target genes from a broad variety of pathways and processes were identified from the comparative sequence analysis (FIG. 3a). Of these, 127 genes were identified as essential *in vitro* in at least one organism. Those genes for which no allelic-replacement mutants could be isolated (despite repeated attempts) remained candidates for further analysis. Almost 100 allelic-replacement mutants were tested in an animal model of virulence and 64 targets were identified that were not essential *in vitro* but which attenuated growth *in vivo* (FIG. 3b). Progression of these targets introduces additional complications. For example, it was not immediately obvious how to evaluate inhibitors of these targets, as by definition they would not be active in standard *in vitro* antibacterial assays. Evaluating leads in *in vivo* infection models was a possible way forward, but would have required intensive additional resource. Broad-spectrum targets of unknown function[14], although clearly offering novelty, likewise carried the liability of finding a biochemical function to assay. As we had a wealth of *in vitro* essential targets that *did* have well-defined, assayable functions, neither virulence targets nor targets of unknown function were taken forward on the basis that life was hard enough without the added complexities of these targets.

There has been no mention of medicinal chemistry so far. In fact, biology dominated the workload of our

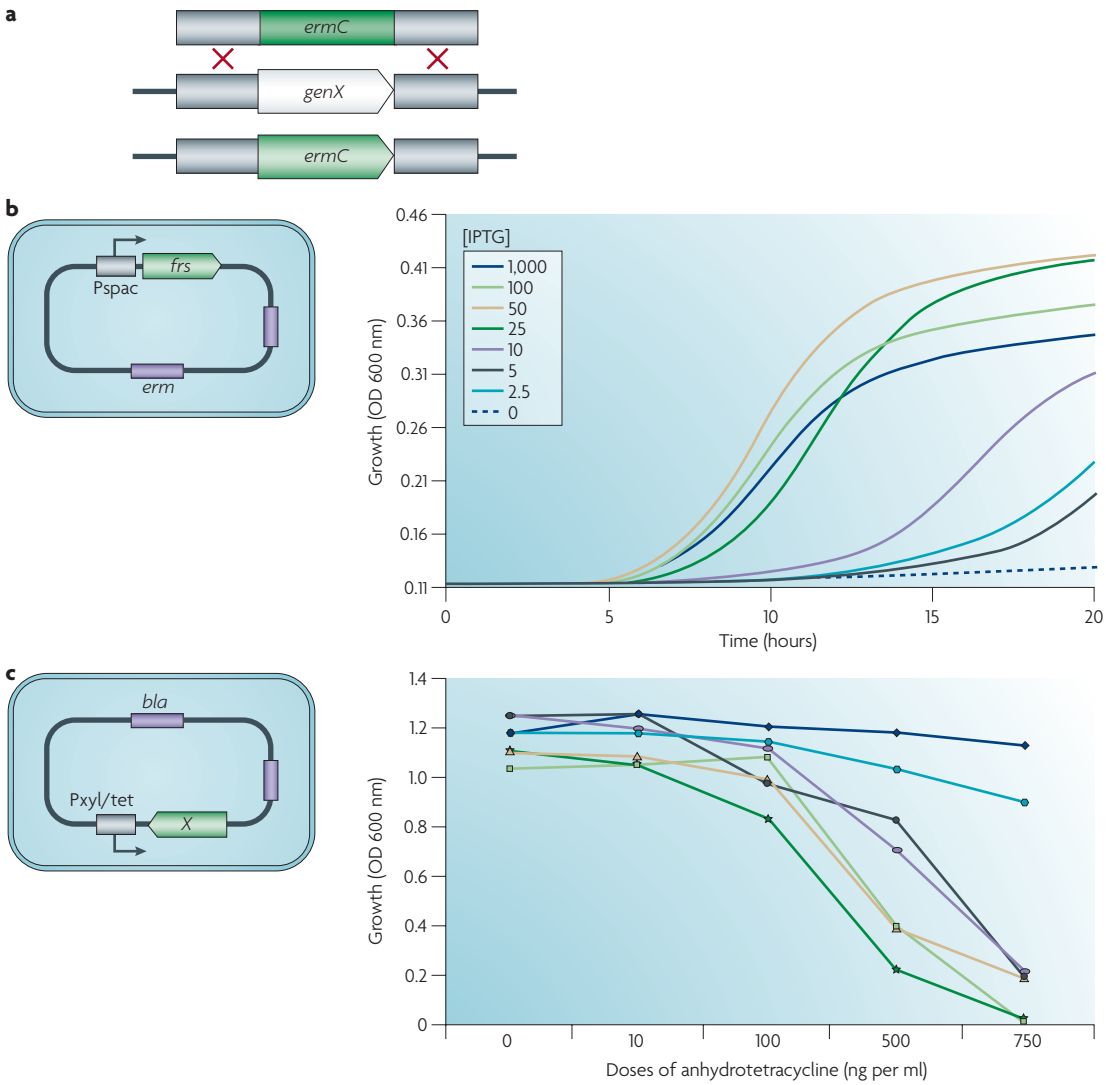


Figure 2 | **Method for testing gene essentiality. a** | Allelic-replacement mutagenesis was performed by transforming *Streptococcus pneumoniae* with linear DNA fragments and selecting for recombinants resistant to the antibiotic marker (ErmC). The result was a swap of the target gene (genX) for a selective marker. **b** | Genes in *Staphylococcus aureus* can be placed under the control of inducible promoters to confirm gene essentiality. IPTG (isopropyl-β-D-thioglactopyranoside) was used at various concentrations to induce expression from the Pspac promoter. **c** | Antisense RNA can be used to downregulate expression of genes both *in vitro* and *in vivo*. The upper blue line is the strain containing the control plasmid alone with no insert. All others represent growth of strains expressing antisense RNA to different gene fragments. For details see REF. 34.

genomics-based discovery. The ratio of biologists to chemists in the antibacterial discovery group was 2:1 during this time. For example, establishing the technology and methodology to regulate gene expression in pathogens, ensure expression occurred *in vivo* and develop approaches to enable single allelic replacements to be performed in just 10 days was only accomplished with a huge initial investment of biology resource. Reagent generation and preparation for the numerous HTS campaigns required many more biochemists and enzymologists. Much effort was spent on the earliest, and most biology intensive, phase of discovery: establishing the credentials of targets. As we shall see, it had limited impact on producing antibacterial development candidates of sufficient quality.

Antibacterial development candidate
A compound that achieves target antibacterial activity *in vitro* (MIC90s) and *in vivo* (infection models), shows a viable therapeutic window based on rodent toxicity, and has physical and pharmaceutical properties suitable for preclinical GLP toxicology studies.

Outcome of target-based HTS campaigns. Compared with mammalian targets, antibacterial targets moved quickly into HTS. Preparing reagents in quantity for the HTS rarely was an obstacle. Cloning and expression of a bacterial gene in a bacterial host is far less involved than finding the proper gene–vector–host combination to express a mammalian gene. In most instances, just 10 litres of bacterial culture yielded enough protein for screening and follow-up assays. Most of the targets were intracellular enzymes for which a broad range of assay methodologies and formats exists. Only 10% of targets failed to reach HTS because of problems associated with generating sufficient active protein(s) or substrate, poor signal-to-noise ratio for the assay or

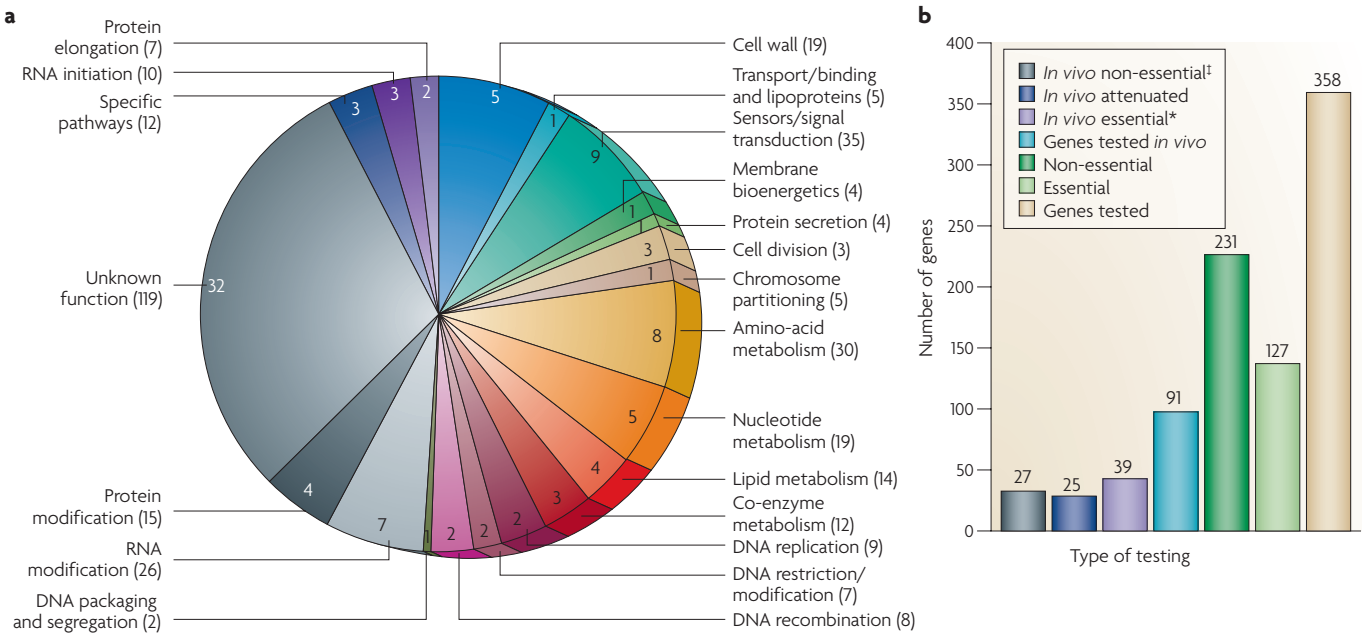


Figure 3 | **Results of gene essentiality testing in *Streptococcus pneumoniae*. a** | The chart shows 358 *S. pneumoniae* genes that were tested for essentiality by allelic-replacement mutagenesis. Figures in brackets show the number of genes tested, and figures in the pie-chart slices represent the percentage of total genes examined. **b** | Genes were considered likely to be essential if they could not be deleted by allelic replacement following several attempts. Some allelic-replacement mutants were examined in a rat respiratory-tract infection model[33]. Two days post-infection, the colony-forming units of the mutants in lungs was determined and compared with wild type. Mutants that were attenuated in the animal model by 4–6 logs (shown by *) are here considered to be *in vivo* essential. Mutants that were attenuated by <2 logs (shown by ‡) are non-essential *in vivo*.

because the assay could not be miniaturized to a robust HTS format. Other therapeutic area targets had higher attrition rates.

Between 1995 and 2001, 67 HTS campaigns on antibacterial targets (TABLE 1) were run against the SmithKline Beecham compound collection (which consisted of 260,000–530,000 compounds). This was an unprecedented concentration of screening resource for a single therapy area. Some targets were screened more than once, because either a better assay was developed or additional chemical diversity was subsequently added to the collection.

A mere 16 HTS gave rise to hits, and only 5 of these resulted in leads (TABLE 1). For the remaining hits, chemical modification failed to generate molecules that met the lead criteria. TABLE 2 illustrates the two fates of these hits. Further derivatization of the undecaprenyl (UDP)-*N*-acetylglucosamine-enolpyruvyl reductase (MurB) lead to neither increased inhibitory potency against the target nor introduced antibacterial activity. Although both of these properties were achieved for ribonuclease P (RNaseP), the antibacterial activity could not be mechanistically linked to inhibition of the enzyme. These hits turned out to be non-specifically toxic to both mammalian and bacterial cells, usually as a result of indiscriminate cell-membrane disruption.

Success in finding lead compounds from the genomics-based efforts was only realized for peptide deformylase (PDF), enoyl-acyl carrier protein reductase (FabI), 3-ketoacyl-acyl carrier protein III (FabH), methionyl tRNA synthetase (MetRS) and phenylalanyl-tRNA synthetase (PheRS) targets. Highly potent inhibitor series for MetRS and FabI with potent antibacterial activity were synthesized[15–17] (TABLE 2), but these turned out to be select-spectrum agents lacking activity either against a suitable range of Gram-positive pathogens or against *S. pneumoniae* and Gram-negative pathogens. Although we were able to enhance the antibacterial activity of the FabH leads, combining this with the necessary drug-like properties proved unattainable. Because the PDF leads were similar to those already identified by other groups[18] they were set aside within GSK in favour of novel leads identified by structure-based design approaches for this target; PheRS leads are still being pursued. Although HTS and chemical modification of hits did generate novel early-stage leads with potent antibacterial activity, their bacterial spectra were very limited. The level of success was unsustainably low in relation to the large effort invested. Later in the review, we discuss how insufficient or improper molecular diversity of the compounds screened was a primary reason for this lack of success.

Genomics blind spots. Apart from the difficulty of obtaining leads, blind spots in genomic analysis led to other surprises. Targets identified by genomics are only truly validated in those particular strains for which sequence comparisons were made and in which genetic knockouts of the putative target were generated. For example, MetRS was selected as a target based on the high sequence homology between the strains of

S. pneumoniae, *S. aureus* and *Escherichia coli* that were sequenced at that time. In the clinic, however, there is considerable variation in the homology of this target within a species. Although the MetRS inhibitors were potent against strains of *S. aureus* and *Enterococcus* sp., the minimum inhibitory concentrations (MICs) of the MetRS inhibitors for a panel of *S. pneumoniae* strains showed a bimodal distribution[19]. As it turned out, some strains of *S. pneumoniae* contain two different genes whose gene products catalyse the same tRNA amino-acylation reaction, only one of which was inhibited by the potent series of MetRS inhibitors that were elaborated from HTS hits. Genomic analysis of the five *S. pneumoniae* genomes available to us at that time was not predictive of the presence of a non-homologous gene that encodes a protein with the same activity as the target gene. Indeed, without the complete genomic sequences of all the various strains of a species, it is impossible to know whether the target gene is found in all strains of the species.

Then there is the case of duplicate genes that have a synthetically lethal relationship. As the target of the clinically useful antibiotic fosfomycin, UDP-*N*-acetylglucosamine-enolpyruvyl transferase (MurA) is pharmacologically validated and worthy of further exploitation. Our work showed that several Gram-positive genomes actually have two *murA* genes, both of which are essential. A compound must inhibit both Mur enzymes in order to kill the organism, a feature that can be more difficult to design or discover from screening (see above)[20].

FabI, an enoyl-ACP-reductase, was known to catalyse a reaction essential for fatty-acid biosynthesis in *E. coli*. When GSK started work on this target in 1995, it was assumed to have the same role in all eubacteria and therefore to be a suitable target for a broad-spectrum agent. However, genes unrelated to *fabI* were discovered that encoded the enoyl-ACP reductase function in other organisms. In *S. pneumoniae*, for example, FabK is responsible for this activity. An inhibitor of FabI would therefore not affect the viability of *S. pneumoniae*, a key respiratory pathogen. Because of the differential essentiality of a given gene among different strains or species[21], genomic analysis must be interpreted carefully when considering which target to select for a broad-spectrum agent. Despite this lack of spectrum, optimization of the hits from the FabI screen was highly successful in producing compounds with potent antibacterial activity (MIC <0.001 μg per ml) against multi-resistant *S. aureus* (TABLE 2)[16,17].

Outcome of whole-cell screening strategies. The lack of tractable leads, together with the known difficulty of converting compounds lacking whole-cell activity into ones with such activity, led us to also run 'empirical' (non-target based) whole-cell antibacterial screens. Although less sensitive than molecular screens, they do interrogate all targets in their physiological context simultaneously, and they select for antibacterial activity from the outset. Compounds can affect the growth of bacteria in a variety of ways, so a secondary assay to eliminate the non-specific nuisance compounds is also required. To drive the lead optimization process, the molecular target must be known, and so rapid and reliable methods for determining the MOA are key to the approach. Assays of macromolecular synthesis (RNA, protein, DNA, peptidoglycan and fatty acids), which comprise more than 70% of druggable targets, were devised for multiple pathogens in high-throughput microtitre format. The techniques of genomics — gene overexpression and underexpression (for example, antisense), resistant mutant analysis and microarray technologies — were applied to investigate the molecular targets of hits[22].

Two empirical antibacterial screens were run, one with the wild type antibiotic sensitive strain *S. aureus* RN4220 and one with a wild-type (efflux competent) strain of *E. coli* (TABLE 1). Up to ~500,000 synthetic compounds were screened at a concentration of 10 μM. The *E. coli* screen did not yield exploitable hits and many nuisance compounds were encountered. The *S. aureus* screen yielded thousands of antibacterials, of which approximately 300 possessed antibacterial activity against *S. aureus* and one other Gram-positive or Gram-negative pathogen, and which were judged at that stage to be chemically tractable. The great majority of these, however, were subsequently ruled out as non-specific membrane-active agents (detergents and uncouplers) by use of a red-blood-cell lysis assay and a *S. aureus* membrane depolarization assay[22]. Such compounds were often lipophilic, positively charged compounds, illustrated by two classes of compounds studied in detail from GSK's whole-cell *S. aureus* screen, the quinoline methanols and the tetrahydro-β-carbolines. Both classes had good antimicrobial activity, with MICs against many Gram-positive and Gram-negative bacteria in the range of 0.125–4 μg per ml. There was no preferential inhibition of a particular macromolecular process (DNA, RNA, protein, cell wall and fatty acids), and no cross-resistance was seen to known target-based resistance mechanisms. However, antibacterial activity was eventually attributed to nonspecific membrane interactions as shown by whole-cell depolarization activity and erythrocyte lysis activity, which correlated with antibacterial activity for analogues in each series. We were able to map quinoline methanol-resistant mutants in *S. pneumoniae* to the *atpC* subunit of the F_0F_1 ATPase, a target limited in essentiality to selected pathogens including some streptococci and *Mycobacterium tuberculosis*[23]. Neither of these classes were further investigated for Gram-positive or broader-spectrum agents as their nonspecific membrane-based mechanisms of action would not deliver exploitable agents across the necessary spectrum of pathogens.

A thiazolidinedione class of compounds showed good antimicrobial activity against Gram-positive bacteria[24], but the MOA could not be ascertained despite considerable effort. The class was not membrane active, assays of macromolecular synthesis failed to reveal any clues, resistant mutant analysis only identified a transporter, and 'ligand fishing' with immobilized inhibitor was unsuccessful. With no MOA, a lack of activity against Gram-negative pathogens and discouraging developability characteristics, a path forward could not be established.

Table 1a | **Outcomes of 70 HTS campaigns of antibacterial targets between 1995–2001**

Target	Homologue	Success of HTS (outcome)
Fatty-acid synthesis		
Phosphopantetheine adenylyl transferase	*S. pneumoniae*	No hits
Acyl carrier protein synthase	*S. pneumoniae*	No hits
Biotin ligase (BirA)	*S. aureus*	No hits
β-Ketoacyl-acyl carrier protein synthase III (FabH)	*S. pneumoniae*	Leads (progressable lead identified)
Enoyl-acyl carrier protein reductase (FabI)	*S. aureus*	Leads (progressable lead identified)
Coupled FabH–FabG	*S. pneumoniae*	Hits (derivatives made, but no leads identified)
Acetyl co-enzyme A carboxylase	*S. aureus*	Hits (derivatives made, but no leads identified)
DNA replication		
DNA polymerase IIIα (DnaE)	*S. aureus*	No hits
Type I topoisomerase	*E. coli*	No hits
Gyrase-dependent DNA replication	*E. coli*	Hits (derivatives made, but no leads identified)
Gyrase-dependent DNA replication	*H. influenzae*	No hits
DnaB helicase	*S. aureus*	No hits
PcrA DNA helicase	*S. aureus*	Hits (derivatives made, but no leads identified)
Protein modification		
Peptide deformylase	*S. aureus*	Leads (progressable lead identified)
Methionine aminopeptidase	*S. aureus*	Hits (derivatives made, but no leads identified)
Two-component signal transduction		
CheY-CheA chemosensor	*E. coli*	Hits (derivatives made, but no leads identified)
Protein elongation		
Ribosomal protein S8–16S rRNA helix 21 interaction	*S. aureus*	No hits
Global transcription–translation assay	*E. coli*	No hits
Global transcription–translation assay	*E. coli*	No hits
Decoding region (aminoglycoside displacement)	*E. coli*	No hits
Peptidyltransferase binding assay	*E. coli*	No hits
Protein termination		
Peptidyl tRNA hydrolase	*S. pneumoniae*	No hits
Peptidyl tRNA hydrolase (rescreen)	*S. pneumoniae*	No hits
RNA elongation		
Bacterial RNA polymerase	*S. aureus*	No hits
Ribonuclease P	*E. coli*	Hits (derivatives made, but no leads identified)
Ribonuclease P	*S. aureus*	No hits
Cell division		
FtsH ATP-dependent protease	*S. aureus*	No hits
FtsZ tubulin-like protein	*S. aureus*	Hits (derivatives made, but no leads identified)
FtsA–FtsZ protein interaction	*S. aureus*	No hits
Glycolytic pathway		
Transketolase	*S. pneumoniae*	No hits
Transketolase (rescreen)	*S. pneumoniae*	No hits
Amino-acid synthesis		
Chorismate synthase	*S. pneumoniae*	No hits
5-Enolpyruvoylshikimate-3-phosphate synthase	*S. pneumoniae*	No hits

The minimum definition of a 'hit' was a chemically tractable, low-micromolar inhibitor of the target and, where appropriate, at least tenfold selectivity against the mammalian version of the target. A 'lead' was a 'hit' that also had antibacterial activity, together with evidence that the mechanism of antibacterial activity was via inhibition of the target screened. No 'leads' were directly identified from HTS. In all cases, the leads evolved following chemical modification of 'hits'. FabG, 3-ketoacyl-acyl carrier protein reductase; HTS, high-throughput screening.

Table 1b | **Outcomes of 70 HTS campaigns of antibacterial targets between 1995–2001**

Target	Homologue	Success of HTS (outcome)
Protein secretion		
SecA subunit of preprotein translocase	*E. coli*	No hits
Signal peptidases	*E. coli*	Hits (derivatives made, but no leads identified)
Signal peptidases (rescreen)	*S. aureus*	No hits
UMP kinase inhibitor	*S. aureus*	No hits
Cell-wall synthesis		
Undecaprenyl (UDP) pyrophosphate synthetase	*S. aureus*	No hits
Penicillin-binding protein-2′ (PBP-2′)	*S. aureus*	No hits
UDP-*N*-acetylglucosamine-enolpyruvyl reductase (MurB)	*S. pneumoniae*	No hits
UDP-*N*-acetylglucosamine-enolpyruvyl transferase (MurA)	*S. pneumoniae*	No hits
UDP-*N*-acetyl muramyl:L-alanine ligase (MurC)	*S. pneumoniae*	No hits
N-acetylglucosamine-1-P uridyl transferase (GlmU)	*S. aureus*	No hits
N-acetylglucosamine-1-P acetyl transferase (GlmU)	*S. aureus*	No hits
N-acetylglucosamine-1-P acetyl transferase (GlmU) (rescreen)	*S. aureus*	No hits
Serine β-lactamase	P99	No hits
Metallo β-lactamase	CfiA	No hits
tRNA synthetases		
F tRNA synthetase	*S. aureus*	
M tRNA synthetase	*S. aureus*	Leads (progressable lead identified)
Y tRNA synthetase	*S. aureus*	Leads (progressable lead identified)
W tRNA synthetase	*S. aureus*	Hits (derivatives made, but no leads identified)
V tRNA synthetase	*S. aureus*	Hits (derivatives made, but no leads identified)
V tRNA synthetase (rescreen)	*S. aureus*	No hits
C tRNA synthetase	*S. aureus*	No hits
D tRNA synthetase	*S. aureus*	No hits
H tRNA synthetase	*S. aureus*	No hits
I tRNA synthetase	*S. aureus*	No hits
K tRNA synthetase	*S. aureus*	No hits
L tRNA synthetase	*S. aureus*	No hits
N tRNA synthetase	*S. aureus*	No hits
P tRNA synthetase	*S. aureus*	No hits
S tRNA synthetase	*S. aureus*	No hits
G tRNA synthetase	*S. aureus*	No hits
T tRNA synthetase	*S. aureus*	No hits
A tRNA synthetase	*S. aureus*	No hits
E tRNA synthetase	*S. aureus*	No hits
R tRNA synthetase	*S. aureus*	No hits
Whole-cell antibacterial assays		
Whole cell antibacterial	*S. aureus*	No hits
Whole cell antibacterial	*E. coli*	No hits
Whole cell antibacterial (rescreen)	*S. aureus*	Three hits (derivatives made, but no leads identified)

The minimum definition of a 'hit' was a chemically tractable, low-micromolar inhibitor of the target and, where appropriate, at least tenfold selectivity against the mammalian version of the target. A 'lead' was a 'hit' that also had antibacterial activity, together with evidence that the mechanism of antibacterial activity was via inhibition of the target screened. No 'leads' were directly identified from HTS. In all cases, the leads evolved following chemical modification of 'hits'. FabG, 3-ketoacyl-acyl carrier protein reductase; HTS, high-throughput screening.

Like biochemical screening of individual antibacterial targets, the success rate of whole-cell antibacterial screening was disappointing. Sorting through the high number of nuisance compounds to define whether the antibacterial activity was a result of either a nonspecific (and potentially cytotoxic) mechanism or a specific target was too burdensome. Given the genetic and biochemical tools available, finding compounds worth studying that were not simply nonspecific cytotoxics was a larger problem than actually elucidating their MOA. The inability of this approach and the target-based strategy to find good lead molecules was probably a consequence of the lack of chemical diversity screened at that time.

Conclusions from GSK's HTS-based approach. From the 70 HTS campaigns run between 1995–2001 (67 target based, 3 whole cell), only 5 leads were delivered, so that, on average, it took 14 HTS runs to discover one lead. Based on GSK screening metrics, the success rate from antibacterial HTS was four- to five-fold lower than for targets from other therapeutic areas at this time. To be sure, this was a disappointing and financially unsustainable outcome, especially in view of the length of time devoted to this experiment and considering that costs per HTS campaign were around US$1 million. Furthermore, multiple high-quality leads are needed given the attrition involved in the lead optimization and clinical development processes required to create a novel antibiotic.

GSK was not the only company that had difficulty finding antibacterial leads from HTS. A review of the literature between 1996 and 2004 shows that >125 antibacterial screens on 60 different antibacterial targets were run by 34 different companies[25]. That none of these screens resulted in credible development candidates is clear from the lack of novel mechanism molecules in the industrial antibacterial pipeline. We are only aware of two compounds targeting a novel antibacterial enzyme (PDF) that have actually progressed as far as Phase I clinical trials, and technically speaking PDF was identified as an antibacterial target well before the genome era. Potential reasons for the poor success rate of antibacterial HTS are discussed below.

Difficulty at this early stage — screening and finding leads — is only the beginning. Even when a lead is identified it can take many years and the synthesis of thousands of derivatives to identify a molecule that has all of the necessary antimicrobial and pharmaceutical properties to warrant testing in humans. For example, one of our series has taken a team of at least 20 chemists more than 5 years to create a broad-spectrum development candidate for the treatment of respiratory community infections.

So why is the lead optimization phase of antibacterials so challenging? Consider that an ideal novel antibacterial to treat community respiratory infections will need to cover seven key pathogens that cause such infections (*S. pneumoniae*, *H. influenzae*, *S. aureus*, *Moraxella catarrhalis*, *Mycoplasma pneumoniae*, *Chlamydia pneumoniae* and *Legionella pneumophila).* To treat such infections, a single compound must inhibit the growth of many different Gram-positive and Gram-negative bacterial species, all of which have different molecular targets, different membrane permeabilities and different metabolic pathways. Making such a compound is a profound chemical challenge: consider that Gram-positive *S. pneumoniae* and Gram-negative *H. influenzae* bacteria share less in common genetically than do humans and paramecia[26]. Moreover, it must also demonstrate an acceptable side-effect profile at the high blood levels typically required to ensure effectiveness against the least susceptible organisms. For example, there is a 1,000-fold difference in peak drug concentration in human plasma between sumatriptan (Imitrex; GSK), a drug for treating migraine, and the antibiotic combination of amoxicillin and clavulanic acid (Augmentin; GSK). (For more information see Drugs @ FDA in Further information.) Finally, the compound must have pharmaceutical properties that allow for a competitive and convenient dosing profile.

Paramecia
Freshwater protozoa of the genus *Paramecium* with an oral groove for feeding.

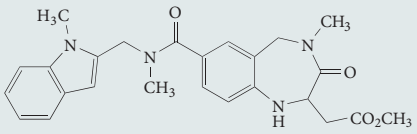
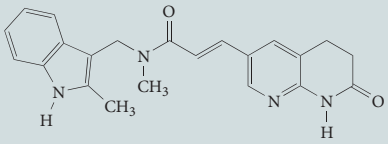
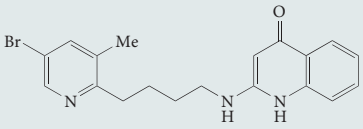
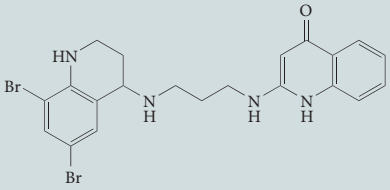
Adapting to unique challenges

With our lacklustre HTS results, it made little scientific or economic sense to simply keep screening more targets. Our experience with the lead optimization phase illustrated that delivering high-quality, broad-spectrum antibacterial development candidates from a single programme requires the commitment of a large and long-term effort. It was clear that we needed to change our approach.

In 2002, GSK overhauled its antibacterial research strategy to address these key challenges. The entire internal focus was shifted to a select number of programmes with late-stage leads (none of these originated from the HTS campaigns run between 1995–2001). Our decision to pursue only broad-spectrum agents limited the choice of targets. For example, agents such as our FabI and MetRS leads, although promising, did not meet the necessary spectrum requirements and were partnered with biotechnology companies (Affinium and Replidyne, respectively). To enable optimal biology and chemistry resourcing of our late-stage programmes, all early-stage programmes (~30) that were in the 'HTS to lead' phases were terminated and in-house HTS for the antibacterial therapeutic area temporarily ceased.

Instead, we wanted to concentrate solely on novel chemical structures — not targets — whose members had excellent *in vitro* and *in vivo* antibacterial activity against both Gram-positive and Gram-negative pathogens and into whose chemical structures might be engineered the physical properties needed for a pharmaceutical product. As these leads were not going to come from screening, we needed alternative approaches. First, we reconsidered known antibacterial molecules to see whether we could improve their antibacterial and developability properties. Along these lines, we have modified the pleuromutilin core structure in ways that have allowed us to bring three derivatives into clinical development. We also found lead molecules the old-fashioned way: screening a small, discrete library of compounds for antibacterial activity. Here we found a novel compound class that inhibits

Table 2 | **Example properties of 'hits' obtained from HTS and outcome of their optimization**

Target	Development status	Structure	Enzyme IC_{50} (μM) in *S. aureus*	MIC (μg per ml) in *S. aureus*	MOA shown?
FABI[16]	Hit		17.1	>64	No
FABI[16]	Optimized lead		0.047	0.06	Yes
Met tRNA synthetase	Hit		0.35	>64	No
Met tRNA synthetase	Optimized lead		0.008	0.5 (MIC90)	Yes
MurB	Hit	Unavailable	4.8	>64	No
MurB	Optimized lead	Unavailable	4.8	>64*	No
Rnase P	Hit	Unavailable	8	1	No
Rnase P	Optimized lead	Unavailable	8	1*	No
Tyr tRNA synthetase	Hit	Unavailable	0.3	>64	No
Tyr tRNA synthetase	Optimized lead	Unavailable	0.04	>64	Yes‡

*Derivatives of HTS hit were made, but none were significantly more active than initial HTS hit. ‡Mode of action was demonstrated by downregulating the FRS target and showing specific sensitization of strain to FRS leads. FRS, phenylalanine tRNA synthetase; HTS, high-thoughput screening; MOA, mode of action; MIC, minimum inhibitory concentration.

bacterial DNA replication, several of whose members have also moved into development. Finally, rational design initiatives have also led to other lead molecules which have reached the development interface.

To support this strategy, we devoted large medicinal chemistry teams to a smaller number of programmes. Our choice has been to work on a select number of programmes very thoroughly rather than on many things inadequately. We realize that we will miss out on some opportunities, but we want to give our selected programmes the best chance of succeeding. We have streamlined, and automated in some cases, the biological characterization of the leads to provide high-throughput *in vitro* antibacterial assessment and more rapid *in vivo* evaluation of promising leads. Antibacterial research does have one great advantage: animal models of infection are predictive of efficacy in humans. One can use the pharmacokinetic/pharmacodynamic parameters established in animal models of infection to predict the likelihood of the drug working in humans, and the necessary therapeutic exposure. As an antibiotic does not have an intended target in humans, careful choice of bacterial target, or demonstration of only weak activity against any human homologues, should avoid mechanism-based toxicity. To capitalize on this advantage, we redesigned our progression plan so that we could use efficacy in animal models of infection as an early decision point in lead optimization. For example, in one programme in which a large proportion of compounds synthesized met *in vitro* antibacterial criteria, we synthesized at risk large quantities of each new molecule (0.5–1 g). Because there was sufficient material to assess both the *in vitro* antibacterial activity (against up to 100 pathogens) as well as the *in vivo* efficacy, decisions could be made on the future direction of medicinal chemistry efforts within 10 days of receiving a new compound, rapidly accelerating the lead optimization process.

In addition, the organization of research at GSK into Centers of Excellence for Drug Discovery (CEDDs) — small, self-contained, therapeutically focused research units — enabled sufficient resources to be deployed to

the antibacterial area, which can sometimes be diluted in large matrix organizations. Rapidly generating key biological data and focusing significant medicinal chemistry resources on a smaller number of well-validated targets has proven to be substantially more productive than either Glaxo Wellcome or SmithKline Beecham were in the past at generating antibiotics with a novel mechanism of action. So far, following this strategy has led to six development candidates with a pipeline poised to deliver further candidates with a novel MOA. We are not declaring success yet, but there has been greater productivity, measured by the number of development candidates, in the past 4 years than in the previous 20. As a further validation of this approach and as a measure of our commitment to infectious diseases, GSK has now created a specific Infectious Diseases CEDD to include antibacterial and antiviral drug discovery, as well as drugs for diseases of the developing world.

We did not entirely give up on the early part of antibacterial R&D. Our compound collection has substantially expanded and improved in quality since we finished the genomics-based screening campaigns. Indeed, we have attempted to create libraries of molecules that might be better suited to antibacterial targets. A few of the targets that had broad-spectrum potential have been re-screened against this larger and more diverse GSK compound collection in collaboration with medium-sized pharmaceutical companies. Hits and leads have been transferred to our alliance partner, who is responsible for producing development candidates that are then taken back by GSK for further development. This approach enables us to maintain an early-stage portfolio in a way that does not compromise internal focus on our advanced lead optimization programmes.

Approaches to improve antibacterial discovery

Our experience and those of others[25] underscores the inadequacy of screening of synthetic libraries of compounds against isolated bacterial targets to generate novel antibacterial leads. A different paradigm for antibacterials is needed.

Selection of HTS targets and screens. Confidence that inhibition of the target will result in cell death and resolution of the infection is the epitome of target validation. With the powerful genetic tools available, there is little excuse not to have this information for all the key pathogens within the desired spectrum. As we have seen, however, it is not trivial to thoroughly validate an untested target (see, for example, the MetRS example above).

Regardless of their cellular location, single enzyme targets are suspect because of the ease of evolving resistance by single point mutation, either in the structural gene, in the promoter or in genes involved in transport or permeabilility. Targeting multiple enzymes that are structurally related or recognize common substrate motifs, and offer the possibility of finding a small molecule that binds to more than one family member, is much preferred[27]. Unless there is a substantial synthetic chemistry effort committed to converting good enzyme inhibitors into permeative antibiotics, screening intracellular protein targets — particularly lone enzymes — promises to be frustrating. Extracellular targets are always attractive, especially for biochemical screens, because penetration of the cellular boundary is not required.

Experience suggests that it is easier to find the cellular target of an antibacterial compound than it is to engineer permeability into an enzyme inhibitor. Therefore whole-cell assays are favoured for finding a lead compound that has a modicum of antibacterial activity, but biochemical assays and genetic studies are vital to determine the MOA of these leads. Using engineered strains that underexpress a particular target is a way to detect compounds that have both target specificity and antibacterial activity[28]. Finally, it should be noted that designing HTS assays to accommodate modern large-scale robotic screening often involves compromises, such as using non-natural substrates or artificial reaction conditions, and defining imperfect hit selection criteria, which inadvertently fail to identify interesting inhibitors. Furthermore, robust proof that the essential function of the enzyme is being effectively captured by the HTS format can be lacking. These aspects are perhaps more of an issue with entirely novel targets.

Some targets are clearly more tractable than others for reasons that are not always obvious. Certain targets (for example, the ribosome and gyrase) have multiple classes of published leads and marketed drugs, whereas other essential gene products have no known inhibitors despite a long history of antibacterial research. Discovering novel strategies of inhibiting pharmacologically validated, tractable targets — DNA replication, the ribosome and cell-wall biosynthesis — is a more rational strategy[29]. Put simply, a good target is better than a new target.

Chemical diversity. An extremely important factor when searching for new drugs is the variety, or diversity, of chemicals available to screen. The chemical collections of most large pharmaceutical companies have been panned for possible antibacterials. The low-hanging fruit from the antibiotic tree has probably already been picked. The synthetic screening collections of different companies probably have substantial overlap, due to the use of similar chemical synthetic methods and acquisition of compounds from the same vendors. New sources of compounds are needed. Combinatorial chemistry was heralded as the cavalry in this regard, but the technology for generating chemical diversity is not quite ready. Indeed, compounds made using combinatorial chemistry occupy a different, and narrower, chemical space compared with marketed drugs and natural products[30]. Known antibacterials do not generally follow Lipinski's 'rule of five'[31], although corporate compound collections are heavily biased towards compounds that do (FIG. 4). Greater molecular diversity and better understanding of which physical chemical properties are important for antibacterials is necessary. To this end, as referred to previously, we have attempted to create chemical libraries that are better suited to finding antibacterial compounds and these have been added to our compound collection.

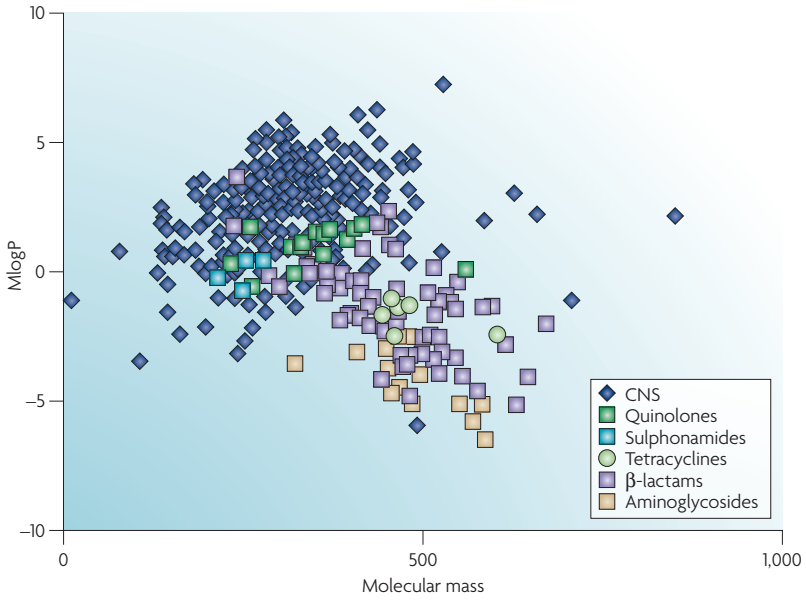


Figure 4 | **The chemical diversity of antibacterials is different to other drugs.** A plot of calculated logP and molecular mass of marketed drugs for central nervous system disorders compared with marketed antibacterial classes. CNS drugs (similar observations for other mammalian target classes; data not shown) closely follow Lipinski's rule of five. Antibacterial molecules are on average more hydrophilic and slightly larger.

Fortunately, nature is not yet tapped out of novel compounds. Bizarre organisms are being isolated from all sorts of extreme biological niches, bringing with them their own chemical defense mechanisms[26]. These naturally occurring organisms, together with recombinant organisms generated using combinatorial genetics[32] and the availability of new chimeric metabolic pathways, promise to deliver an abundance of new compounds. We are enthusiastic advocates of natural product screening to search for novel antibacterial leads.

More chemists needed. The only way to overcome the challenges of multifactorial antibacterial lead optimization is to expand the number of chemical derivatives. We now employ roughly two chemists for each biologist in the antibacterial therapeutic area, a fourfold turn-around from the days when genomics dominated our activities. That these large teams are needed for the long haul (3–5 years) could be the biggest challenge as they require commitment to antibacterial discovery that biotechnology companies and even big pharma could find difficult to justify on the basis of financial return only.

Perspectives on antibacterial research

FIGURE 1 illustrates the timescale and probability of success for each step of antibacterial development using a combination of GSK and industry averages. Industry data illustrate that, on average, 16 Phase I starts are required for one antibacterial product. There are no more than two to three novel-mechanism systemic antibiotics in Phase I studies, and these are Gram-positive-spectrum agents or community respiratory-tract infection drugs. According to these metrics, an additional 12 Phase I starts are needed — a fourfold increase in investment — between now and 2008 to generate one novel-mechanism antibacterial by 2012.

The scenario for the tougher Gram-negative hospital pathogens is more worrisome[5]. Efflux-mediated resistance, which vitiates the activity of a broad range of structural classes, is formidable in Gram-negative bacteria. Right now, there are no novel MOA antibacterials in Phase I, nor are there even good preclinical leads with promising Gram-negative activity. Assuming aggressive timelines of 3–5 years to deliver a development candidate and 6 years to compete clinical testing, agents for Gram-negative infections could be 9–11 years away (FIG. 1). Taking the attrition into account, it could be as long as 10–15 years before we see a novel mechanism agent for treating Gram-negative hospital infections.

Attrition metrics suggest that the current industry pipeline has a low probability of delivering a single novel-mechanism antibiotic. Companies that remain committed to this area, such as GSK, will need to continuously introduce development candidates into the clinic until (at least) one crosses the finish line as a registered medicine. However, to assure ourselves that novel-mechanism antibiotics will be available for public health, substantially more compounds need to be produced and tested in human studies. This is not going to happen until more drug hunters, both in academia and at companies, engage and apply greater investment to the area.

Concluding remarks

The goal of this review was to focus specifically on the scientific challenges of antibacterial research from the GSK perspective. Some of the challenges encountered were part of a learning curve, and a function of incomplete knowledge at the time. However, many more of the technical difficulties still remain, such as acquisition of biologically relevant chemical diversity, and achieving activity across a diverse spectrum of pathogens, including highly challenging Gram-negative pathogens, with safe drugs. Improvements in the success rate for molecular target HTS will be needed before this is a robust discovery platform for antibacterials. In the meantime, at GSK we have concentrated our effort on lead optimization of novel lead classes from alternative sources. We are mindful of other environmental factors but, from our perspective and as emphasized in this review, the scientific challenges of delivering novel mechanism antibiotics are equally difficult. The painful reality of drug discovery is that things go wrong. This is reflected in the low probability of success for creating an antibacterial worthy of approval for clinical use. The pipeline of novel-mechanism antibacterials is still empty and will remain that way for a considerable time. In conclusion, our experience suggests that synthesizing novel chemical structures that interact with and block established targets in new ways is a robust strategy.

1. House of Lords Select Committee on Science and Technology Seventh Report. *Resistance to Antibiotics and Other Antimicrobial Agents* [online], <http://www.parliament.the-stationery-office.co.uk/pa/ld199798/ldselect/ldsctech/081vii/st0701.htm> (Stationary Office, London, UK, 1998).
2. Shlaes, D. M. *et al*. Antibiotic discovery: state of the state. *ASM News* **70**, 275–281 (2004).
3. Talbot, G. H. *et al*. Bad bugs need drugs: an update on the development pipeline from the antibacterial availability task force of the IDSA. *Clin. Infect. Dis.* **42**, 657–668 (2006).
4. Wenzel, R. P. The antibiotic pipeline — challenges, costs, and values. *N. Engl. J. Med.* **351**, 523–526 (2004).
5. Infectious Disease Society of America. *Bad Bugs, No Drugs* [online], <http://www.idsociety.org/pa/IDSA_Paper4_final_web.pdf> (2004).
Seminal review by key opinion leaders on the shrinking investment by big pharma in antibacterial discovery, and the growing challenge of resistant pathogens.
6. Tickell, S. The Antibiotic Innovation Study: Expert Voices on a Critical Need [online], <http://soapimg.icecube.snowfall.se/stopresistance/Innovation%20study%20april%20low%20res.pdf> (2005).
7. Nathan, C. Antibiotics at the cross roads. *Nature* **431**, 899–902 (2004).
8. Kaplan, W. & Laing, R. *Priority Medicines for Europe and the World* [online], <http://mednet3.who.int/prioritymeds/report/index.htm> (World Health Organization, Geneva, 2004).
9. Brown, J. R. & Warren, P. V. Antibiotic discovery: is it in the genes? *Drug Discov. Today* **3**, 564–566 (1998).
10. Chan, P. F., Macarron, R., Payne, D. J., Zalacain, M. & Holmes, D. J. Novel antibacterials: a genomics approach to drug discovery. *Curr. Drug Targets — Infect. Dis.* **2**, 109–119 (2002).
11. Chan, P. F. *et al*. Characterization of a noval fucose-regulated promoter (P*fcsK*) suitable for gene essentiality and antibacterial mode of action studies in *Streptococcus pneumoniae*. *J. Bacteriol.* **185**, 2051–2058 (2003).
12. Zhang, L. *et al*. Regulated gene expression in *Staphylococcus aureus* for identifying conditional lethal phenotypes and antibiotic mode of action. *Gene* **255**, 297–305 (2000).
13. Yin, D. *et al*. Identification of Antimicrobial targets using a comprehensive genomic approach. *Pharmacogenomics* **5**, 101–113 (2004).
14. Zalacain, M. *et al*. A global approach to identify novel broad-spectrum antibacterial targets among proteins of unknown function. *J. Mol. Microbiol. Biotechnol.* **6**, 109–126 (2003).
15. Jarvest, R. L., *et al*. Nanomolar inhibitors of *Staphylococcus aureus* methionyl tRNA synthetase with potent antibacterial activity against Gram-positive pathogens. *J. Med. Chem.* **45**, 1959–1962 (2002).
16. Payne, D. J. *et al*. Discovery of a novel and potent class of FabI directed antibacterial agents. *Antimicrob. Agents Chemother.* **46**, 3118–3124 (2002).
17. Fan, F. *et al*. Defining and combating the mechanisms of triclosan resistance in clinical isolates of *Staphylococcus aureus*. *Antimicrob. Agents Chemother.* **46**, 3343–3347 (2002).
18. Aubert, K. & Zalacain, M. 3 Peptide deformylase inhibitors. *Prog. Med. Chem.* **44**, 109–143 (2006).
19. Gentry, D. R. *et al*. Variable sensitivity to bacterial methionyl-tRNA synthetase inhibitors reveals subpopulations of *Streptococcus pneumoniae* with two distinct methionyl-tRNA synthetase genes. *Antimicrob. Agents Chemother.* **47**, 1784–1789 (2003).
An example illustrating the risk associated with exploitation of non-clinically validated targets for antibacterial discovery.
20. Du, W. *et al*. Two active forms of UDP-*N*-acetylglucosamine enolpyruvyl transferase in Gram-positive bacteria. *J. Bacteriol.* **182**, 4146–4152 (2000).
21. Heath, R. J. & Rock, C. O. A triclosan-resistant bacterial enzyme. *Nature* **406**, 145 (2000).
22. Payne, D. J., Gwynn, M. N., Holmes, D. J. & Rosenberg, M. Genomic approaches to antibacterial discovery. *Methods Mol. Biol.* **266**, 231–259 (2004).
23. Andries, K. *et al*. A diarylquinoline drug active on the ATP synthase of *Mycobacterium tuberculosis*. *Science* **307**, 223–227 (2005).
24. Heerding, D. A. *et al*. New benzylidenethiazolidine-doines as antibacterial agents. *Bioorg. Med. Chem. Lett.* **13**, 3771–3773 (2003).
25. Chan, P. F. *et al*. Finding the gems using genomic discovery: antibacterial drug discovery strategies — the successes and the challenges. *Drug Discov. Today: Ther. Strategies* **1**, 519–527 (2004)
26. Pace, N. R., A molecular view of microbial diversity and the biosphere. *Science* **276**, 734 (1997).
27. Wong, K. K. *et al*. Engineering a cell-free murein biosynthetic pathway: combinatorial enzymology in drug discovery. *J. Am. Chem. Soc.* **120**, 13527–13528 (1998).
28. DeVito, J. A. *et al*. An array of target-specific screening strains for antibacterials discovery. *Nature Biotechnol.* **20**, 478–483 (2002).
29. Wong, K. K. & Pompliano, D. L. in *Resolving the Antibiotic Paradox: Progress in Understanding Drug Resistance and Development of New Antibiotics* (eds Rosen, B. P. & Mobashery, S.) (Plenum, New York, 1999)
30. Feher, M. & Schmidt, J. M. Property distributions: differences between drugs, natural products, and molecules from combinatorial chemistry. *J. Chem. Inf. Comput. Sci.* **43**, 218–227 (2003).
31. Lipinski, C. A., Lombardo, F., Dominy, B. W. & Feeney, P. J. Experimental and computional approaches to estimate solubility and permeability in drug discovery and development settings. *Adv. Drug Deliv. Rev.* **23**, 3–25 (1997).
32. Walsh, C. T. Combinatorial biosynthesis of antibiotics: challenges and opportunities. *Chembiochem* **3**, 125–134 (2002)
33. Throup, J. P. *et al*. A genomic analysis of two-component signal transduction in *Streptococcus pneumoniae*. *Mol. Microbiol.* **35**, 566–576 (2000).
34. Ji. Y. *et al*. Identification of critical staphylococcal genes using conditional phenotypes generated by antisense RNA. *Science* **293**, 2266–2269 (2001)

Acknowledgements
We thank the numerous scientists from all parts of GSK who have contributed to the data discussed in this review and thank C. Edge (GSK, Molecular Discovery Research) for figure 4.

Competing interests statement
The authors declare competing financial interests: see Web version for details.

FURTHER INFORMATION
Centers for Medicines Research: http://www.cmr.org
Drugs @ FDA: http://www.accessdata.fda.gov/scripts/cder/drugsatfda/index.cfm
Access to this links box is available online.