

# The Use of Structure-Guided Design to Discover New Anti-Microbial Agents: Focus on Antibacterial Resistance

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**Abstract:** Serious attempts to address antibiotic resistance, a worldwide public health concern, have recently become more intensive. In hospital settings, resistance to antibacterial agents has been recognized by clinicians for several decades. Resistant strains are now isolated on a daily basis from patients with community-acquired infections further elevating the level of concern among public health officials. The pharmaceutical industry has generally focused its attentions on chronic therapeutic indications in recent years (e.g. cardiovascular and metabolic diseases), but will likely be forced to re-engage in antibacterial discovery efforts as therapeutic options diminish for the treatment of infections caused by multi-drug resistant pathogens. The ability to squeeze additional utility out of known classes of antibacterial agents has become limited and antibacterial discovery scientists will need to focus on new approaches and targets. These new approaches will need to include strategies that explicitly address resistance up front and simultaneously attempt to facilitate the slower development of resistance as new compound classes enter clinical use. One approach that can be a useful component of antibacterial discovery efforts and prospectively address resistance is structure-guided design (SGD). This review will describe several recent examples in which SGD was applied as part of a multidisciplinary effort to address antibacterial resistance. These include dihydrofolate reductase inhibitors, broad-spectrum  $\beta$ -lactamase inhibitors, novel oxazolidinones, aminoglycoside mimetics, peptide deformylase inhibitors, and inhibitors that simultaneously target DNA gyrase and topoisomerase IV.

**Keywords:** Antibiotic resistance, antibiotic drug discovery, resistance mechanisms, structure-guided design, dual targeting

## INTRODUCTION

The pharmaceutical industry is falling behind in the race to develop antibiotics to treat infections caused by emerging drug-resistant pathogens, with fewer and fewer novel antibacterial agents entering into clinical use [1-4]. Indiscriminate use of antibiotics over many years has created a situation where “superbugs” are no longer the rarity, but rather commonly factored into the clinician’s empiric antibiotic selection. The prevalence of multi- and pan-resistant pathogens in both hospital and community settings is of great concern. Gram-negative bacteria posing the most serious treatment challenges include *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Stenotrophomonas maltophilia* and *Acinetobacter baumannii*, with increasing incidence of drug-resistant isolates, and fewer active antibiotics from which to select [5-7]. Among Gram-positive pathogens, the drug-resistant pathogens of greatest concern are methicillin-resistant *Staphylococcus aureus* and *Staphylococcus epidermidis*, vancomycin-resistant *Enterococcus faecium* and *Enterococcus faecalis*, as well as multidrug-resistant *Mycobacterium tuberculosis* [8, 9]. Given the current global resistance crisis, addressing the medical need for new antibiotics with activity against drug-resistant pathogens should be a high priority for the pharmaceutical industry.

Antibiotics are used to treat diseases caused by a wide variety of bacterial pathogens. Since their introduction into

widespread use in the early 1940’s, a good part of the clinical success of antibiotics is due to the fact that most bacterial pathogens are highly related at the level of their essential metabolic machinery, where most antibiotics act. In recent years, the explosion of microbial genomic information has revealed that there theoretically exists a wide variety of *potential* unexploited targeting opportunities in essential metabolic pathways and processes [10]. Today, antimicrobial drug-discovery scientists can use information garnered from genome sequencing, knowledge of three dimensional structure, and predictive modeling, in the selection of theoretically “druggable antibiotic targets” with high potential for broad antibacterial spectrum and low potential for inhibition of host machinery.

Despite the abundance of theoretical targeting opportunities in the bacterial genome, there has been limited success in converting these opportunities into successful drug development programs. This is in part due to the unique challenges in developing novel antibacterial agents in contrast to other classes of therapeutics. Variability in antibiotic susceptibility among bacterial pathogens is a key challenge in antibacterial discovery/development and is not only due to genetic variations at the level of the molecular target, but also to intrinsic and acquired resistance mechanisms that pose significant impediments to effectiveness of antibiotics. A better understanding of the molecular basis of many important antibiotic resistance mechanisms has highlighted opportunities where structure-guided design (SGD) can be applied as a powerful tool in the discovery of novel antibacterials to counter these mechanisms.

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## FACTORS AFFECTING ANTIBIOTIC SUSCEPTIBILITY

The majority of clinically used antibiotics essentially target three macromolecular biosynthetic pathways: cell wall, nucleic acid (DNA and RNA), and protein. Bacteria can become less sensitive, or resistant, to a wide variety of chemically unrelated antibacterial agents by three general mechanisms: mutational, acquired, and intrinsic.

### Mutational Resistance

Mutations in the bacterial genome may impact the structure or regulation of the molecular target, efflux pumps or permeability factors, conferring clinically relevant antibiotic resistance phenotypes [11]. The frequency at which antibiotic resistance will arise in a given bacterial population is generally reliant on the level of antibiotic exposure, the expression of acquired and intrinsic mechanisms, and the number of ways in which an organism's genome can mutate to resistance while maintaining viability under the selection conditions [12-14]. When mutations in the bacterial genome specifically result in key amino acid changes in the binding site of an antibacterial agent, SGD can be a useful tool in attempting to "design around" or re-optimize binding interactions that may have been perturbed by a particular mutation.

Typically during infection, bacterial loads can reach approximately  $10^{10}$  organisms [13]. Antibiotic resistance rates are usually defined as the frequency that phenotypically resistant mutant organisms will arise in a given population in culture [12]. Therefore, in larger bacterial populations, there is greater chance that an antibiotic resistant mutant will be present and survive antibiotic exposure. It has been noted that the majority of successful monotherapeutic antibiotics are "multi-targeting", inhibiting more than one essential target. This suggests that slower resistance development attributed to the reduced likelihood that a double mutant will be present during an infection may be a key factor for prolonging the clinical utility of an antibacterial agent [15]. As another strategy to suppress the emergence of antibiotic resistant organisms during the treatment of infection, Drlica has suggested the idea of dosing antibiotics to maintain a "mutant prevention concentration (MPC)" [13, 14]. The MPC is defined as the minimal inhibitory concentration (MIC) of the least susceptible single-step mutant. This concept is most applicable to antibiotic classes such as the fluoroquinolones for which target-based resistance mutations observed in laboratory experiments are the same mutations observed in the clinic. In such cases, one can estimate the *in vivo* MPC from *in vitro* experiments [16]. The MPC is expected to be less applicable for other antibiotic classes in which acquired resistance is the major clinically relevant resistance mechanism [17].

### Acquired Resistance

The widespread use of antibiotics derived from natural products has led to the evolution of acquired resistance mechanisms in some clinical pathogens. These resistance mechanisms include covalently altering the molecular target, covalently inactivating the antibiotic, expression of a trans-

missible efflux pump, or providing an exogenous bypass mechanism. They are thought to have evolved as defenses against antimicrobial compounds produced in environmental bacterial populations. One application of SGD to address acquired resistance might be in the optimization of novel antibacterial agents that are less susceptible to known inactivating enzymes or that possess interactions with the molecular target that are unaffected by such covalent target modifications. The most prevalent types of clinically acquired resistance mechanisms for aminoglycoside,  $\beta$ -lactam, and macrolide antibiotics are encoded by genes present in transmissible extrachromosomal genetic elements such as plasmids or transposons. These mechanisms rapidly disseminate in bacterial populations and are constantly evolving in the scope and degree in which they confer antibiotic resistance. Just as antibiotic exposure applies pressure to select for mutations in the genome of a given pathogen, it also applies pressure to select for variants of acquired resistance mechanisms against wider and wider varieties of antibacterial agents. Thus, the commonly employed "next generation" strategy of deriving new antibiotics from pre-existing classes is likely to select for the next generations of the very resistance mechanisms the new antibiotics are designed to prevent.

Aminoglycoside antibiotics such as gentamicin and tobramycin bind directly to the ribosome and inhibit bacterial translation. Enzymes capable of inactivating aminoglycoside antibiotics by O-phosphorylation, N-acetylation, or adenylation are usually found on transmissible plasmids in both Gram-negative and Gram-positive pathogen populations [18,19]. These covalent modifications render aminoglycosides unable to efficiently bind to their molecular target on 16S rRNA ("A-site"). In addition to drug-inactivation mechanisms, a series of methylases capable of covalently modifying a key nucleotide in the A-site target of aminoglycosides, disrupting drug-target interactions, have been described recently in Gram-negative pathogens and found to be associated with transposon and transmissible plasmids [20,21]. The antibacterial activities of the macrolide-lincosamide-streptogramin B and oxazolidinone families of translation inhibitors are affected by acquired efflux-mediated mechanisms as well as transmissible methyltransferases that inhibit drug binding by covalently modifying the 23S rRNA target. While both of these resistance mechanisms have been identified in a multitude of organisms, the clinical impact is most relevant in Gram-positive bacteria such as *streptococci* and *staphylococci* [22-24]. For the cell wall inhibiting  $\beta$ -lactam antibiotic class (including cephalosporins, carbapenems and monobactams), high-level resistance can occur via production of secreted  $\beta$ -lactamases that hydrolyze this class of antibiotics. Over 530  $\beta$ -lactamase enzymes have been reported to date; these enzymes can be encoded by genes present on the chromosome, transmissible plasmids or transposable elements and vary in their substrate specificity for various subclasses of  $\beta$ -lactam antibiotics [25].

Recently, two new plasmid-based mechanisms of fluoroquinolone resistance found in Gram-negative clinical isolates have shown that synthetic antibacterial agents such as the quinolones are also vulnerable to the emergence of acquired resistance [26].

## Intrinsic Resistance

Most bacterial pathogens are capable of reducing their susceptibility to antibiotics by altering the permeability of their cell envelopes, upregulating a battery of multi-drug efflux systems, and switching to phenotypically antibiotic-tolerant states [27-30]. Chromosomally encoded multi-drug efflux pumps are present in both Gram-positive and Gram-negative organisms and significantly contribute to clinically relevant reduced susceptibility to a wide spectrum of structurally unrelated antibiotic classes. In addition to the broad substrate recognition properties of multi-drug efflux pumps, the outer membrane itself has proven to be a highly significant barrier to the design of novel Gram-negative antibiotics. For Gram-negative pathogens such as *Escherichia coli* and *P. aeruginosa*, antibiotics not only need to avoid efflux mechanisms, but also traverse outer membrane lipopolysaccharide or transport through porins to gain access to their intracellular targets. While many researchers have attempted to elucidate the physicochemical properties that promote efficient antibiotic uptake into bacteria, the ability to incorporate this knowledge into novel, synthetic antibacterial compounds has been difficult.

## HOW WILL WE DISCOVER THE ANTIBACTERIAL AGENTS OF TOMORROW?

Ideally, new chemical classes of antibacterial agents with new mechanisms of action would be the optimal approach towards combating resistance. This has been quite challenging for reasons stated above and modification of known classes of antibacterials still remains an active area of research and development. While genomics has offered some new targets to consider, translation into practice has been quite limited thus far. Classic approaches used in the discovery of antibacterial agents have also fallen short. However, the past two decades have shown a maturity in SGD to the point where it has been routinely applied as a useful lead discovery and optimization tool in a variety of therapeutic areas [31]. It should, therefore, not be surprising that this approach has also found some utility in the discovery and optimization of new antibacterial agents. Since 2005 there have been several hundred publications wherein SGD was used in some capacity in antibacterial discovery programs [32]. This is consistent with data from 2005 suggesting that approximately 250 structures existed in the PDB at that time for a variety of potential antibacterial targets [33]. A review published in 2006 [34] nicely describes some of the work in the preceding years. In this section we will attempt to highlight some of the more recent work and we will focus on some interesting examples of SGD that specifically and prospectively address resistance.

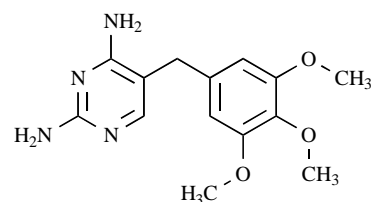
## HIGHLIGHTED EXAMPLES OF SGD THAT SPECIFICALLY ADDRESS RESISTANCE

Perhaps it is not surprising that the examples chosen to highlight below involve well-known, validated and essential bacterial targets. These targets have been the focus of antibacterial discovery efforts for some time and the application of SGD represents a logical progression in terms of both necessity and maturity of SGD. It is clear that high-throughput screening (HTS) itself has provided limited re-

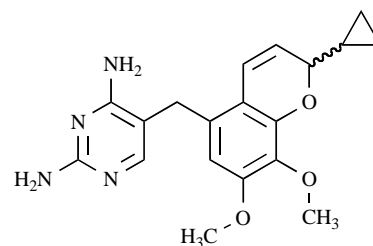
turn on investment with some estimates suggesting that the success rate from antibacterial HTS was 4- to 5- fold lower than for other therapeutic targets [35]. The examples below highlight cases wherein SGD was employed as part of a multifaceted approach to address specific aspects of resistance.

## Designing Around Specific Target Site Mutations: Iclaprim and Dihydrofolate Reductase

Dihydrofolate reductase (DHFR) is an essential bacterial enzyme involved in tetrahydrofolate biosynthesis and has been a drug target for over 40 years [36]. Trimethoprim (TMP), Fig. (1), is a DHFR inhibitor that ultimately causes bacterial death via prevention of nucleic acid synthesis. DHFR is one of the earliest targets (of any therapeutic class) to which SGD has been applied. Elegant work by researchers at Burroughs Wellcome employed structural information to better understand the enzyme mechanism of DHFR as well as the key determinants of binding for the diamino-pyrimidine class of which TMP is the charter member [37-38]. They also used SGD in an attempt to improve the potency and pharmacological profile of TMP [39].



Trimethoprim (TMP)

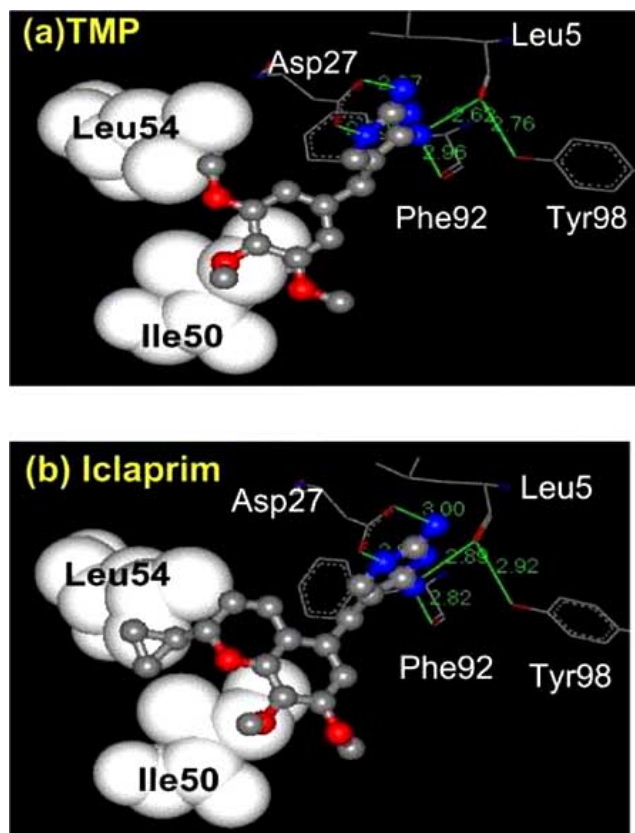


Iclaprim

Fig. (1). Chemical structure of Dihydrofolate reductase inhibitors.

Through long-term clinical use, the emergence of bacterial strains resistant to TMP has occurred through point mutations in the TMP binding site as well as DHFR bypass enzymes [40]. One such single site target-based mutation that has caused clinically relevant TMP resistance in *S. aureus* is the substitution of phenylalanine with tyrosine at position 98 [41-42]. The crystal structure of this mutant enzyme suggested that this mutation results in loss of a hydrogen bond strength between the 4-amino group of TMP and the carbonyl oxygen of Leu5, Fig. (2a) [40]. Researchers at Roche used this detailed structural knowledge in conjunction with molecular modeling approaches to design modified diamino-pyrimidines that would compensate for the loss of binding affinity due to this single site substitution of Phe98 to Tyr98. The result of their efforts is a compound, iclaprim, Fig. (1), that gains new hydrophobic interactions between the iclaprim cyclopropyl group with Ile50 and Leu54, Fig. (2b) [40, 43]. In terms of DHFR inhibition, Iclaprim is equipotent to

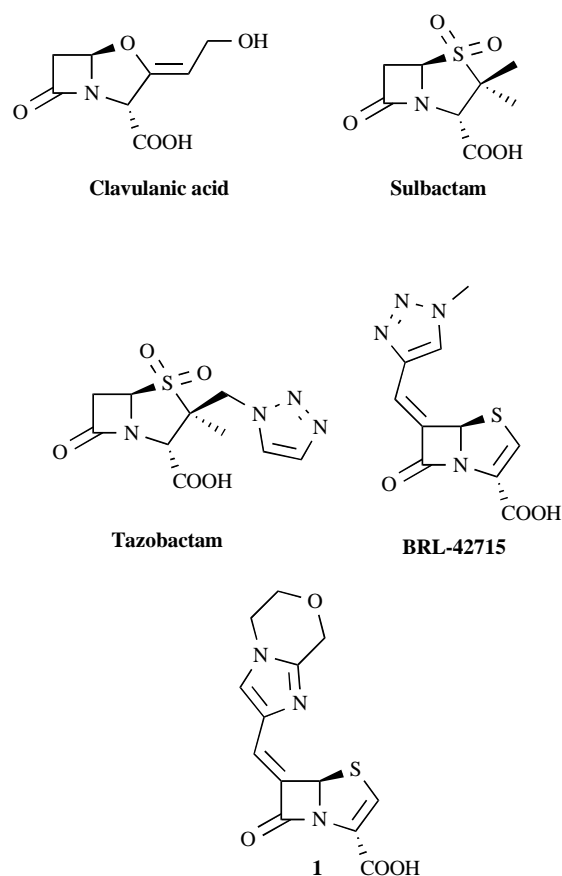
TMP ( $IC_{50} < 10$  nM against *S. aureus* and *E. coli*). Antibacterial potency has also been improved over a range of Gram-positive and Gram-negative organisms (e.g. MRSA  $MIC_{90} = 0.06$   $\mu\text{g/mL}$  vs.  $8.0$   $\mu\text{g/mL}$  for TMP)[43]. Iclaprim was licensed to Arpida and has recently completed a Phase III clinical trial in complicated skin and skin structure infections.



**Fig. (2).** Differences in binding to mutated *S. aureus* DHFR between trimethoprim and iclaprim. Iclaprim (b) shows additional hydrophobic contacts with Ile50 and Leu54 relative to trimethoprim (a), reprinted from ref. [40].

### Design of Broad-Spectrum $\beta$ -Lactamase Inhibitors Based on Knowledge of a Key Reaction Product

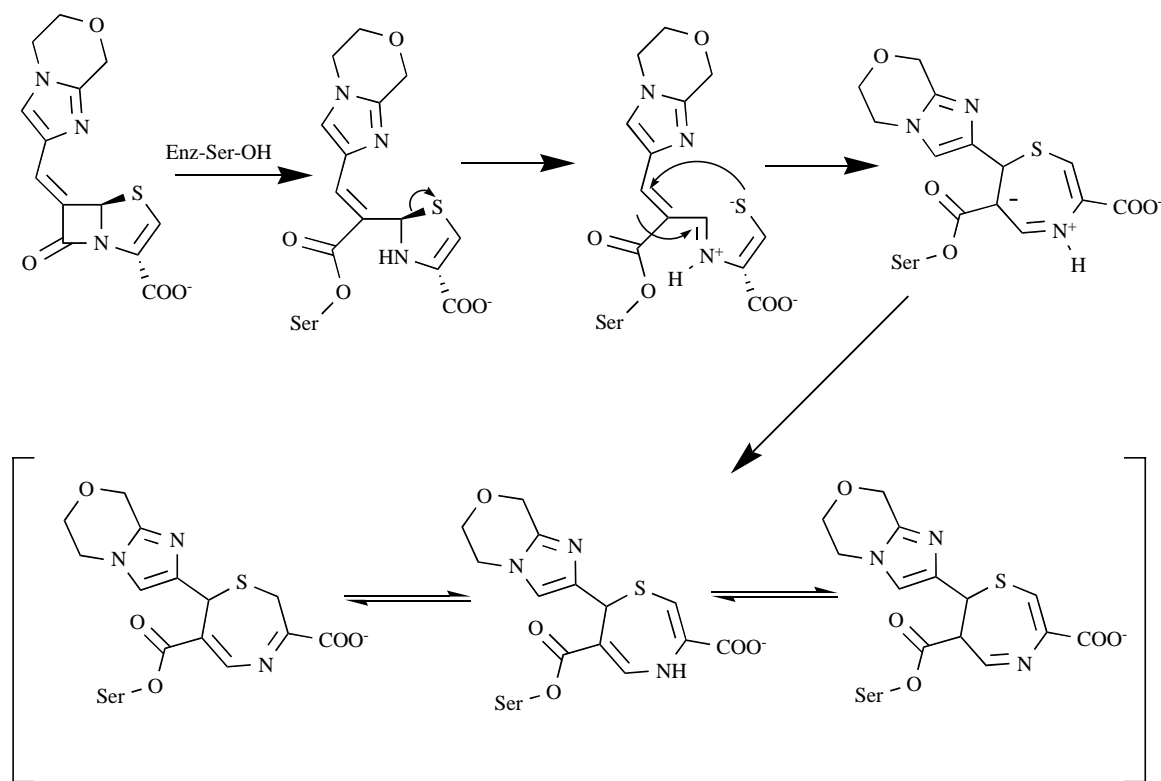
Among the most common types of acquired resistance examples are the  $\beta$ -lactamases. These enzymes are divided into two categories, serine- $\beta$ -lactamases (classes A, C, D) and zinc metallo- $\beta$ -lactamases (class B), based on mechanism and active site machinery [44]. Much effort has gone into the discovery of  $\beta$ -lactamase inhibitors that act as mechanism-based irreversible inactivators and that can be coadministered along with a  $\beta$ -lactam antibiotic. Clinically and commercially successful compounds such as clavulanic acid, sulbactam, and tazobactam, Fig. (3), are effective inhibitors of class A  $\beta$ -lactamases with some limited effectiveness against class D, but with poor inhibitory potency against class C  $\beta$ -lactamases [45]. The extensive use of these  $\beta$ -lactamase inhibitors has resulted in the emergence of new  $\beta$ -lactamase enzymes with broad substrate specificity [46]. Second generation  $\beta$ -lactamase inhibitors that might possess an expanded spectrum of activity have, therefore, been pursued by a variety of research groups.



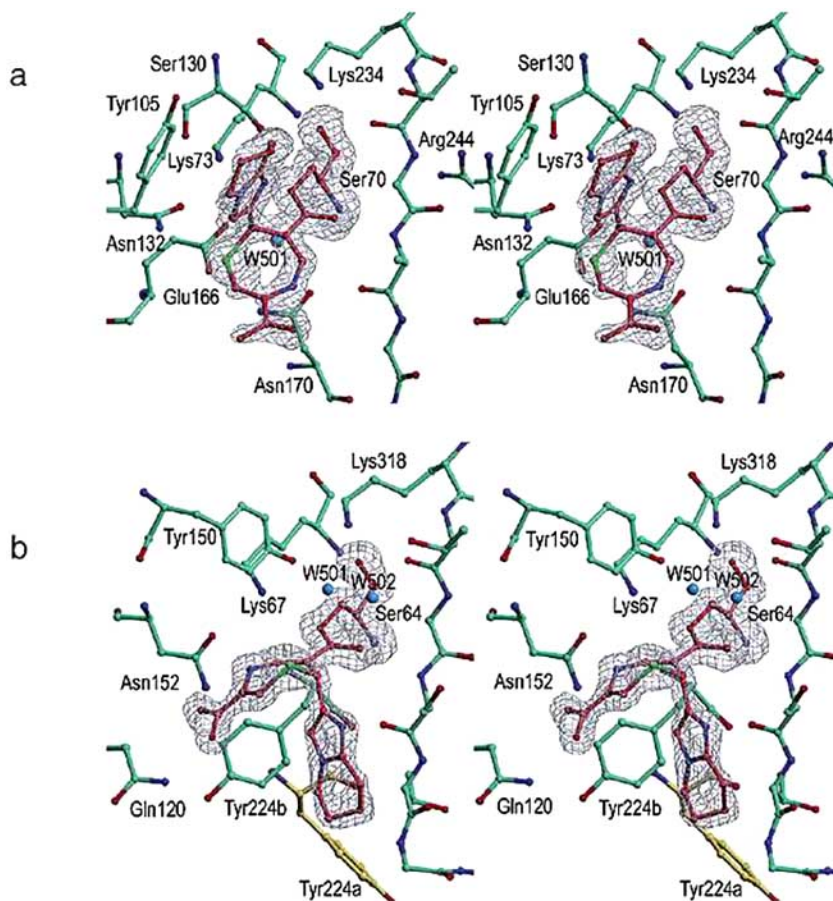
**Fig. (3).** Chemical structure of  $\beta$ -lactamase inhibitors

The Wyeth group has been working on a class of 6-methylidene penems with some success in this regard. Previous work at Beecham Research Laboratories had attempted structural modifications of the penem nucleus and identified BRL-42715, Fig. (3), as a representative of a novel class of compounds containing an alkyldiene moiety at the C6 position of the penem ring system. This series possessed potent  $\beta$ -lactamase inhibitory potency and showed a broader spectrum than previous  $\beta$ -lactamase inhibitors [47], however was not developed due to chemical instability and short half-life in humans [48]. Elegant work by the Wyeth group utilizing electrospray ionization mass spectrometry [48] and X-ray crystallography [49] elucidated the detailed mechanism of compound (1), bound to representatives of both class A (SHV-1) and class C (GC1)  $\beta$ -lactamases. They determined that these 6-methylidene penems were undergoing a rearrangement to dihydro-1, 4-thiazepines Fig. (4).

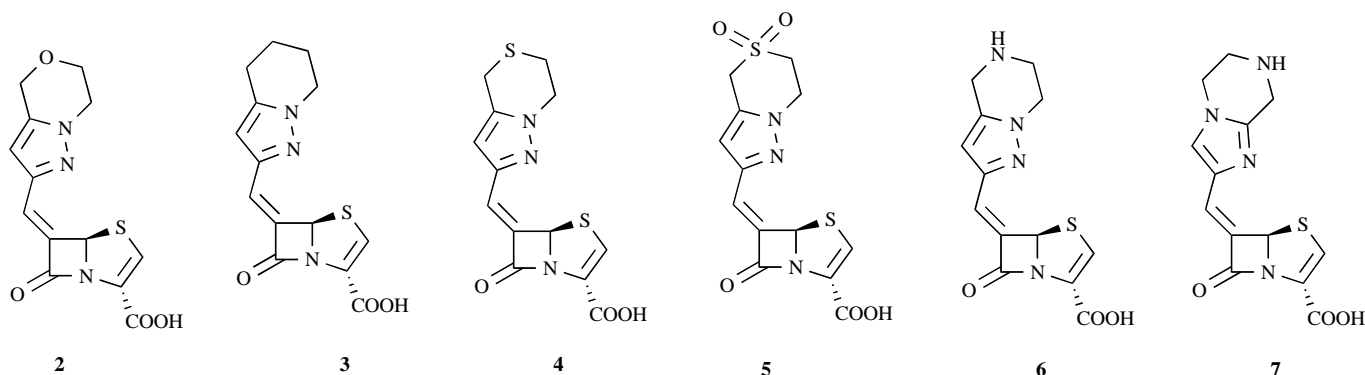
These high-resolution crystal structures further showed that the dihydrothiazepine intermediate is oriented differently in each complex Fig. (5). This observation is significant in the context of designing new 6-methylidene penem analogs [46, 50, 51] since the stereochemical outcome of the stereogenic C7 center can have a dramatic impact on binding. Using the crystallographic information for the rearranged product of (1), Venkatesan, *et al.* designed and modeled a series of 6-methylidene penems containing [6, 5] fused bicycles into both SHV-1 and GC1 and synthesized analogs based on this work, Fig. (6) [46]. These compounds



**Fig. (4).** Proposed rearrangement mechanism of 6-methylidene penem (1) to dihydro-1,4-thiazepines in  $\beta$ -lactamase active site.



**Fig. (5).** Stereoview highlighting the different orientation of the dihydrothiazepine rearrangement intermediate of (1) in: **a.** SHV-1  $\beta$ -lactamase and, **b.** GC1  $\beta$ -lactamase, reprinted from ref. [49].



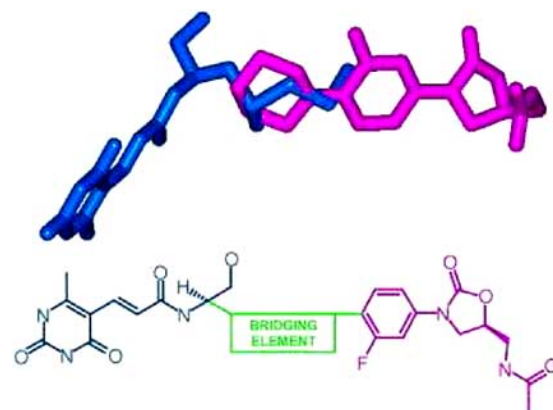
**Fig. (6).** 6-methylidene penems designed based on knowledge of the rearrangement mechanism described in the text and in Figs. (4) and (5).

were found to be potent, broad-spectrum  $\beta$ -lactamase inhibitors (of both class A and C  $\beta$ -lactamases). When combined with piperacillin, (**3**) showed similar antibacterial potency to (**1**) with MIC values between 1-4  $\mu\text{g}/\text{mL}$  against several piperacillin-resistant organisms expressing different  $\beta$ -lactamases. Compounds (**1**) and (**3**) additionally demonstrated *in vivo* efficacy in a murine acute lethal infection model against TEM-1-producing *E. coli*, with  $\text{ED}_{50}$  values in the range of 20-30 mg/kg when dosed intravenously. Calculation of interaction energies suggested that the formation of the 1,4-thiazepines in both class A and C will favor a C7-R stereochemistry for this class of compounds [46].

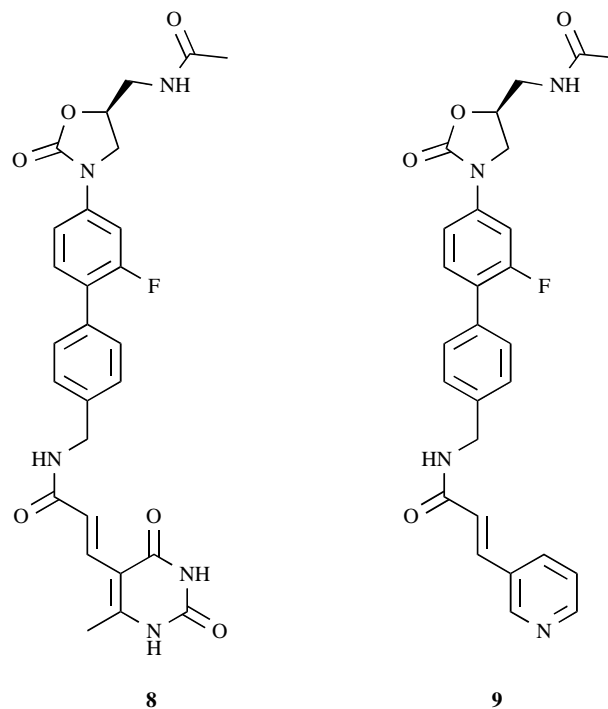
### Novel Oxazolidinones Effective Against Drug-Resistant Bacteria

Both the 30S and 50S ribosomal subunits have gained increased visibility as antibacterial targets as the amount of structural information available for the specific binding domains has increased. A recent review nicely describes current efforts in this area [52]. This paper also provides a nice example of the work done at Rib-X pharmaceuticals combining binding knowledge of sparsomycin and linezolid in the 50S subunit to design hybrid compounds that are effective against target-based drug-resistant (including linezolid-resistant) bacteria. The key to this work was the use of the structural information and modeling to identify an optimal bridging element between the retained features of sparsomycin and linezolid. Fig. (7) shows two examples of such hybrid molecules in which a biaryl was identified as an optimal bridging unit. These researchers also had some success in their attempts to replace the thymine moiety of sparsomycin with simple heterocycles, Fig. (7b), by further cycles of SGD [52]. They were ultimately able to identify biaryl oxazolidinone hybrids (e.g. RX-A<sub>667</sub>) with increased potency against *Haemophilus influenzae* (MIC = 0.5  $\mu\text{g}/\text{mL}$  vs. 16  $\mu\text{g}/\text{mL}$  for linezolid), linezolid-resistant *enterococci* (MIC = 1.0  $\mu\text{g}/\text{mL}$  vs. 32  $\mu\text{g}/\text{mL}$  for linezolid) as well as other Gram-positive organisms that also possessed pharmacokinetics consistent with QD or BID dosing [52].

The following example of SGD that will be discussed involves design of compounds targeted against the 30S ribosomal subunit.



**b.**



**Fig. (7).** **a.** Relative binding orientations of sparsomycin (magenta) and linezolid (blue) with the 50s rRNA stripped away for clarity. The design hypothesis is denoted below the 3D structures, reprinted from ref. [52]. **b.** Compounds resulting from the design with a biaryl as the optimal bridging unit.

### De Novo Design of Novel Aminoglycoside Mimetics

The decoding site (A-site) in 16S rRNA of the 30S ribosomal subunit is well established as the antibacterial target for the aminoglycosides (AGs). The aminoglycosides are very effective antibacterial agents, especially against Gram-negative infections. However, toxicity, poor oral bioavailability and resistance are of concern for these natural product antibacterials [53]. There has long been a desire for improved ligands targeting A-site rRNA that might overcome some of these limitations and recent structural information of several aminoglycosides bound to the A-site [54-56] can provide a starting point for the design of such compounds.

Researchers at Anadys Pharmaceuticals have used the available structural information to determine the minimal substructural features that contribute to aminoglycoside binding [57-58]. They have determined the 2-deoxystreptomamine (2-DOS) moiety, Fig. (8), to be essential for the antibacterial activity of the AGs. Their design goal was to use the structural information to identify a 2-DOS replacement with fewer stereocenters that would be synthetically easier than the chemistry required for the synthesis of AG analogs. During the course of their investigation several 2-DOS mimetics including acyclic analogs [59], aminomethylpiperidines [60], and aminoazepanes [61] were designed using stan-

dard molecular modeling techniques (e.g. molecular mechanics and molecular dynamics). Perhaps the most successful replacement to come out of this work was a cis-3, 5-diamino-piperidinyl (DAP, Fig. (8)) moiety that retained the signature cis-1, 3-diamino fragment of 2-DOS while reducing the number of stereocenters from five to two. Attachment of the DAP moiety to a triazine core, Fig. (8), resulted in a class of compounds that proved amenable to further optimization (i.e. 3,5-diamino-piperidinyl trianes, DAPT<sub>s</sub>). Several compounds from the DAPT class were found to bind to decoding site RNA, inhibit translation *in vitro*, and demonstrate antibacterial potency against Gram positive and Gram-negative bacteria, including *P. aeruginosa*. Compound (10), Fig. (8), was shown to possess modest antibacterial activity against aminoglycoside-resistant *S. aureus* strains (gentamicin MIC > 64 µg/mL) with an MIC of 16 µg/mL and also demonstrated *in vivo* efficacy in a mouse model of *E. coli* infection [57] when dosed intravenously.

### Design of Slow Binding Macrocyclic Peptide Deformylase Inhibitors

Peptidomimetics targeted against metalloenzymes have remained challenging from a general drug design perspective due to both the peptidic nature of the molecules as well as

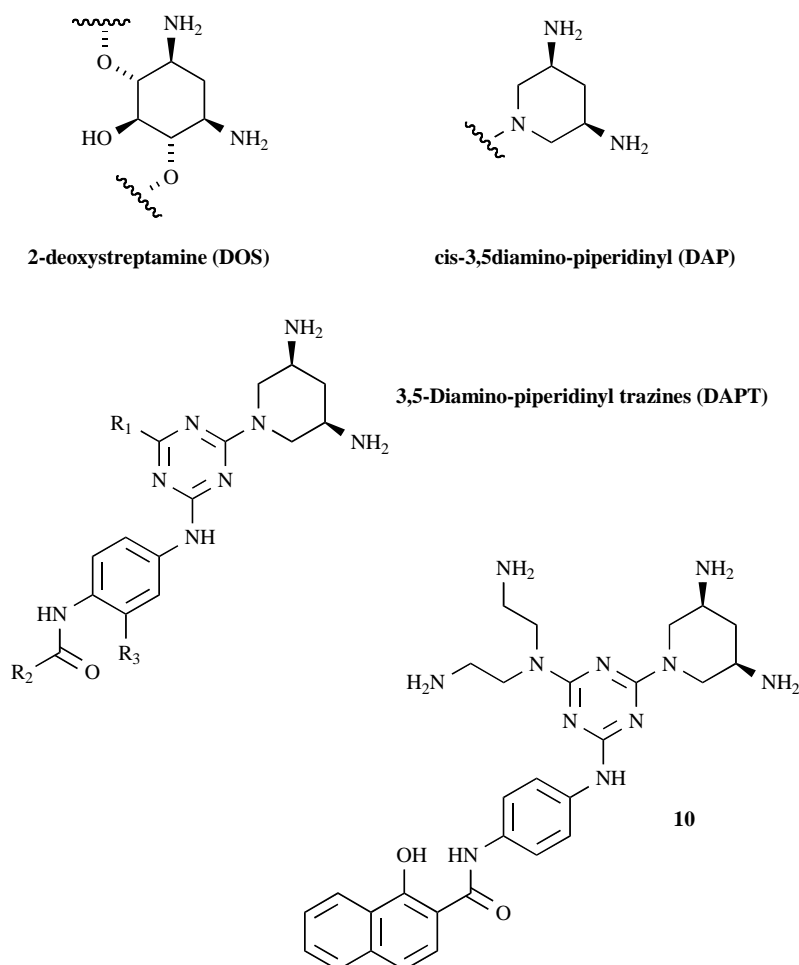


Fig. (8). Aminoglycoside mimetic design.

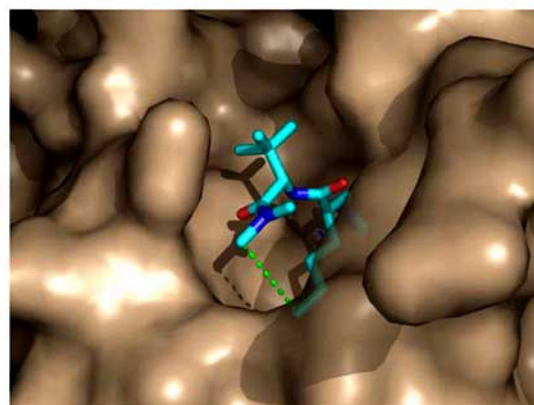
the general lack of “drug-likeness” of many metal-binding groups [62]. Peptide deformylase (PDF) is a somewhat unique metallopeptidase in that it utilizes a  $\text{Fe}^{+2}$  ion to enable amide bond hydrolysis. PDF catalyzes the hydrolytic removal of the amino terminal formyl group from N-formylmethionine as newly synthesized polypeptides emerge from the ribosome [63]. Since PDF is an essential bacterial enzyme, it is believed that PDF inhibitors would represent a new class of antibacterial agents [64-66]. The availability of structural information [67-69] has enabled the design of both peptidic and nonpeptidic inhibitors [34].

Some of the initial structures revealed that peptidic inhibitors are bound in an extended conformation with the P1' and P3' side chains oriented on the same face, making contact with the enzyme surface, Fig. (9a), [67]. While the P2' side chain is facing solvent, the P1' n-butyl group of (11) Fig. (10), is buried in a hydrophobic portion of the binding site. The observation that the P1' and P3' groups were in reasonably close proximity led to the design idea of cyclizing these two groups to form a macrocycle, Fig. (10) [70]. It was reasoned that such a cyclization might serve to rigidify and preorganize the bioactive conformation thereby increasing binding affinity to PDF as well as improving selectivity against other metalloproteases. Molecular modeling suggested that a nonyl linker would be in the optimal range to link the P1' C $\alpha$  carbon with the P3' amino group and maintain the extended conformation of the peptide backbone. Compound (12), Fig. (10), exemplifies this design and is shown modeled into the *E. coli* PDF active site in Fig. (9b). This compound (a 15-membered macrocycle) was shown to be a potent inhibitor of PDF ( $K_i^* = 0.33$  nM against *E. coli* PDF) as were similar 16- and 17-membered macrocycles. Further, these cyclized analogs exhibited slow binding kinetics to PDF whereas corresponding acyclic analogs all acted as simple competitive inhibitors under the same conditions. When compared to their acyclic counterparts, these 15-17 membered macrocycles displayed 20-50-fold greater inhibitory potency ( $K_i^* = 0.22 - 0.33$  nM vs. a  $K_i = 11$  nM for (11)) [71]. Other cyclized inhibitors in this series that possessed P2' groups other than t-butyl glycine also showed improved selectivity vs. MMPs 1, 2, 3 and 9 (The t-butyl glycine, itself, also imparts selectivity against these same MMPs.) In general, the cyclized analogs showed antibacterial potencies similar to their uncyclized counterparts (e.g. MICs 2-4, 16, >32, 0.5  $\mu\text{g}/\text{mL}$  for (12) against *S. pneumoniae*, *S. aureus*, *E. faecalis*, *H. influenzae* respectively, vs 8, 16, 32, 0.25  $\mu\text{g}/\text{mL}$  for (11)). These compounds were shown to be inactive against *E. coli*. Importantly, the cyclized inhibitors showed improved stability against proteolytic degradation. It might be expected that such a novel chemotype that exhibits slow binding kinetics and is directed at a novel target (i.e. PDF) would foster a slower development of target-based resistance as well as a reduced propensity towards efflux, however, this remains to be proven in this instance.

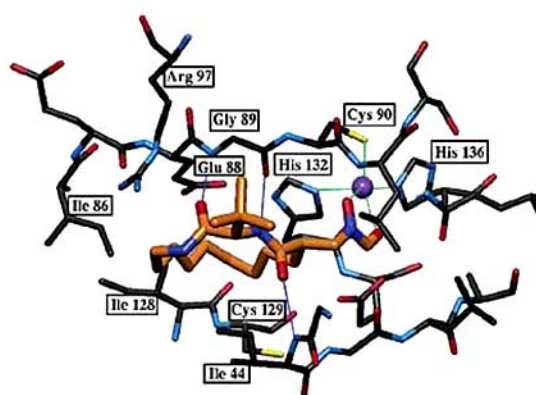
#### Design of Dual-Targeting Agents Against DNA Gyrase and Topoisomerase IV

As mentioned above, multi-targeting offers another approach to new antibacterial agents with potential for the slow development of resistance. Our own work has been directed

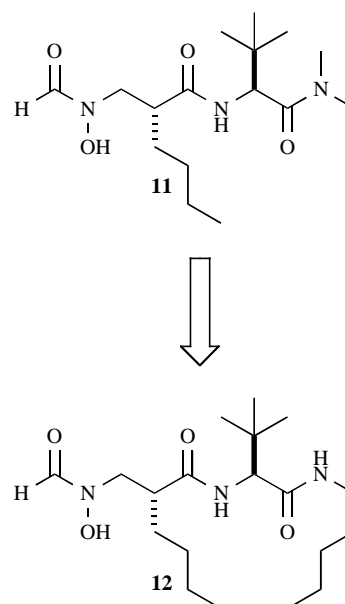
a.



b.



**Fig. (9).** a. Binding of compound (11, PDB access code 1G27) to PDF highlighting the proximity of the P1' and P3' side chains oriented on the same face, making contact with the enzyme surface. b. Proposed binding mode for compound 12 docked into PDF, reprinted from ref. [70].

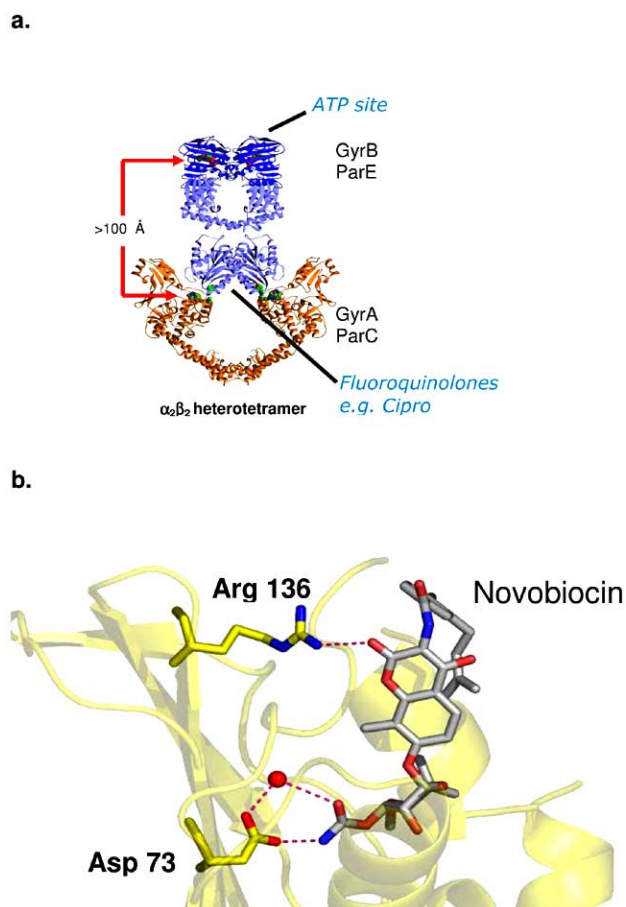


**Fig. (10).** Design concept for cyclizing P1' and P3' group of compound 11 to compound 12 which is a 15-membered macrocycle.

towards the design of small molecules that simultaneously inhibit DNA gyrase as well as topoisomerase IV (topoIV). Bacterial DNA gyrase and topoisomerase IV are well-characterized clinically validated targets of the fluoroquinolone antibiotics. The fluoroquinolones exert their antibacterial activity through inhibition of the catalytic (DNA binding) subunits. However, inhibition of these enzymes through binding to the ATP sites of these essential enzymes has been less successfully exploited [72]. We have discovered a new chemical class of benzimidazole ureas [73] that bind to the ATP sites of both of these enzymes (GyrB subunit of gyrase and ParE subunit of topoIV), thus inhibiting their role in DNA replication. These enzymes act in concert to maintain a healthy balance between the superhelical storage form of DNA and the partially unwound form necessary for DNA replication. Gyrase is the only bacterial enzyme capable of introducing negative supercoils ahead of the replication fork while the primary function of topo IV is decatenation of daughter chromosomes behind the replication fork [72].

Gyrase and topoIV are  $\alpha_2\beta_2$  tetramers with the GyrB/ParE containing ATP sites sitting atop the DNA binding domain, Fig. (11a). This homology model [74] suggests that the ATP binding site is  $>100$  Å away from the quinolone-resistance determining region (QRDR) where most known fluoroquinolone-resistance mutations map, thus reducing the risk of cross-resistance with the fluoroquinolones. Additional insights that helped define our design criteria to prospectively minimize the development of resistance were initially provided by structural information of the natural product, novobiocin, bound to the GyrB subunit of *E. coli* [75] and *S. aureus* [76]. Novobiocin, Fig. (12), is a prototypical natural product coumarin GyrB inhibitor that exhibits antibacterial potency against a variety of bacteria [77]. Analysis of the structure of novobiocin bound to *E. coli* GyrB, Fig. (11b) illustrates two key binding features that we viewed as essential to maintain. First, we rationalized that it would be important to maintain/enhance the hydrogen bond network between any new inhibitor class with Asp73 and a highly conserved water. Our design concept postulated that if this set of interactions could be made with part of the molecular scaffold, it would contribute to greater ligand efficiency since the scaffold would be involved in providing a significant portion of the binding energy. This would, thus, provide an optimization path to low molecular weight inhibitors. Molecular modeling suggested that such a strong set of interactions involving a molecular core containing both a hydrogen bond donor and acceptor, separated by two bonds, would enable the molecule to sit lower in the binding site (relative to novobiocin) and maximize greater hydrophobic interactions in the bottom portion of the binding site. By accomplishing this, a compound would contact many of the same residues as the adenine portion of ATP [73]. One might further expect this to contribute to a significantly reduced selection of resistant mutants since any mutations in this portion of the binding site would likely lead to nonviable bacteria. Fig. (11b) also highlights a hydrogen bond between Arg136 and the coumarin ring of novobiocin. We viewed that maintenance of this interaction would also be essential towards potent inhibition of gyrase and topoIV. This portion of the binding site is close enough in space ( $\sim 10$  Å) relative to the Asp73/conserved water portion of the binding site to allow a

relatively small synthetic molecule to optimally achieve both sets of interactions.



**Fig. (11).** a. Homology model of DNA gyrase and topoIV  $\alpha_2\beta_2$  heterotetramer, illustrating the target ATP site (contained within GyrB and ParE) and highlighting its relationship to the fluoroquinolone binding site (in GyrA and ParC). b. Key hydrogen bonds between the natural product novobiocin and *E. coli* GyrB.

The above design criteria were then applied to the selection of potential hits from both virtual and high-throughput screening. Fig. (13) shows the design cycle that was followed towards the optimization of a novel class of dual GyrB/ParE inhibitors. This design cycle involved identification of a hit that is consistent with the above design criteria, followed by multiple iterations of modeling, determination of new X-ray structures, medicinal chemistry and biological testing. Approximately 30,000 compounds, pre-filtered for drug-like properties, [78] were used as the screening set. While several different scaffold classes showed inhibition of ATPase activity against the GyrB subunit, compound (13), Fig. (12), appeared to allow a clear optimization path enabled by this structural information. This hit was a  $2$   $\mu$ M gyrase inhibitor with no topoIV inhibitory activity and was inactive microbiologically. Docking of (13) into the X-ray structure from which novobiocin was removed immediately suggested two types of structural modifications, Fig. (14). Firstly, it appeared that modification of the carbamate oxygen of (13) to nitrogen (urea) might change a seemingly re-

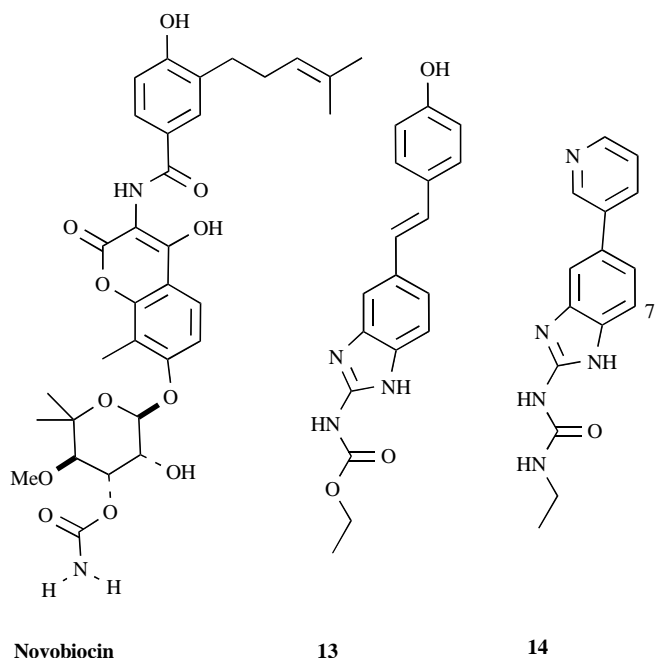


Fig. (12). Chemical structure of GyrB inhibitors

#### Design Cycle for Dual GyrB/ParE Antibacterial Agents

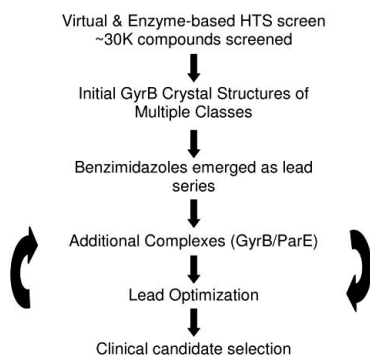


Fig. (13). Iterative structure-guided design cycle employed in the discovery and optimization of the benzimidazole class of dual GyrB/ParE inhibitors.

pulsive interaction into an attractive one that would further contribute to the extensive hydrogen bond network described above and in Fig. (14). This model further suggested that the benzimidazole urea scaffold would be positioned deep in the ATP binding site. The second design principle to emerge from this initial analysis was that the hydrogen bond interaction between Arg136 and the exocyclic carbonyl oxygen of the novobiocin coumarin ring, Fig. (11b) could potentially be mimicked by a heterocyclic hydrogen bond acceptor directly attached to the benzimidazole urea core, Fig. (14). The resultant compound, (14), Fig. (12) and Fig. (14), provided a 15-fold improvement in  $K_i$  against *S. aureus* gyrase (0.13  $\mu\text{M}$ ) relative to (13) and possessed some weak inhibitory potency against *E. coli* topoIV with a  $K_i$  of 2.3  $\mu\text{M}$  (for reference, novobiocin possesses  $K_i$  values of 0.01 and 0.11  $\mu\text{M}$ , respectively, against *S. aureus* gyrase and *E. coli* topoIV). Compound (14) showed modest antibacterial potency with an MIC of 16  $\mu\text{g/mL}$  against *S. aureus* and *H. influenzae* and 2  $\mu\text{g/mL}$  against *Streptococcus pneumoniae* (comparable values for novobiocin against these organisms were 0.125, 2.0, and 0.063  $\mu\text{g/mL}$ , respectively). This arylbenzimidazole urea then became the chemotype to further optimize in an attempt to further improve the topoIV inhibitory potency.

Exploration at various positions on the arylbenzimidazole core proceeded with the guidance of structural information [73]. As knowledge of the SAR evolved, it became clear that substitution at the 7-position (14), Fig. (12), was the most productive towards improving both topoIV inhibitory activity and antibacterial potency. At this time, no structure existed for the ParE subunit, however there appeared to be an evolving trend that planarity of the C-7-substituent was required for optimal topoIV inhibitory activity. This trend can be illustrated in Table (1) by compounds (15-17). Compound (15) contains a methoxyester at the C-7 position and is predicted by quantum mechanical rotational barrier calculations to be coplanar with the benzimidazole core [73]. Coplanarity is also predicted for compound (16), whereas (17) is predicted to be out of plane by approximately  $60^\circ$  relative to the benzimidazole core. While all three compounds are potent gyrase inhibitors with roughly equivalent  $K_i$  values, Table (1), coplanarity appears to be required for potent inhibition of topoIV as exemplified by a 30-fold increase in  $K_i$  for (17)

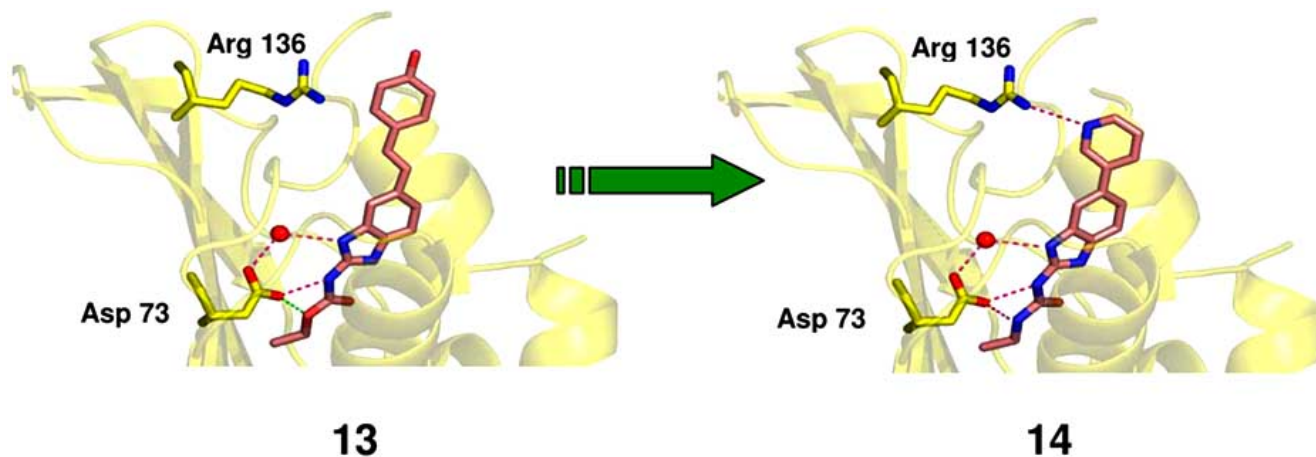
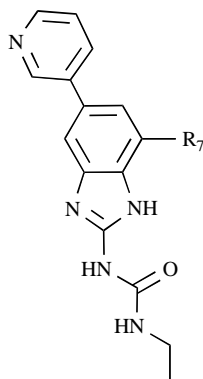


Fig. (14). Models of compound 13 (left panel) and 14 (right panel) docked into *E. coli* DNA gyrase highlighting the potential for 14 to make a hydrogen bond with Arg136 as well as more effective hydrogen bonds to a network involving Asp73 and a conserved water.

Table 1. Enzyme Inhibition Data and Antibacterial Potency for Selected R7 Substituents



Compound		15	16	17
	R7			
K <sub>i</sub> (nM)	<i>S. aureus</i> gyrase	0.008	0.015	0.017
	<i>E. coli</i> topoIV	0.035	0.046	1.3
MIC <sub>90</sub> (μg/mL)	<i>S. aureus</i>	0.063	0.063	4.0
	<i>S. pneumoniae</i>	0.016	0.008	0.5
	<i>E. faecalis</i>	0.5	1.0	>16

relative to (15) and (16). More importantly, compounds with coplanar C-7 substituents showed exceptional antibacterial potency against several organisms, Table (1). This observation allowed routine rotational barrier calculations to guide medicinal chemistry and evaluate many different substituted aryls at the C-7 position. Subsequently, the X-ray structure of novobiocin bound to the topoIV ParE subunit was solved in our group [79]. Additional structures solved with C-7 aryl containing benzimidazole ureas showed that the portion of the binding site accommodating the C-7 aryl group appeared to be slightly narrower in topoIV ParE than in gyrase GyrB, Fig. (15). This relatively subtle structural difference was largely due to the substitution of a single amino acid in topoIV (Met74) relative to gyrase (Ile78) and caused a different amino acid side chain packing in this portion of the binding site. The net result of this relatively narrowed portion of the binding site in ParE is the requirement for a greater degree of coplanarity of any 7-aryl substituent for optimal inhibitory potency against topoIV. The ability of several of these compounds (eg. (15), (16)) to possess an intramolecular hydrogen bond further imparts ligand preorganization in terms of this required coplanarity. The availability of these new ParE structures combined with the rotational barrier calculations enabled further optimization towards compound (18), Fig. (16). This compound showed equipotent enzyme inhibitory activity against both enzymes as well as excellent antibacterial potency, Table (2), [80]. Compound (18) was further shown to possess low *in vitro* resistance frequencies [80, 81] and to maintain antibacterial potency against commonly encountered resistance pheno-

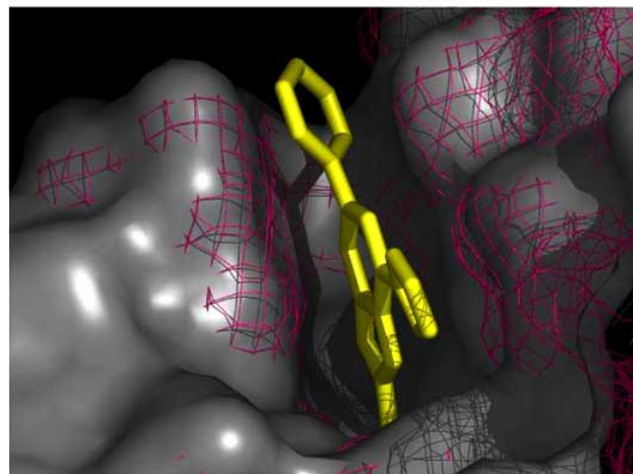
Gyrase (GyrB)  
TopoIV (ParE)

Fig. (15). Molecular surface of GyrB ATP binding site (solid gray) from crystallographic complex of a closely related analog of compound 16 (yellow). The corresponding, slightly narrower portion of the ParE ATP site is shown as a purple mesh.

types including fluoroquinolone- and multi-drug-resistant strains [80]. Compound (18) demonstrated efficacy when dosed intravenously in a rodent model of skin infection and orally in a rodent model of pneumococcal pneumonia [73].

**Table 2. Enzyme Inhibition Data and Antibacterial Potency for Compound 18.**

<b>K<sub>i</sub> (nM)</b>	<i>S. aureus</i> gyrase	14
	<i>S. aureus</i> topoIV	< 6
	<i>E. coli</i> gyrase	< 4
	<i>E. coli</i> topoIV	23
<b>EC<sub>50</sub> (μM)</b>	Human topoII	> 25
<b>MIC<sub>90</sub> (μg/mL)</b>	<i>S. aureus</i>	0.12
	<i>S. pneumoniae</i>	0.03
	<i>E. faecalis</i>	0.06

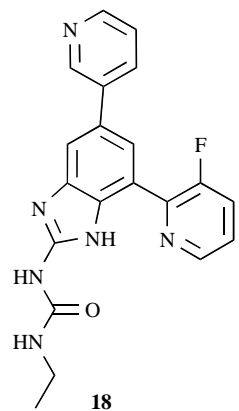
**Table 3. Additional Examples of Structure-Guided Design Applied to Fight Resistance**

Target	SGD Description	Resistance-Thwarting Mechanism	Ref
tRNA guanine transglycosylase (TGT)	De novo design using crystal structure of TGT ( <i>Z. mobilis</i> )	Novel compound class, novel target	83,84
β-lactamase	SG-library design to explore a series of boronic acid non-β-lactam inhibitors of AmpC	Inactivation enzyme inhibition, prolong activity of β-lactams	85
β-lactamase	Rational design of 4-substituted tricyclic carbapenems (trinems). Inhibitors of both class A and C β-lactamases	Inactivation enzyme inhibition, prolong activity of β-lactams	45
A-site of 16S RNA	Design of paromomycin analogs containing an ether functionality at C2' that can contact residues involved in tobramycin binding and reorient the compounds in the binding site.	Different contacts in A-sites, potential to avoid some mutational "hot-spots." Additional functionality offers potential to avoid deactivation enzymes	86
A-site of 16S RNA	Design of aminoglycoside analogs that are conformationally-restrained towards A-site binding while simultaneously destabilizing the conformation required to bind AG-inactivation enzymes	Compound(s) no longer susceptible to enzymatic inactivation	87
Histidine kinase inhibitors of bacterial two-component signaling systems (TCS)	Structure Based Virtual Screening to identify inhibitors that disrupt YycG/YycF TCS essential in <i>S. epidermidis</i> .	Novel compound class, novel target, multi-targeting potential	88
ZipA/FtsZ	NMR-based fragment screening approach combined with crystallography. Identification of novel hits that disrupt protein-protein interactions between ZipA and FtsZ.	Novel compound class, novel target	89
DNA Gyrase	Structure Based Virtual Screening to identify small molecules that target sites distinct from those of the fluoroquinolones. These sites include the ATP site as well as a previously unexplored site formed at the dimer interface of subunit A.	Novel compound class, novel target, multi-targeting potential	90

This example represents the prospective design of dual targeting agents of a novel chemical class with a novel antibacterial mechanism of action. The initial chemical design criteria and subsequent optimization were enabled by the early and frequent use of structural information. This has

ultimately led to a new class of potent antibacterial agents with low *in vitro* resistance frequencies and the potential for clinical effectiveness.

Additional recent examples of note are summarized in Table (3).



**Fig. (16).** Chemical structure of an optimized dual targeting GyrB/ParE inhibitor

## LOOKING AHEAD

Additional SGD opportunities will undoubtedly increase as more structural information becomes available for interesting antibacterial targets. One such opportunity addresses a key mechanism of intrinsic resistance, efflux-mediated resistance. While efflux can be an issue for both Gram-positive and Gram-negative bacteria, it has proven to be more clinically relevant for Gram-negative organisms because of synergy with reduced compound uptake due to the enhanced permeability barrier present in these organisms [28]. This form of intrinsic resistance has led to efforts in recent years to discover efflux pump inhibitors (EPIs) that could be co-administered with existing antibacterial classes [82]. The availability of X-ray structural information [82, and references contained within] offers the possibility to design EPIs targeted against specific efflux pump families. Additionally, this structural information could be used to ‘design in’ the potential for a given compound class to be a poorer efflux substrate. If structural information were also known for a compound (or compound class) against its intended molecular target, then readily testable hypotheses could be generated as to which portions of a compound might be modifiable to diminish efflux while maintaining key binding features required for the primary target.

## CONCLUSIONS

The current resistance crisis has the potential to render our current armament of antibacterial agents ineffective. Of particular concern is the spread of these singly and multi-drug resistant strains into the community [91-94]. The concept of “staying one step ahead” of resistance by continuing to produce successive generations of antibacterial classes that have been in use for years is really nothing more than a band-aid. While this may buy us some time, it is clear that we need to use all of the tools at our disposal to discover new structural classes of antibacterial agents targeted against less exploited bacterial processes. The examples discussed in this review reflect our view that SGD is one such tool that will be applied more routinely and in a prospective manner to help battle this significant global health concern.

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