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In the battle against the increasing threat of bacterial resistance, antibacterial drug discovery must exploit the most effective tools. Structure-based design offers the promise of rapid progression from target through to investigational new drug.

Antibacterial drug discovery and structure-based design

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Bacterial resistance continues to develop and pose a significant threat, both in hospitals and, more recently, in the community. A focus on other therapeutic areas by the larger pharmaceutical companies has left a shortfall in the pipeline of novel antibacterials. Recently, many new structures have been studied by structure–genomics initiatives, delivering a wealth of targets to consider. Using the tools of structure-based design, antibacterial discovery must exploit these targets to accelerate the process of drug discovery.

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Antibacterial research, too long the poor relation of lifestyle drug discovery, is once again a topic of heated discussion and cutting edge research. Reports of communally and hospital-acquired infection from multidrug-resistant bacteria continue to make the news and vancomycin, historically the last line of defence in treatment, shows signs of fallibility. The decline of antibacterial research by many of the big pharmaceutical companies has led to a shortfall in new and better agents to fight the present threat of drug resistance [1]. Where is the next generation of antibacterials?

Antibacterials remain attractive commercially, with growth in this field continuing for hospital-related products and also with the spread of resistance into the community [2,3]. After many years of exhaustive medicinal chemistry studies on the modification of known antibacterial scaffolds, it is becoming increasingly difficult to deliver new leads. The focus of much antibacterial research has, therefore, moved to the identification of novel chemical classes and novel bacterial targets. To this end, many smaller companies have taken up the challenge [4]. One anticipated advantage of developing drugs with novel mechanisms of action is that latent resistance mechanisms will not be present in nature, thus slowing the rate of bacterial adaptation to a new antibacterial class. Many of the companies embracing the challenge of antibacterial discovery are actively pursuing techniques such as structure-based design, to bring new agents to the market faster and at reduced cost.

The demand for novel chemotherapeutic antibacterial agents that overcome current resistance mechanisms has been paralleled by the wealth of structural data from the structure–genomics initiatives and bioinformatics data from genome sequencing [5,6]. Key pointers in the identification of druggable targets are: a gene must be essential for the survival of the bacterial cell; and the bacterial gene must not have a close homologue in the human genome. With unprecedented numbers of novel atomic resolution structures, the antibacterial research community is now faced with multiple opportunities for structure-based targets [7,8]. The number and variety of

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structures available for study in the antibacterial field also provides an excellent testing ground for the refinement of structure-based design techniques.

The methods exploiting structural biology in drug discovery have been reviewed comprehensively elsewhere and so will not be covered in detail here [9]. This review describes several recent applications of structure-based drug discovery in the antibacterial arena and some of the promising new structural information for future research. In the sections below, a brief description of the tools available for structure determination and design is followed by sections describing the recent advances made on several targets in key areas of the bacterial cell cycle.

Structure determination and rational design

X-ray crystallography remains the gold standard for structure elucidation, affording high precision and the ability to look at complex systems, such as the ribosome [10]. Crystallography has its limitations, the production of protein can be challenging (especially for membrane proteins) and not all proteins are readily crystallized, often requiring extensive protein engineering before crystals can be delivered. The models produced by the crystallographer also have limitations that must be recognized [11]. NMR and homology modelling offer alternative routes to structure-based design. Improvements in NMR techniques and the increasing use of automation in spectral assignment have enhanced the targets available for NMR study, as well as the throughput [12]. The wealth of structural data from structure–genomics initiatives has made the choice of homologues available for the modeller wider, giving greater applicability and automation to homologue modelling [13]. The science of *de novo* structure prediction from sequence also continues to develop with the promise of being able to predict structure from primary sequence [14].

Structure-based design can be applied in a variety of methods once a structure is available [15]. Identification of chemical starting points for lead optimization can be achieved through fragment screening, virtual docking of commercial or virtual compounds or *de novo* design [16–27]. These approaches require detailed scoring of interactions and this is an evolving area where significant advances are continually being made [28]. With increased computational power, the use of molecular dynamics (MD) and molecular mechanic Poisson–Boltzmann-simulated annealing (MM-PBSA) calculations is improving the quality of *in silico* scoring, although a direct correlation between experimental and *in silico* binding remains a challenge [29].

Fundamentally, in medicinal chemistry programmes, structure-driven design is an enrichment tool, aiding the synthetic chemist in the prioritization of compounds throughout the lead optimization process. Structure-driven design is an excellent tool for designing increases in potency and improving selectivity. Used in conjunction with ADME–Tox and metabolite identification, a structure can focus chemistry on regions suitable for modification, improving stability or bulk properties such as solubility, without affecting potency. Compound **1** (Table 1) shows the oxazolidinone linezolid, the first new class of antibacterial to reach the

TABLE 1

Recent antibacterial agents rationalized by structure-based design

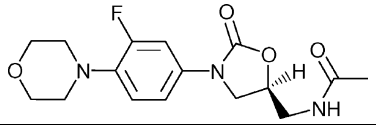
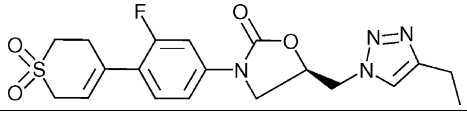
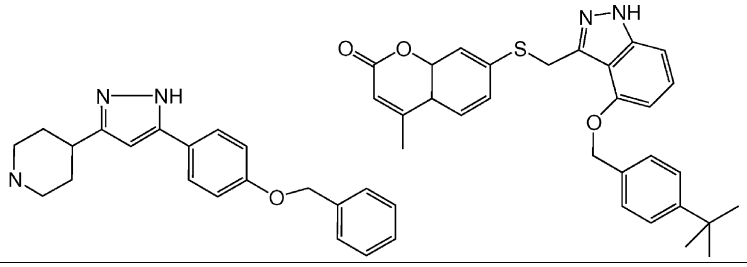
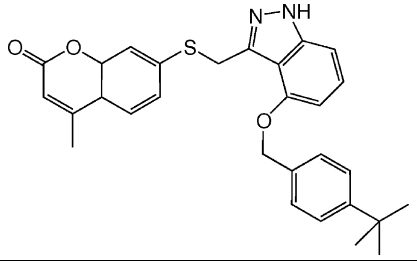
Compound number	Antibacterial agent	Target	Activity (IC_{50}, unless otherwise stated)	Refs
1		Ribosome	1.8 μM	[30]
2		Ribosome	NA	[31]
3		Gyrase B	128 μg ml^{-1}	[41–44]
4		Gyrase B	0.25 μg ml^{-1}	[41–44]

TABLE 1 (*Continued*)

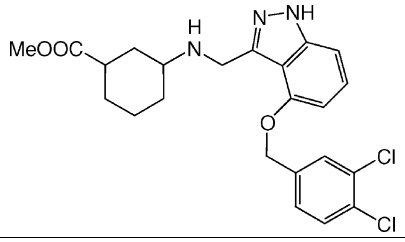
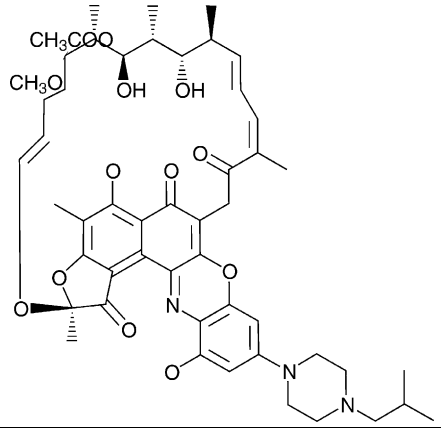
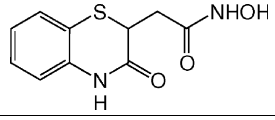
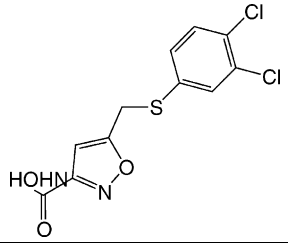
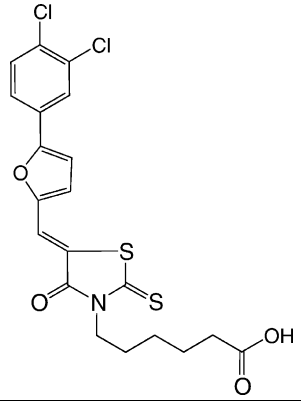
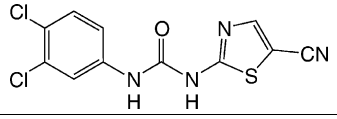
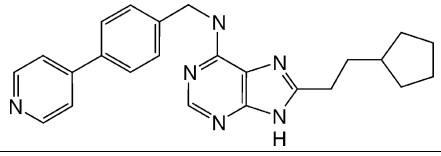
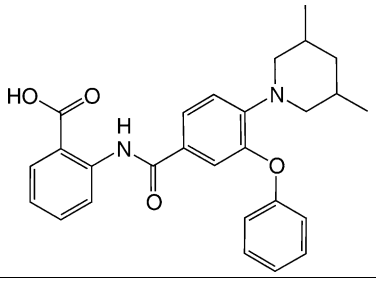
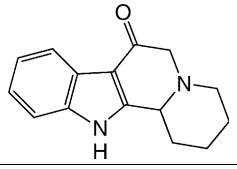
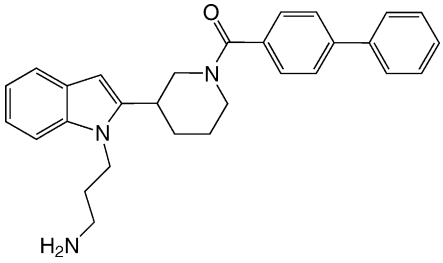
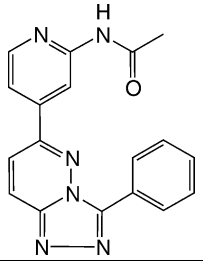
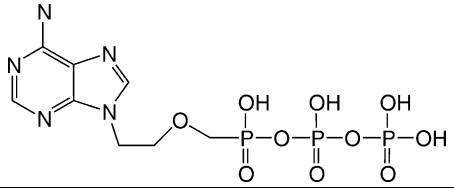
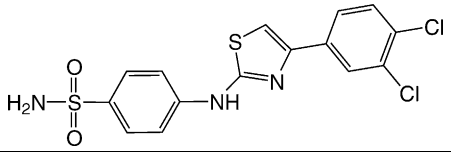
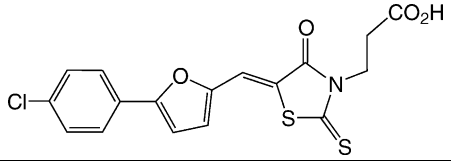
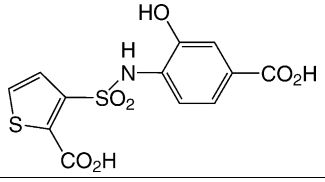
Compound number	Antibacterial agent	Target	Activity (IC_{50}, unless otherwise stated)	Refs
5		Gyrase B	1.0 μg ml^{-1}	[41–44]
6		RNA polymerase	NA	[54]
7		Peptide deformylase	<0.005 μM	[66]
8		Peptide deformylase	2 μM	[67]
9		Peptide deformylase	0.89 μM	[68]
10		MurA and MurB	12.6 μg ml^{-1} and 4.5 μg ml^{-1}, respectively	[70]
11		MTA nucleosidase	K_i = 0.0028 μM	[76]

TABLE 1 (*Continued*)

Compound number	Antibacterial agent	Target	Activity (IC_{50}, unless otherwise stated)	Refs
12		FabH	0.27 μM	[79]
13		ZipA	1170 μM	[87,88]
14		ZipA	296 μM	[89]
15		ZipA	K_d = 79 μM	[90]
16		Edema factor	K_i = 0.027 μM	[94]
17		Edema factor	10–15 μM	[97]
18		Lethal factor	K_i = 0.8 μM	[102]
19		AmpC	K_i = 1 μM	[112]

market in the past 30 years and the first ribosome drug not derived from natural products [30]. In the design of the next generation of oxazolidinones, the potential side-effects of amine oxidase inhibition of this compound class are being addressed by structure-based design of molecules with enhanced selectivity against monoamine oxidase-A and -B (MAO-A and MAO-B) [31]. Compound **2** (Table 1) is an example of a substituted triazole analogue of linezolid. Compound **2** retains good antibacterial activity and higher K_i values against MAO-A. The SAR of the substitution pattern of the triazole series has been rationalized through docking calculations against homology models of MAO-A and MAO-B.

In diversity-driven hit identification via HTS, structure-based design can also play a role. Before an HTS campaign against MurG, an antibacterial target in the cell wall biosynthesis pathway, Hu *et al.* used the available crystal structure of a glycosyl donor complexed to MurG to design modifications allowing introduction of a fluorophore [32]. The design was successful with the *N*-acetyl glucosamino derivative acting as a substrate and the development of an assay suitable for HTS.

Structure-based drug discovery in the antibacterial field has been comparatively less successful than in other fields, such as antivirals, given the number of requisite bacterial structures available for study [33,34]. This is, in part, because of the difficulties of cell penetration. Structure-based design can deliver improvements in potency and, where required, selectivity but other tools are required to aid in the design of cell penetration. *In silico* topological modelling of chemical structural information from known antibacterials can be demonstrated to identify novel chemical scaffolds, not included in the original training set, with antibacterial activity [35]. In the future, *in silico* tools might be able to steer the structure-based designer to gross antibacterial properties suitable for cell penetration, whereas the 3D information of the target complex allows increases in potency. Modelling in conjunction with mutagenesis studies on the archetype non-specific enterobacterial porin OmpF with β-lactams suggests a combination of the anionic residues at the pore eyelet, and steric controls of antibacterial flux across the porin. A more fundamental understanding of transport mechanisms might aid the future design of antibiotics with improved cell-penetration characteristics [36].

Transcription of genetic code

Transcription of the bacterial genetic code requires the manipulation of DNA within the cell and synthesis of RNA. Key enzymes involved in transcription are the DNA topoisomerases and RNA polymerase.

The topoisomerases are DNA-binding proteins that control bacterial DNA topology, chromosome function and maintain DNA supercoiling [37,38]. All topoisomerases cleave DNA, forming a transient phosphodiester bond between a broken strand and a tyrosine of the protein. DNA topology is modified during the lifetime of the covalent intermediate and the enzyme is released on religation of the DNA. Those enzymes that cleave one strand of DNA are classified as type I, whereas those that cleave both strands of DNA and generate a staggered double-strand break are classified as type II. DNA gyrase and topoisomerase IV are both type II topoisomerases and essential proteins in the bacterial lifecycle. These proteins function as heterotetrameric complexes (A_2B_2 for DNA gyrase and C_2E_2 for topoisomerase IV) and have been validated as therapeutic drug targets by several successfully marketed antibacterials, with the quinolones and fluoroquinolones recognized as a common line of defence in antibacterial chemotherapy [39]. This particular class of antibacterial agent interacts with the A-subunit of DNA gyrase (GyrA) and/or the C-subunit of topoisomerase IV (ParC), although the design of such inhibitors has not been guided by structural information. Recently, the small-angle X-ray scattering of full-length GyrA has been obtained and this could, in combination with further modelling studies, deliver new opportunities for drug design, increasing our understanding of the mechanism of action of the quinolone and fluoroquinolone class of antibacterials [40].

By contrast, small-molecule inhibitors that target the B-subunit of DNA gyrase (GyrB) or the E-subunit of topoisomerase IV (ParE) have not, to date, generated a marketed antibacterial. This situation could change in due course, because significant effort has been deployed to access crystallographic information on this target and the availability of crystal structures (to drive the medicinal chemistry process) appears to have been a prerequisite for recent advances [41–44]. Compound **3** (Table 1) shows moderate activity in a supercoil assay against type II topoisomerase and some antibacterial activity (IC_{50} of 128 μg ml^{-1} against gyrase and an MIC of 64 μg ml^{-1} against *Staphylococcus aureus*, FDA 209P). By comparison, compound **4** (Table 1) has excellent potency against GyrB but weak antibacterial activity, attributed to poor cell penetration (IC_{50} of 0.25 μg ml^{-1} against gyrase and MIC >128 μg ml^{-1} against *S. aureus*, FDA 209P). Combining the cell-penetration properties of compound **3** with the potency of compound **4** gives compound **5** (Table 1). This compound shows excellent antibacterial activity against Gram-positive *staphylococci* and *enterococci* (IC_{50} of 1 μg ml^{-1} against gyrase and an MIC of 4 μg ml^{-1} against *S. aureus*, FDA 209P, as well as an MIC of 4 μg ml^{-1} against *Enterococus faecalis*). Docking studies of compound **5** against GyrB suggest regions for chemical modification to further-increase potency. The pharmacophore for GyrB is well described and includes the role of highly conserved water molecules in the ATP-binding site [45]. The flexibility and functionality of the target has been studied in detail and this information has led to the postulation of alternative binding sites, adjacent to the known binding sites, for targeting in rational design [46]. More recently, the release of the crystal structure of *Escherichia coli* topoisomerase IV ParE indicates a strong correlation with the structure of GyrB, suggesting inhibitors with dual GyrB and ParE inhibition could be possible (Figure 1) [47]. A dual mechanism of antibacterial activity would be advantageous, because it would be expected to enhance the stability of a novel antibacterial against the rapid development of resistance through target mutagenesis.

At the core of transcription of the genetic code lies RNA polymerase (RNAP), which essentially synthesizes all cellular RNA molecules [48]. Several high-resolution structures for RNAP from *Thermus aquaticus* and *Thermus thermophilus* have recently been solved [49–52]. The RNAP core is a multiprotein complex made up of $\alpha_2\beta\beta'$ subunits with poorly conserved and loosely associated ω and δ proteins. Only upon interaction with the family of proteins called the σ-factors does the holoenzyme complex initiate transcription. The similarities between the thermophile's RNAPs and those of other pathogenic bacterial species make the structural and func-

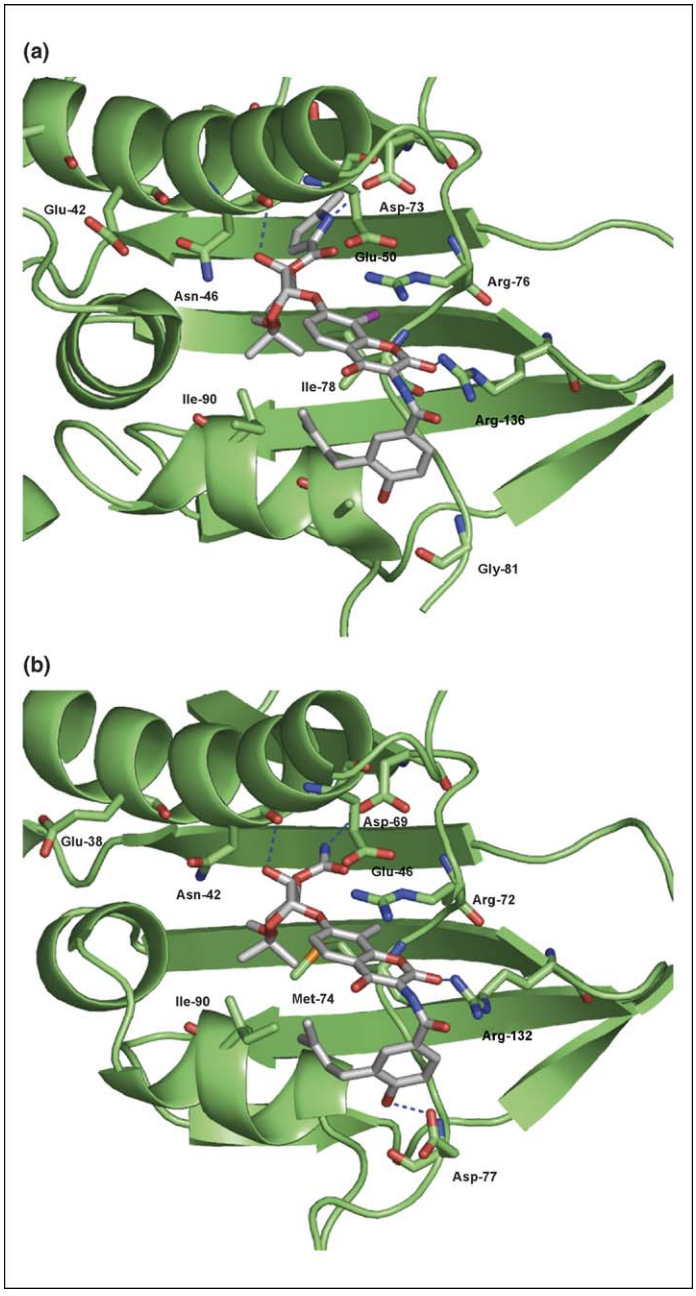


FIGURE 1

The structural comparison of Gyrase B and ParE. (a) The 24 kDa Gyrase (Gyr) B fragment from *Escherichia coli* in complex with chlorobiocin (Protein Data Bank entry 1KZN, protein ribbon in orange) and **(b)** the 24 kDa ParE fragment from *E. coli* in complex with novobiocin (Protein Data Bank Code 1S14). The high degree of structural conservation between these antibacterial targets at their ATP sites indicates the potential for the design of dual inhibitors. The protein backbone is shown as a ribbon diagram. Key residues are represented in stick form with carbon atoms (green), nitrogen atoms (blue) and oxygen atoms (red). Metal ions are shown as solid spheres. For clarity the carbon skeleton of the inhibitor is shown in grey. H-bonds are shown in dashed blue (representations were generated using PyMOL).

tional information suitable for modelling. The design of small peptides has been reported, they promise to compete with the binding of the transcription factor σ^A offering new routes to antibacterial activity [53]. These new designs could, in the future, complement the current rifampicin-style RNAP antibacterials, such as the subtype antibacterial rifalazil, compound **6** (Table 1), currently in clinical trials and showing efficacy against resistant mutants of *Chlamydia trachomatis* [54].

Translation of genetic code

Error-free translation of the genetic code is essential for cell viability. The machinery of translation, the ribosome, is one of the most exploited areas of antibacterial chemotherapy. Recently, other targets such as aminoacyl-tRNA synthetase and peptide deformylase, which are involved in protein synthesis and post-translational modification, respectively, have attracted widespread attention.

The bacterial ribosome is the key site for drugs targeting translation. Several clinically important antibiotics interfere with protein synthesis via this target (e.g. aminoglycosides, macrolides and oxazolidinones). The ribosome is predominantly made up of RNA, therefore ribosomal drugs represent the only current clinically accepted RNA-targeting small-molecule therapies. Over the past few years, several 3D structures of antibiotics bound to their ribosome targets have been determined [10]. These first structures have allowed detailed analysis of the binding sites and mechanisms of inhibition of the bacterial ribosome. In light of the new structural information, improvements are promised in the current therapies, whether it is in the aminoglycosides targeting the decoding site or the macrolides, streptogramins, chloramphenicols and oxazolidinones targeting the peptidyl-transfer centre [55]. Exploiting *in silico* screening tools, the X-ray crystallographic information on the 30S ribosome subunit has been successful for identifying novel ribosomal ligands [56]. Using the crystal structure of paromomycin complexed to the decoding region of 16S rRNA [57], docking studies, exploiting software previously trained using known ribosome crystal structures, identified ribosome ligands with K_i values in the low μM range. It is possible to imagine that *in silico* screening could be able to identify not only novel chemotypes targeting the ribosome, but also novel binding sites – as yet unexploited in antibacterial drug discovery.

In the process of translating the genetic code, accurately associating the 21 amino acids to their appropriate anticodon triplets is crucial. These reactions are catalyzed by aminoacyl-tRNA synthetases and have been widely targeted for antibacterial intervention [58]. Two classes of aminoacyl-tRNA synthetase exist in bacteria. Drugs such as indolmycin and TAK-083 successfully inhibit class I Trp tRNA synthetase and show good antibacterial properties against bacteria, such as *Helicobacter pylori*, that carry only the class I aminoacyl-tRNA synthetase [59]. However, human pathogens such as *Streptomyces coelicolor*, which contain the auxillary class II Trp tRNA, are resistant to indolmycin [60]. Other pathogens such as *Streptoccocus pyogenes* contain only class II Trp tRNA. The structure of a homologue of class II aminoacyl-tRNA synthetase from *Deinococcus radiodurans* could offer a route to structure-based development of antibiotics against pathogens carrying only the class II aminoacyl-tRNA synthetase, and combination therapies for pathogens containing both classes of tRNA synthetase [61].

Peptide deformylase (PDF) is an essential enzyme catalyzing the hydrolytic removal of the N-terminal formyl group from the methionine of nascent ribosome-synthesized polypeptides [62,63]. Two PDF inhibitors, VIC-104959 (LBM415) and BB-83698, have progressed to Phase I clinical trials [64]. PDF is an Fe metallohydrolase; however, an active, stable Ni surrogate is

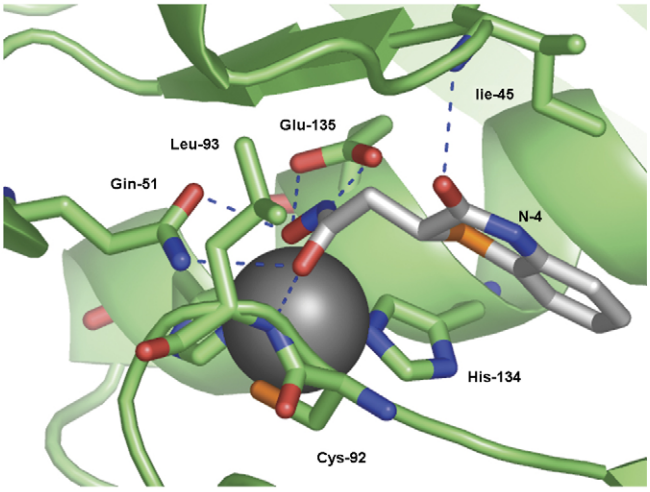


FIGURE 2

Nonpeptidic inhibition of peptide deformylase. The structure of a nonpeptidic inhibitor of peptide deformylase, compound **7**, bound to *Staphylococcus aureus* Ni–peptide deformylase (PDF), Protein Data Bank entry 1S17. The solvent exposed *N*-4 position of the benzothiazinone heterocycle, modified to improve antibacterial activity, is highlighted (representations were generated using PyMOL).

often used in the laboratory because of the instability of the ferrous ion in PDF – caused by rapid, irreversible oxidation to the inactive ferric species [65]. Research in this area continues to deliver potent inhibitors with antibacterial activity and enhanced stability over earlier pseudopeptidic inhibitors, as well as improved selectivity over the potentially harmful human matrix metalloproteinases (MMPs). A crystal structure (Figure 2) was obtained for compound 7 (Table 1), a potent inhibitor of *S. aureus* Ni–PDF (IC_{50} <5 nM), in complex with *Pseudomonas aeruginosa* Ni–PDF [66]. The identified hit failed to show significant antibacterial activity (with an MIC of 8 μg ml^{-1} against *Moraxella catarrhalis*, NB50012 strain). The low antibacterial activity of compound **7** was thought to be caused by poor cell penetration. The crystal structure of the Ni–PDF complex of compound **7** was used to identify regions for modification, enhancing antibacterial activity without the loss of potency against the target. A pentyl substituent on the solvent-exposed *N*-4 of the benzothiazinone of compound **7** retains potency (IC_{50} <5 nM against *S. aureus* Ni–PDF) and shows improved antibacterial properties with an MIC of 2 μg ml^{-1} against *M. catarrhalis* NB50012. Another non-peptide scaffold identified as a PDF inhibitor is compound **8** (Table 1, IC_{50} = 2.0 μM against *S. aureus* Ni–PDF) [67]. Compound **8** showed poor antibacterial activity, with an MIC of 16 μg ml^{-1} against an *E. coli* strain (EC-AH547) with reduced efflux activity. Docking studies investigating the crystal structure of PDF have suggested the direction of future synthesis – to increase potency by exploring the P2′ and P3′ pockets of the PDF site. A further non-peptide scaffold that inhibits PDF, compound **9**, has been identified in a virtual screen against soybean PDF2 (IC_{50} = 0.89 μM) [68]. Unsurprisingly, given the virtual screen was against the soybean active-site, the compound shows less activity against *E. coli* PDF (IC_{50} >30 μM). However, the success of the virtual-screening approach to soybean PDF suggests a virtual screen against a bacterial active site might be worthwhile.

Cell wall biosynthesis

Peptidoglycan is an essential bacterial cell wall polymer, composed of alternating units of UDP-*N*-acetylglucosamine and UDP-*N*-acetylmuramic acid, cross-linked via short peptide chains, and its role is unique to bacteria. The complex pathway for cell wall biosynthesis is well understood, conserved in Gram-positive and Gram-negative bacteria, and all the enzymes involved have, therefore, been considered potential targets for new antibiotics. The later polymerization steps of peptidoglycan are exploited by the antibacterial β-lactams, vancomycin and bacitracin. The early steps of biosynthesis of the peptidoglycan cytoplasmic precursor, *N*-acetylglucosamine-*N*-acetylmuramyl pentapeptide, (the Mur pathway) have been less exploited to date [69]. Structures are available for each of the enzymes in the Mur family of proteins and offer a rich area for research into novel antibacterials. A series of phenylthiazolyl urea and carbamate derivatives have been reported with activity against MurA and MurB. Compound **10** (Table 1) is an example of the thiazolyl urea series (IC_{50}s: 12.6 μg ml^{-1} *E. coli* MurA; 4.5 μg ml^{-1} *E. coli* MurB; and MIC = 1 μg ml^{-1} *S. aureus* GC 1131 MRSA). Docking studies were used to identify possible binding poses to MurB, and areas for further chemical elaboration of these series were proposed. However, it has been postulated that their antibacterial activity might only be, in part, caused by their inhibition of cell wall biosynthesis [70]. For MurB, an *in silico* approach to hit identification has been reported, almost a million commercial compounds have been screened for docking- and shape-complementarities to the available crystal structure [71]. 400 compounds were subsequently assayed against *Haemophilus influenzae* MurB and, after removal of promiscuous and intractable inhibitors, six hits of interest were identified with IC_{50}s ranging 4–76 μM. Similarity searching was employed to identify compounds structurally similar to best inhibitor and of the 50 compounds tested, two showed IC_{50}s <10 μM. A crystal structure of the complex of the most-potent of these compounds bound to MurB was used to purchase a further 300 analogues – to furnish SAR within the compound class before committing to a synthetic-chemistry campaign.

Crystal structures of the penicillin-binding protein (PBP) family, involved in the transglycosylation and transpeptidation steps of cell wall biosynthesis, are also available [72]. This remains a fertile area for research as resistance to the classic β-lactam inhibitors of cell wall biosynthesis emerges. A detailed understanding of the mechanism of β-lactam inhibition of PBPs can only aid in future drug discovery. In *ab initio* quantum mechanical and molecular mechanical (QM/MM) calculations, qualitative agreement was achieved with experimental kinetics for the rate of hydrolysis of β-lactams in a PBP, compared with a class C β-lactamase [73]. Close examination of the catalytic mechanism can only help future structure-based design to circumvent resistance developed through mutation of the PBP targets.

Metabolic pathways

Several metabolic pathways have been demonstrated as targets for antibacterials. In the folate pathway, dihydrofolate reductase

TABLE 2

The status of metabolic pathway targets recently described in the literature

Target	Organism	Status	Refs
Anthranilate synthase	*Serratia marcescens*	Virtual screen completed	[137]
Dihydrodipiclinate reductase (DHPR)	*Escherichia coli*	Structure-based design and NMR studies performed	[138]
EthR repressor of the *ethA* gene	*Mycobacterium tuberculosis*	X-ray structure determined	[139]
Methionine *S*-adenyltransferase (MAT)	*M. tuberculosis*	Homology model generated	[140]
Thymidine monophosphate kinase	*M. tuberculosis*	Virtual screen completed	[141]
Thymidine monophosphate kinase	*M. tuberculosis*	*De novo* drug design reported	[27]
Thymidylate synthase	*Lactobacillus casei*	Molecular dynamics completed	[142]
Thymidylate synthase	*L. casei*	Virtual screen completed	[143]
UDP-(3-O-(*R*-3-hydroxymyristoyl))-N-acetylglucosamine deacetylase (LpxC)	*Aquifex aeolicus*	X-ray structure determined	[144]

(DHFR) is well documented in several therapeutic areas, including antibacterials [74]. In a comparison of the effectiveness of diversity-driven library design and focused library design, *S. aureus* DHFR structure was used as the docking template. By prioritizing the focused library, based on the scores and binding modes of docked conformations, the focused set delivered a higher hit-rate and more-potent inhibitors than the diversity-driven library [75]. In a recent article on S-adenosylhomocysteine–methylthioadenosine nucleosidase (SAH–MTA nucleosidase), a highly conserved essential target in a range of pathogenic bacteria, the advantages of having a crystal structure early in the design process is described [76]. Inhibition of MTA nucleosidase activity is expected to attenuate the expression of virulence factors. Additionally, the accumulation of MTA nucleosidase substrates inhibits various bacterial methyltransferases, affecting the recycling of adenine and methionine, which is necessary for DNA and protein synthesis. The binding of a purine analogue was found to be different to adenine binding in the active site. Armed with this information, the structure-based design was able to deliver compound **11**, with a K_i of 2.8 nM and MIC ~12 μg ml^{-1} (no species data are reported). Attempts to improve the affinity of compound **11**, by introducing hetero atoms to the phenyl ring – reducing internal steric clashes observed in the crystal structure of **11** bound to MTA nucleosidase, were unsuccessful. The reasons behind this failure are unclear but demonstrate that we remain someway short of fully understanding of the process of ligand recognition.

Table 2 lists some of the recent publications on antibacterial targets in a range of biosynthetic pathways. Metabolic pathways, such as fatty acid biosynthesis and the non-mevalonate pathway, have come under increasing scrutiny with regard to representing targets that are unique to bacteria.

Fatty acid biosynthesis is an essential metabolic process in all life-forms and is required for cell viability and growth, as well as being a target of interest in antibacterial research [77]. Eukaryotes utilize large multifunctional proteins (termed type I fatty acid synthetases), whereas prokaryotes have several discrete enzymes (type II fatty acid synthetases), presenting an opportunity for type-specific selectivity. The first committed step of the biosynthetic process in bacteria involves β-ketoacyl-ACP synthase FabH, a highly conserved enzyme across Gram-positive and Gram-negative organisms and the target for the antifungal compounds cerulenin and thiolactomycin [78]. Nie *et al.* [79] have published a comprehensive study of hit identification and validation against FabH. A combination of substructure, 2D similarity and pharmacophore searches complemented by virtual docking resulted in the selection of 2500 compounds (for testing against FabH) from commercial-supplier collections. Those with IC_{50} <10 μM were then taken forward to crystallography. Structure determination was combined with modelling and synthesis to deliver significant advances in potency, generating low-nanomolar inhibitors, for example compound **12** (Table 1), which has an IC_{50} of 0.27 μM against *E. faecalis* and an MIC of 5.6 mg ml^{-1} against *S. aureus* MRSA 703. During the course of the study, unforeseen structural differences in some pathogenic FabH enzymes highlighted the importance of a detailed structural understanding of the FabH active-sites to aid the design of broad-spectrum antibacterials. FabI, the target of the antituberculosis drug isoniazid and the widely used antimicrobial triclosan, remains of considerable interest in antibacterial drug discovery [80,81]. Using high-resolution structures as a template to guide inhibitor design, FabI inhibitors with antibacterial activity against methicillin-resistant *S. aureus* (MRSA) and vancomycin-resistant *S. aureus* (VRSA) have been identified [82].

The non-mevalonate methylerythritol phosphate pathway for isoprenoid biosynthesis is essential in Gram-negative bacteria, but is not found in humans. The first enzyme in the pathway, 1-deoxy-d-xylulose-5-phosphate reductoisomerase (DXR), is the rate-limiting step and the target for the antimalarial compound fosmidomycin [83]. The recent release of a high-resolution structure of *E. coli* DXR, and detailed docking analysis of fosmidomycin, will undoubtedly lead to further structure-based design against this target – and the remaining targets in the non-mevalonate pathway look potentially interesting for future study [84,85].

Cell division

The bacterial cell division cycle represents an underexploited area of research in the search for new antibacterials. Inhibition of cell division would ultimately lead to cell death; however, a detailed understanding of the events involved in cell division is still under

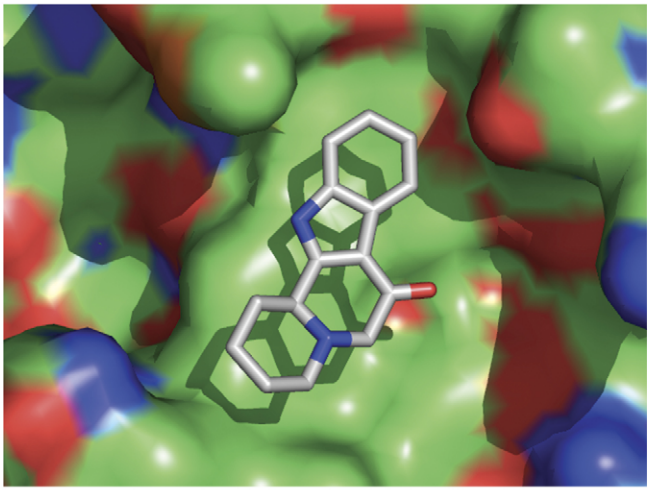


FIGURE 3

Fragment screening of inhibitors of bacterial cell division. The structure of the fragment inhibitor, compound **13**, is shown bound to ZipA at the FtsZ binding site (Protein Data Bank entry 1SIJ). The ZipA binding site is shown as a surface representation to highlight the predominantly shape–fit-driven binding of compound **13**. The surface is colour-coded by element (green for carbon, blue for nitrogen and red for oxygen), showing the predominantly hydrophobic nature of the binding site (representations were generated using PyMOL).

scrutiny. Currently, one of the most studied enzymes with a crucial role in bacterial cell division is FtsZ. FtsZ forms a polymeric ring that constricts when cells divide. Nucleotide hydrolysis is the rate-limiting step in bacterial cell division; therefore competitive inhibitors binding at the GTP site could offer a route to novel antibacterial candidates [86]. FtsZ recruits a series of proteins in the sequential steps required for *E. coli* cell division and inhibition of the protein–protein interaction between FtsZ and ZipA has been reported as a potential route to novel antibacterial therapy [87]. Combining crystallographic information (Figure 3) obtained from the protein–inhibitor complexes of fragment hits of ZipA, such as compound **13** (IC_{50} = 1170 μM and MIC = 128–256 μg ml^{-1}, Table 1), improvements in potency have been observed inhibiting the ZipA–FtsZ binding event. Whether this activity is solely related to ZipA–FtsZ remains unclear but this study has led to further rounds of designing modulators of the protein–protein ZipA interactions [88]. The indoloquinolizinone scaffold of compound **13** offers limited scope for chemical diversification. Replacing this scaffold with other scaffolds, such as the 2-(piperidin-3-yl)-1*H*-indole of compound **14**, allows for an explosion in the combinatorial chemistry [89]. Compound **14** demonstrates an IC_{50} of 296 μM and, in a *zipA* gene-knockout *E. coli* strain (completed with ZipA on a thermosensitive plasmid), demonstrates an MIC of 6.25 μg ml^{-1}.

More-recent studies have used the bioactive conformation of an alternative class of inhibitor (taken from the protein–inhibitor crystal structure) to identify further hits by shape comparison, so-called scaffold hopping. The method uses a calculated 3D volume for the query molecule, searching a multi-conformer database for molecules with similar shapes. This was followed by evaluating van der Waals contacts between the hits and the protein to remove compounds with obvious steric clashes. The new scaffolds would have been missed by 2D similarity searches but offer new chemical starting points that lack the developmental issues associated with the original hit series. Compound **15** (Table 1) is a new scaffold, identified by the shape–fit comparison. Although the K_d for compound **15** is weaker than the original HTS hit (79 μM compared with 7 μM), the compound is smaller and does not show the same cytotoxic effects, and has less intellectual property concerns [90].

Targeting virulence factors

Virulence factors are a collection of essential bacterial genes expressed at defined periods of the infection cycle. The transient expression of virulence factors during the infection cycle has made them less attractive as targets for broad-spectrum antibacterial drug discovery [91]. However, in the fight to identify novel antibacterial targets against specific and 'difficult' pathogens, such as *Bacillus anthracis*, virulence factors have generated considerable interest. Spore-forming *B. anthracis* secretes three major toxins: edema factor (EF), protective antigen (PA) and lethal factor (LF) [92]. These key virulence factors of anthrax pathogenesis have come under close scrutiny in the hunt for effective postexposure agents in the treatment of anthrax inhalation.

EF is an adenylcyclase that raises the concentration of the secondary messenger, cyclic adenosine-3′,5′-monophosphate (cAMP), to pathological levels. Inactivating the mutation of EF reduces the survival of germinated anthrax spores, indicating an active role of EF in anthrax infection. Docking studies have identified specific binders against the ATP site of EF, with IC_{50}s as low as 60 μM for specific inhibition [93]. The crystal structure of the cellular metabolite of the anti-hepatitis B compound adefovir, compound **16** (Table 1 and Figure 4a) 9-[2-(phosphonomethoxy)ethyl]-adenine diphosphate (PMEApp, K_i = 27 nM), elucidates the molecular basis for the 10,000-fold-higher affinity EF has to PMEApp over its natural substrate, ATP [94].

EF is activated 1000-fold by binding to calmodulin (CaM) and the structure of the EF–CaM complex reveals a protein–protein interaction distinct from other CaM-binding proteins, suggesting that a small molecule interacting with the EF–CaM binding site might not interfere with other CaM-binding events [95,96]. A combination of cell-based assays, that monitor EF-induced morphology changes in murine andrenocortical cells, and surface-plasmon resonance spectroscopy, observing the blocking of EF to immobilized CaM, has identified compound **17** (Table 1) as an inhibitor of EF–CaM binding, with an IC_{50} of 10–15 μM. Analysis by a combination of photolabelling and mass spectroscopy of protease-derived fragments of EF has identified potential binding sites for small-molecule interaction [97].

PA performs the role of a common binder–promoter to EF and LF, and the first step in anthrax intoxication involves PA binding to the anthrax-toxin receptor of the host cell [92]. PA is then cleaved into two fragments with the large PA_{63} fragment undergoing self-assembly, binding to EF or LF and allowing passage via several steps into the cytosol. Peptides generated (by phage display) against PA_{63} inhibit LF binding and their NMR structures could offer a route to peptidomemetics and future therapy [98].

LF is an MMP that can cleave and inactivate a family of mitogen-activated kinase kinases, including mitogen-activated kinase,

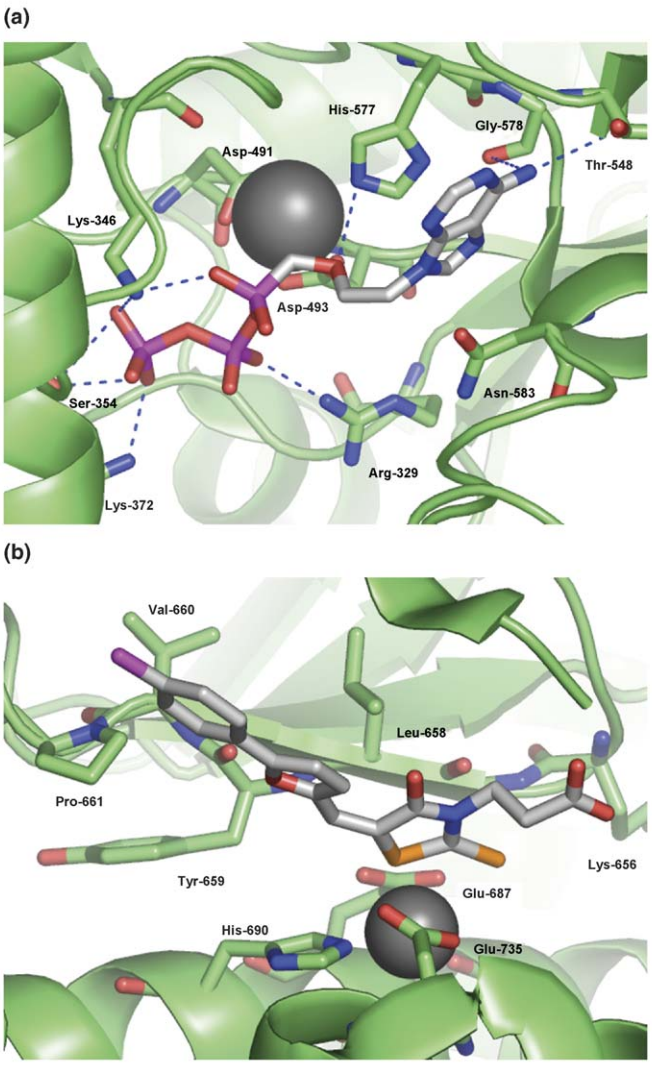


FIGURE 4

Small-molecule inhibition of the virulence factors of *Bacillus anthracis*. (a) The crystal structure of the metabolite of the anti-hepatitis B compound adefovir bound to the anthrax edema factor (Protein Data Bank entry 1PK0). The higher affinity of PMEApp for edema factor (EF) (compared with ATP) results from the additional H-bonds between the methoxy group and His-577, as well as the close association of adenine with Asn-583. **(b)** The crystal structure of an inhibitor derived from an NMR fragment screen, compound **18**, bound to the anthrax lethal factor (Protein Data Bank entry 1ZXV). The small molecule interacts with the zinc atom via a sulfur atom. Additional interactions are hydrophobic in character (representations were generated using PyMOL).

extracellular signal-regulated kinase kinase and p38 kinase [99,100]. Their inactivation will probably result in overexpression of certain lymphokines in macrophages and cell death through a type of sceptic shock [101]. To allow rapid entry into medicinal chemistry, LF has been assayed via an NMR fragment screen using a ^{19}F labelled peptide (Ac-A-R-R-K-K-V-Y-P-NH-Ph-CF_3). Cleavage of the peptide at the P–X effects the ^{19}F chemical environment, allowing the LF kinetics to be studied by monitoring the signals of uncleaved peptide and reaction product. Subsequent crystal structures have been used to rationalize the potency of designed inhibitors [102]. Compound **18,** with a $K_i = 0.8$ μM, (Table 1 and Figure 4b) was found to be the most potent analogue and taken into a study in mice – co-administered in combination with ciprofloxacin (the conventional antibacterial treatment). The combination therapy showed 40% protection against the Sterne strain of *B. anthracis*, compared with only 20% protection in the conventional ciprofloxacin regime and 100% mortality in the untreated control.

Responding to resistance

There are several mechanisms that bacteria employ to achieve resistance to antibiotics. The most significant are: mutations in the drug target-site; overproduction of the drug target; the modification of cell permeability by the loss or mutagenesis of porins; the upregulation of expression of efflux pumps that export the drugs; and inactivation of drugs by enzymatic degradation [103–107].

For the β-lactams, cephalosporins and carbapenems changes in cell permeability and efflux contribute to reduced levels of drug reaching the target. The mutation of the target, the PBP, plays a role in reducing affinity [72,108]. However, the most significant threat to β-lactams has evolved through the degradation of the drugs by the overexpression of one of the four classes of β-lactamase enzymes (A–D), which deactivate the drugs by hydrolyzing the lactam bond. With significant structural data available on the β-lactamases (examples of all four classes represented in the Protein Data Bank), interest continues in designing compounds with enhanced stability to β-lactamase degradation [109,110]. The class A penicillinases have been studied in detail to understand the evolution of their spectrum of activity, by molecular dynamic simulation [111]. The results identify the underlying reasons behind the Class A preference for binding penicillin over cephalosporins, caused, in part, by penicillin's greater efficacy in binding to the oxyanion and carboxylate pockets simultaneously. This level of understanding will aid future design of antibacterial agents.

The β-lactam scaffold is a natural product and bacteria have had extensive opportunities to evolve sophisticated mechanisms of resistance. To circumvent the rapid appearance of drug resistance, interest has flourished on the identification of non-β-lactam leads that inhibit β-lactamase but do not upregulate the β-lactamase enzymes [112]. Following on from previous work employing virtual screening to identify hits against the Class C β-lactamase AmpC, derivatives have been synthesized that retain the desirable lead-like properties while increasing potency, by up to 26-fold, by targeting polar interactions (e.g. compound **19,** $K_i = 1$ μM, Table 1 and Figure 5). In comparison to traditional β-lactam inhibitors, such as clavulanate and cefoxitin, compound **19** inhibits β-lactamase without inducing upregulation.

Aminoglycosides are another series of natural product-derived antibacterial agents where a variety of resistance mechanisms reside in the biosphere. These agents bind to the 30S ribosomal subunit, inhibiting initiation of protein synthesis or causing code misreading [113]. The most prevalent mechanism of resistance in aminoglycoside-resistant clinical strains occurs through the expression of enzymes capable of acetylating either the 6′- or 3′-amino substitutents [114]. It is interesting to postulate whether the elucidation of the structures of aminoglycoside *N*-acetyltransferase, such as that of *Salmonella enterica*, will lead to new inhibitors preventing the covalent modification of aminglycosides [115]. Structures of this type might become the basis of design for future

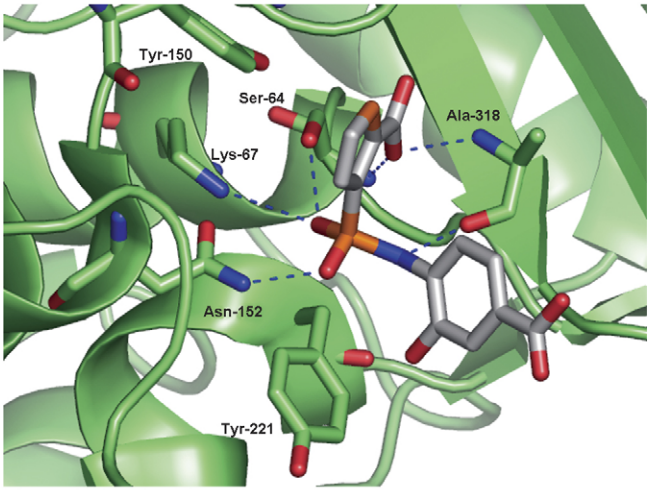


FIGURE 5

Non-β-lactam inhibitors of β-lactamase. The crystal structure of the non-β-lactam inhibitor, compound **19**, bound to *Escherichia coli* β-lactamase AmpC (Protein Data Bank entry 1XGJ). The binding of compound **19** is the result of a series of H-bonds, shape complementarity and an edge-to-face interaction between Tyr-221 and the 2′-hydroxy benzene of compound **19** (representations were generated using PyMOL).

inhibitors returning pathogen sensitivity to hitherto compromised aminoglycoside agents.

Multidrug resistance in bacteria can be achieved by active efflux of structurally unrelated compounds by membrane proteins – multidrug resistance proteins (MDRs) [116]. The problem of efflux is common to anti-infective and anticancer studies and the identification of chemosensitizers to rescue efflux-compromised drugs is of significant clinical interest [117,118]. On the basis of bioenergetic and structural data, multidrug transporters can be divided into two classes. Secondary multidrug transporters use the transmembrane electrochemical gradient of protons or sodium ions to power the expulsion of drugs. ATP-binding cassette (ABC) transporters exploit ATP hydrolysis to fuel drug extrusion from the cell. Most bacterial multidrug efflux systems known to date are sensitive to agents that disrupt the transmembrane proton-motive force, indicating that they are dependent on the electrochemical gradient for activity. However, specific antibacterials are transported out of cells by ABC-type efflux proteins [119–122]. From a structural biology perspective, membrane proteins present a significant challenge. Membrane proteins are often difficult to overexpress in the quantities and purity required for 3D crystallization and, even when suitable protein is available, the amphiphilic nature of the proteins makes crystallization nontrivial. Structural data are limited to <100 unique membrane protein structures. However, the number of new membrane structures is continuing to rise steadily and the available structures include several examples of efflux transporters that have stimulated interest in modelling studies of membrane transporters [123].

Determination of the structures of ABC transporters (MsbA of *E. coli* and *Vibrio cholera* and BtuCD of *E. coli*) has given insight into the structure and function of ABC pumps [124–127]. The crystal structures of several proton-driven transporters (all from *E. coli*) are available and include AcrB, an antiporter belonging to the resistance-nodulation cell-division (RND) class, LacY and GlpT, members of the major facilitator (MF) class, and EmrE, a member of the small-multidrug-resistance (SMR) transporter class [128–131]. Unfortunately, none of these structures was determined with bound ligand and they provide only limited information on the substrate–transporter interaction.

At this time, perhaps the most informative study of the broad specificity of ligand binding of efflux transporters comes from the study of regulation of efflux expression. BmrR is a transcriptional regulator of the *B. subtilis* multidrug transporter Bmr (and BmrR is encoded by a gene immediately downstream of *bmr*). Ligand-binding to BmrR increases the affinity of BmrR to the *bmr* promoter, enhancing transcription of the *bmr* gene [132]. The ligand-binding C-terminal portion of BmrR retains comparable ligand-affinities to the full-length protein and has been studied by X-ray crystallography [133]. The structural evidence indicates the broad specificity of BmrR ligand-binding, essential for its function as an upregulator of Bmr, is driven by van der Waals forces and π-stacking through hydrophobic and aromatic residues, rather than hydrogen bonds [134]. The inherent flexibility in ligand recognition of efflux transporters is potentially a significant challenge for structure-based design of chemosensitizers.

More recently, structures of interest for rational design include the publications of the trimeric TolC protein of *E. coli* [135] in complex with hexamine cobalt and the periplasmic adaptor MexA from *P. aeruginosa* [136]. Combined, these structures represent an example of two-thirds of the tripartite multidrug efflux pump, which removes small molecules such as antibacterials from Gram-negative bacteria. The entrance to the TolC 140 Å pore, which spans the outer membrane and periplasm, of the TolC family is highly conserved and could be a useful target for the future development of inhibitors against multidrug-resistant pathogenic bacteria. The inhibited form of TolC shows little structural difference to nonliganded protein, suggesting it might be a robust model for structure-based design. Equally, by analogy to other structures, the β-barrel of MexA (capped by an α-helix) might be involved in ligand binding and represents a new target for inhibition of the efflux pumps of pathogenic bacteria.

Conclusions

There remains a constant need for new antibacterial agents and this is driven by the emergence of resistance. With the large companies moving out of antibacterial drug discovery there is an opportunity for new companies to deliver. For the companies championing the field of antibacterial research the challenge is how best to pursue new targets.

To reach the goal of novel antibacterials, whether against validated or novel bacterial targets, many researchers are exploiting structure-based design tools as an effective route for drug discovery. Where a structure of the drug target is available, fragment or virtual screening can be used to give rapid and novel entry points to medicinal chemistry programmes. Structure-based design then allows the prioritization of chemical synthesis, with medicinal chemistry focusing on key synthetic stratagems in an efficient manner. In practice, only a few compounds should need to be synthesized and tested. With the structure of the drug target acting as the template, vast numbers of molecules can be tested *in silico*, enriching the final selection of small numbers of active

molecules to be synthesized. When combining bioinformatics with structural biology, essential areas of protein structure can be exploited in the molecular design process, leading to further potential benefits in the clinic by potentially slowing the emergence of resistance from drug-target mutagenesis. However, the key to modern, efficient drug discovery is to deploy multivariable optimization and for researchers to have all the tools for a given problem at their disposal. For novel antibacterial design, rapid increases in potency and selectivity, driven by the close interaction of structural biologists, molecular modellers and medicinal chemists, can be achieved. These advances need to be complemented with pragmatic usage of cellular assays, early ADME–Tox and *in silico* tools – to improve antibacterial activity, allowing efficient and rapid progress from target to new drug.

A challenge remains to convert *in vitro* potency, driven forward by structure-based design, to *in vivo* antibacterial activity. The questions of cell penetration and efflux require careful consideration. A lack of antibacterial activity in compounds with good IC_{50}s against a target is often attributed to poor cell-penetration. A better understanding of the processes of drug entry and expulsion from bacteria is essential if novel compounds designed *in silico* are to deliver antibacterial activity and move forward into the clinic.

The structural genomic initiatives are amassing a wealth of data, fuelling the process of novel antibacterial drug discovery and, in the fullness of time, new compounds should, therefore, emerge into the market place. Whether these new antibacterial compounds will then be made available for clinical use or held in reserve remains to be seen.

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