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ATP-Binding Site of Bacterial Enzymes as a Target for Antibacterial Drug Design

Veronika Škedelj, Tihomir Tomašić, Lucija Peterlin Mašič, and Anamarija Zega*

University of Ljubljana, Faculty of Pharmacy, Aškerčeva 7, 1000 Ljubljana, Slovenia

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1. Introduction

The remarkable ability of bacteria to develop resistance to antibacterial agents is the reason for the continued need to search for new antibacterial targets and develop novel antimicrobial agents.[1,2] Despite bacterial threats to public health, at present, only five major pharmaceutical companies, AstraZeneca, GSK,[a] Merck, Novartis, and Pfizer, have active antibacterial drug discovery programs.[1,3] The painful reality of antibacterial drug discovery is the low probability of success in developing an antibacterial drug that can be approved for clinical use.[4] Over the past 40 years only two new structural types of antibacterial drugs, daptomycin and linezolid, have been introduced to the clinic, following their discovery using empirical screening methods.[5]

Since the late 1990s, the rise of bacterial genomics, by which new targets have been identified, promised to rejuvenate the search for synthetic antibacterial agents. However, the target-based high-throughput screening (HTS) approach that could be an important generator of chemical diversity of antibacterials and has been fruitful in other therapeutic areas has had limited success.[6] There may be a combination of reasons for the low HTS hit rates. In the field of antibacterial drug discovery a HTS hit is defined as a chemically tractable, low-micromolar inhibitor of the target with at least 10-fold selectivity against the human version of the same target, while a lead is a hit with antibacterial activity, together with evidence that the mechanism of antibacterial activity is achieved via inhibition of the target screened.[6] Analysis of the chemical properties of known antibacterial drugs shows that they occupy a unique property space that is different from that of drugs in other therapeutic areas and that they do not generally possess the physicochemical properties required for oral bioavailability although corporate compound collections are heavily biased toward compounds that do.[3,7,8] The ability therefore to apply the extensive drug discovery project with compound classes commonly screened against human target families, such as kinases, is limited.[6] However, given the current level of uncertainty about target relevance in an infected host and about the ability of an antibacterial drug to get to its target, compound libraries developed for other therapeutic areas, in combination with whole-cell screening assays, may be just as likely to harbor hits as compound libraries developed for antibacterial screening. Recently, Miller and co-workers from Pfizer showed the utility of repurposing external compound libraries by screening them in a whole-cell antibacterial assay. They found that a series of pyridopyrimidines (Figure 7), originally derived from a program targeting eukaryotic protein kinases, were also active against a subset of Gram-negative bacteria. By use of genetic and biochemical tools, it was shown that these compounds target the ATP-binding site of the bacterial enzyme biotin carboxylase (BC). These results and other examples that we will describe in this paper demonstrate the feasibility of finding ATP-competitive antibacterials with good selectivity profiles and indicate that the ATP-binding site of bacterial enzymes can be a promising target for antibacterial drug design.[9]

Bacterial genomes encode hundreds of ATP-binding proteins. These include well-validated targets like DNA gyrase and a host of new or underexplored enzymes. However, in the field of antibacterial drug discovery, the design of inhibitors targeting the ATP-binding site of bacterial enzymes has been a taboo theme for a long time. To show in vivo activity, ATP-competitive inhibitors must first be able to compete with the ATP concentration in the bacterial cell.[10−13] For different bacterial strains the ATP content varies from 0.3 to 9 fg of ATP per cell[13] (about 0.6−18 mM) but mostly is in the same range as in human cells where the intracellular ATP concentration is in the range of 1−10 mM.[12] Consequently, researchers have argued that developing compounds with sufficient potency to compete with the high intracellular concentrations of ATP in bacterial cells would be too challenging. However, the examples of successful human protein kinase inhibitors that are mostly competitive antagonists of ATP provide an encouraging prospect that the high intracellular ATP concentration is not necessarily an absolute obstacle.[14]

In order to achieve selectivity for bacterial targets, strategies to identify novel classes of antibacterial agents have tended to focus on genomic targets that share little or no similarity to human proteins. The target must be essential and highly conserved among various bacterial strains and absent, different, or nonessential in humans. Therefore, the main condition for a successful approach to designing a selective

*To whom correspondence should be addressed. Phone: +386-1-4769673. Fax +386-1-4258031. E-mail: anamarija.zega@ffa.uni-lj.si.

[a] Abbreviations: ACCase, acetyl-CoA carboxylase; AMPCPP, adenosine 5′-(α,β-methyleno)triphosphate; AMPPNP, adenosine 5′-(β,γ-imido)triphosphate; ATP, adenosine 5′-triphosphate; BC, biotin carboxylase; Ddl, D-alanine-D-alanine ligase; FGFR, fibroblast growth factor receptor; GHKL, gyrase, Hsp90, histidine kinase, MutL; GSK, GlaxoSmithKline; GyrB, DNA gyrase subunit B; HTS, high-throughput screening; ITC, isothermal titration calorimetry; MIC, minimal inhibitory concentration; MNEC, maximum noneffective concentration; ParE, DNA topoisomerase subunit E; PDB, Protein Data Bank; ProBiS, Protein binding sites; SBDD, structure-based drug design; PS, pantothenate synthetase; TopoIV, DNA topoisomerase IV; VEGFR, vascular endothelial growth factor receptor; VS, virtual screening.

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Table 1. ATP-Binding Bacterial Enzymes Studied

enzyme	enzyme classification	PDB code[20]	cofactor/inhibitor	reference
		Aminoglycoside-Modifying Enzymes		
APH(3′)-IIIa	transferase EC no.: 2.7.1.95	1J7U	AMPPNP	73
		GHKL: ATPase/Kinase Superfamily		
EnvZ	transferase EC no.: 2.7.13.3	1BXD	AMPPNP	42
PhoQ	transferase EC no.: 2.7.13.3	1ID0	AMPPNP	50
		3CGY	**6**	47
DNA gyrase	isomerase EC no.: 5.99.1.3	1EI1	AMPPNP	27
		1AJ6	**1**	30
		1KIJ	**1**	28
		1KZN	**2**	29
topoisomerase IV	isomerase EC no.: 5.99.1.-	1S16	AMPPNP	38
		1S14	**1**	38
		ATP-Grasp Fold Superfamily		
biotin carboxylase	ligase EC no.: 6.3.4.14	2J9G	ADP/AMPPNP	61
		2V58	**14**	9
		2V59	**15**	9
		2W6M	**31**	60
		2W6N	**32**	60
		3JZF	**24**	68
		3JZI	**29**	68
D-Ala-D-Ala ligase	ligase EC no.: 6.3.4.14	1IOW	ADP	65
		Cytidylyltransferase Superfamily		
pantothenate synthetase	ligase EC no.: 6.3.2.1	1N2B	AMPCPP	63
		1N2H	pantoyl adenylate	64
		3COW	**47**	79
		3IOC	**49**	80
		3IOD	**50**	80
		3IME	**36**	62
		3IUE	**35**	62
		3IVX	**39**	62

ATP-competitive inhibitor is dissimilarity of the ATP-binding domains of bacterial and eukaryotic enzymes. However, in many cases, although there is no significant primary sequence homology between the enzymes, the structures of their ATP-binding domains are largely superimposable. The conserved nature of ATP-binding sites in eukaryotic and prokaryotic protein classes has led to skepticism about the possibility of making an inhibitor that selectively targets the bacterial enzyme ATP-binding site. The similar issue of specificity arose for protein kinase inhibitors that target the ATP-binding site that is highly conserved among these enzymes. However, knowledge of the structure of the binding site and different enzyme conformations, together with approaches, such as design of compounds that bind not just in the ATP-binding pocket but also in a neighboring hydrophobic pocket, has led to a number of inhibitors that display reasonable selectivity for a particular kinase.[15,16]

Making incremental improvements to existing scaffolds is a good short-term strategy for refilling the antibiotic pipeline, but a presumably more sustainable way to combat resistance is to discover new scaffolds. The huge array of inhibitors designed for targeting ATP-binding sites of eukaryotic targets, such as protein kinases, present in pharmaceutical compound libraries, constitutes a pool of potential antibacterial scaffolds. These compounds can be mined for their activity against structurally related bacterial targets, following structure-based and computational methodologies applied to ensure selectivity for bacterial enzymes.[9]

Targeting bacterial enzymes that are structurally related or recognize common substrate motifs and offer the possibility of finding a small molecule that binds to more than one family member can be a good strategy for avoiding resistance.[17] Enzymes that catalyze the early cytoplasmic steps of stepwise bacterial cell wall biosynthesis, a series of ATP-dependent amino acid ligases (MurC, MurD, MurE, and MurF), are a good example of such potential antibacterial targets. The ATP-binding sites of these enzymes are structurally very similar (Supporting Information Table S3) and an inhibitor that recognizes the homologous binding motifs will probably bind to more than one enzyme in the pathway. In this way, modest inhibition of several enzymes could reduce flux through a pathway more effectively than potent inhibition of a single enzyme. Furthermore, the frequency of target-mediated resistance to such a compound would be negligible, since mutations conferring resistance would need to occur in at least two different target genes during a single generation.[18,19]

The medicinal chemistry literature describing inhibitors that target the ATP-binding site of bacterial enzymes is comprehensive, but there are few crystal structures of bacterial enzyme ATP-binding pocket−inhibitor complexes. The ATP-binding enzymes discussed in this Perspective (Table 1) were selected from among those for which such structural data are available. In the following sections, we will describe approaches used to discover ATP-competitive inhibitors of bacterial enzymes and the challenge of selectivity, as it is one of the main problems associated with the design of ATP-competitive inhibitors.

2. Approaches Used for Discovering Inhibitors Targeting the ATP-Binding Site of Bacterial Enzymes

2.1. Natural Products. In the past, many successful antibacterial agents have been sourced from empirical screening

of natural products, and today more than two-thirds of the antibacterials in clinical use are natural products or their semisynthetic derivatives.[21,22] One of the first bacterial enzyme inhibitors that occupy the ATP-binding site were the natural coumarin antibiotics novobiocin **1**, clorobiocin **2**, and coumermycin A_1 **3** (Figure 1), produced by *Streptomyces* species,[23] which inhibit bacterial DNA synthesis by interrupting the supercoiling reaction catalyzed by DNA gyrase.[24]

Novobiocin **1** inhibits the GyrB subunit of DNA gyrase, which catalyzes ATP hydrolysis. The ATP-competitive mechanism of GyrB inhibition of **1** was corroborated by solving the crystal structure of *Escherichia coli* 24 kDa N-terminal part of GyrB (GyrB24) in complex with **1** (PDB code 1AJ6), which shows that the binding site of **1** partially overlaps with the ATP-binding site (Figure 2a).[25] In detail, the noviose sugar moiety of **1** and the adenine ring of ATP (Figure 2b and Figure S4 in the Supporting Information) interact with the GyrB24 fragment in a similar fashion. The overall protein structure of GyrB24·**1** and the 43 kDa N-terminal fragment of GyrB (GyrB43)·AMPPNP (adenosine-5′-(β,γ-imido)-triphosphate) (PDB code 1EI1) complexes differ particularly in the ATP-lid conformation. While this loop is in a closed conformation, forming a lid over the ATP-binding site in GyrB43·AMPPNP complex, it adopts an open conformation and moves away from the active site in the case of the GyrB24·**1** complex.[26,27] In the crystal structure of *Thermus thermophilus* GyrB43·**1** complex (PDB code 1KIJ) this loop adopts a slightly different open conformation that indicates that the binding of ATP to GyrB is accompanied by conformational changes within the protein.[28] The binding mode of **2** (Figure 1) (PDB code 1KZN) is very similar to that of **1**. The introduction of the methylpyrrole ring leads to a 20-fold better binding affinity for **2** (K_d = 1.2 nM)[29] than for **1** (K_d = 28 nM), which can be attributed to additional hydrogen bonds and hydrophobic contacts of the methylpyrrole ring in the GyrB active site.[30−32]

Because of its excellent antibacterial activity against Gram-positive pathogens, novobiocin was used for the treatment of infections with methicillin-resistant *Staphylococcus aureus* but was later withdrawn from clinical use because of its toxicity.[34] Novobiocin was the first and until

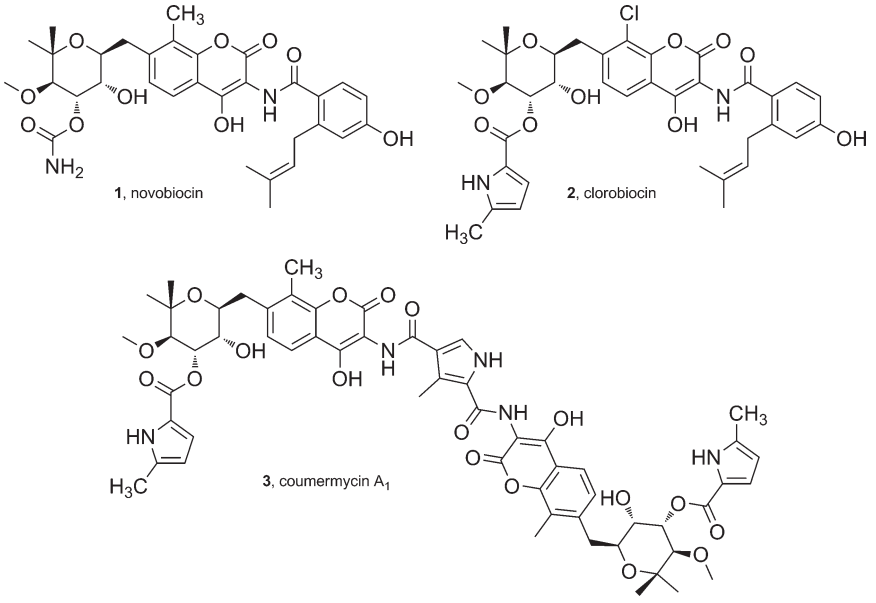

Figure 1. Natural coumarin antibiotics.

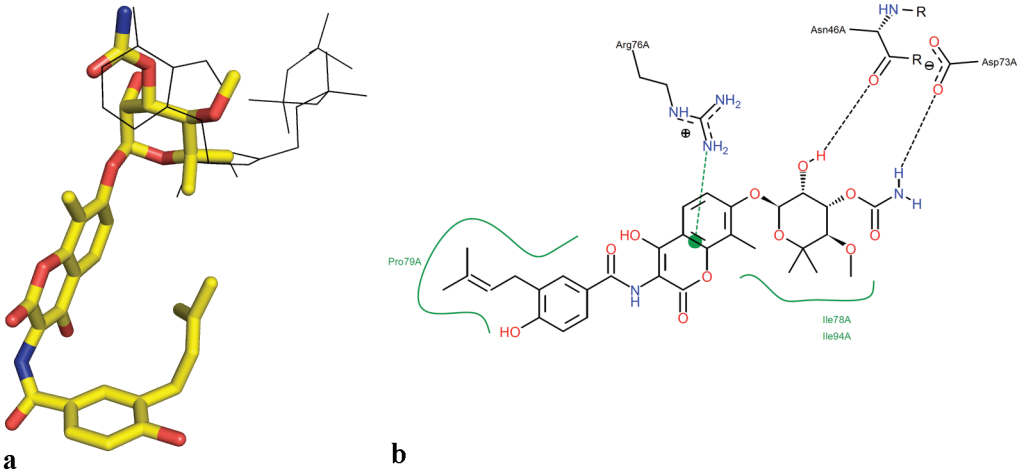

Figure 2. (a) Superposition of **1** (as sticks, PDB code 1AJ6) and AMPPNP (as lines, PDB code 1EI1) in the GyrB active site. (b) Interactions between **1** and GyrB24 amino acid residues (PDB code 1AJ6) as obtained with PoseViewWeb.[33]

now the only ATP-competitive inhibitor of bacterial enzyme ever used in the clinic. Later several novel derivatives were synthesized to improve the physicochemical properties and enhance the in vitro and in vivo effectiveness of the parent compounds.[35−37]

Additionally, compound **1** also inhibits DNA topoisomerase IV (TopoIV) (IC_{50} = 210 nM),[38] bacterial histidine kinase EnvZ (K_d = 120 ± 20 μM),[39] and eukaryotic target Hsp90,[40] which all share a unique structural ATP-binding motif, called the Bergerat fold, and belong to the GHKL phosphotransferase superfamily of enzymes.[41] EnvZ is a transmembrane protein with histidine kinase activity in its cytoplasmic region and acts as an osmosensor in *E. coli*. The three-dimensional structure of the EnvZ·AMPNP complex was determined by NMR (Figure S2 in the Supporting Information).[42] Transferred nuclear Overhauser effect experiments and saturation transfer difference experiments suggest binding to EnvZ in a conformation and orientation similar to those with GyrB.[39] TopoIV is a closely related bacterial enzyme to DNA gyrase from the type II topoisomerase family that utilizes the energy of ATP hydrolysis to relax positive and negative DNA supercoils and to decatenate daughter chromosomal DNA. The ParE subunit of TopoIV is structurally and functionally similar to GyrB and contains an ATPase active site in the N-terminal domain (Figure S5 in the Supporting Information) that dimerizes on ATP binding. The X-ray structure of **1** in complex with the 24 kDa N-terminal fragment of ParE (PDB code 1S14) revealed that **1** binds in a very similar conformation in the two proteins.

Cyclothialidines **4** and **5** (Figure 3) are another group of DNA gyrase inhibitors from *Streptomyces* species. Cyclothialidine **4** is a potent ATP-competitive inhibitor of GyrB with weak antibacterial activity, probably due to poor penetration into the bacterial cell.[31,43] The crystal structure of GyrB24 complexed with **5** has been determined at 2.0 Å resolution and shows that the adenine ring of ATP and the resorcinol ring of **5** are involved in a similar hydrogen bond network and hydrophobic interactions with the protein, which explains the ATP-competitive mechanism of inhibition. The majority of the protein−ligand interactions are formed between the substituted resorcinol ring of **5** and GyrB24.[44]

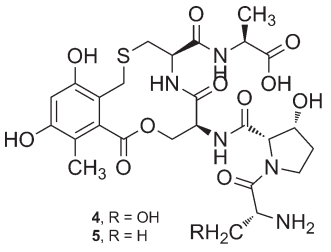

Figure 3. Cyclothialidine DNA gyrase inhibitors.

Radicicol **6** (Figure 4b), a macrocyclic antifungal antibiotic, originally isolated from the fungus *Monosporium bonorden*, inhibits the activities of DNA topoisomerase VI,[45] *Saccharomyces cerevisiae* Sln1 sensor kinase (IC_{50} = 127 μM),[46] *Salmonella typhimurium* sensor kinase PhoQ (K_d = 715 ± 78 μM),[47] the bacterial homologue of Hsp90 (HtpG),[48] and yeast Hsp90 (K_d = 19 nM)[49] by competing with ATP for binding to the Bergerat fold. The crystal structure of the PhoQ catalytic domain from *S. typhimurium* complexed with **6** (PDB code 3CGY) was determined to 2.6 Å and confirmed that **6** binds into the ATP-binding pocket of PhoQ (Figure 4a). The resorcinol ring of **6** and the adenosine ring of AMPPNP[50] are located at similar positions (Figure 4a and Figure S3 in the Supporting Information). On the other hand, the orientation and interactions of the macrocycle of **6** and the phosphates of AMPPNP are very different. Although **6** also inhibits eukaryotic Hsp90, there is an important difference in the binding orientation and affinity, probably due to differences in the ATP-lid region, which provides the possibility for the design of PhoQ-specific inhibitors.[47]

The advantage of antibiotic natural products may be that some of them have been naturally selected to possess desirable antibacterial properties, but on the other hand, they are often very large molecules that do not possess druglike properties and are not bioavailable after per oral application.[51,52] Nevertheless, natural products with known bioactivity form one group of the privileged motifs and can provide good starting points for further synthetic modifications leading to compounds with improved physicochemical properties and biological activity.[53]

2.2. Protein Kinase Inhibitors. Eukaryotic protein kinase inhibitors are widely employed as leads for drug design, and because they mostly target an ATP-binding site, they can be very suitable as starting compounds for the design of ATP-dependent bacterial enzyme inhibitors. For targets with high

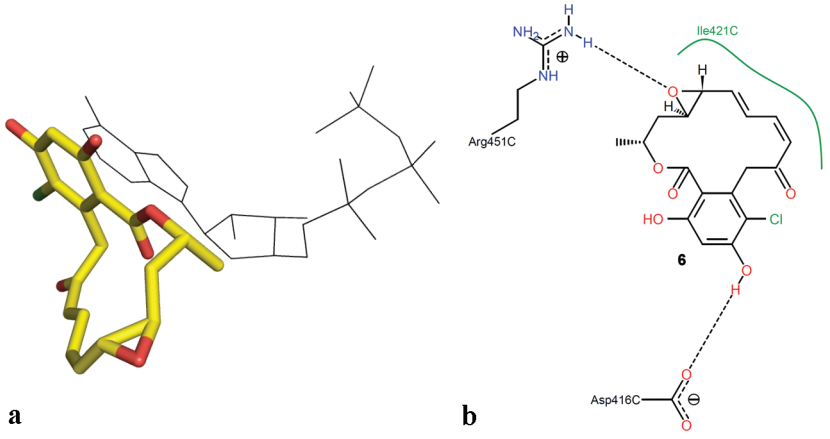

Figure 4. (a) Superposition of **6** (as yellow sticks, PDB code 3CGY) and AMPPNP (in black, PDB code 1ID0) in the PhoQ active site. (b) Schematic representation of interactions between **6** and PhoQ active site residues (PDB code 3CGY) as obtained with PoseViewWeb.

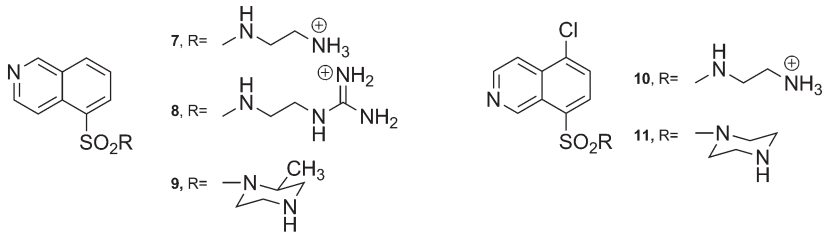

Figure 5. Isoquinolinesulfonamide inhibitors **7**−**11** of aminoglycoside-modifying enzyme APH(3′)-IIIa.

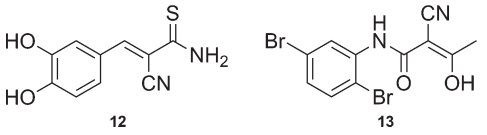

Figure 6. ATP-competitive Ddl inhibitors based on protein kinase inhibitor scaffolds.

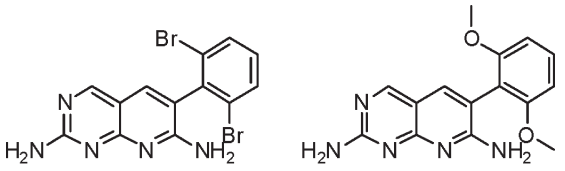

Figure 7. Pyridopyrimidine-based inhibitors of BC based on the protein kinase inhibitor pharmacophore.

sequence and structure homology to known human targets, a potent inhibitor is more likely to be found within the chemical space covered by synthetic pharmaceutical compound libraries.

Recent studies on some bacterial enzymes support the pursuit of antibacterials from leads discovered in eukaryotic drug discovery programs. Studies on aminoglycoside kinase inhibitors that target the ATP-binding site started after the determination of the crystal structure of APH(3′)-IIIa that belongs to aminoglycoside phosphotransferases responsible for development of clinical resistance to aminoglycoside antibiotics.[54] The fact that the three-dimensional structure of APH(3′)-IIIa is quite similar to that of eukaryotic protein kinases, especially in the ATP-binding domain, has led to investigation of the sensitivity of aminoglycoside kinases to known ATP-competitive inhibitors of protein kinases. Isoquinolinesulfonamides **7**−**11** were found to be ATP-competitive inhibitors of APH(3′)-IIIa with K_i values between 49 and 730 μM (Figure 5). Structural studies reveal that these inhibitors bind to the ATP-binding site of protein kinases by forming a hydrogen bond between the nitrogen of the isoquinoline and the main chain amide hydrogen of the linker peptide that joins the N- and C-terminal domains. An analogous hydrogen bond is also observed between the N1 of the adenine ring of AMPPNP and the backbone NH group of Ala93 in APH(3′)-IIIa (Figure S1 in the Supporting Information). Therefore, it can be inferred that isoquinolinesulfonamides **7**−**11** would bind to APH(3′)-IIIa in a similar fashion.[55]

Additional evidence supporting this approach comes from a recent report in which a small set of ATP-competitive eukaryotic protein kinase inhibitors was screened for inhibition of D-alanine-D-alanine ligase (Ddl).[56] Ddl catalyzes the ATP-dependent formation of D-Ala-D-Ala dipeptide, which is an essential building block for bacterial cell wall biosynthesis.[57,58] On the basis of the similarities between

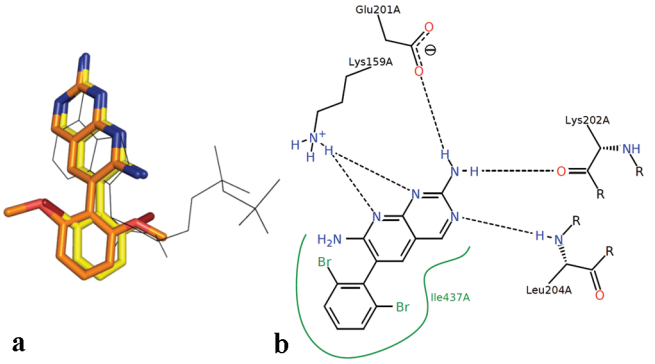

Figure 8. (a) Superposition of inhibitors **14** (in yellow) and **15** (in orange) (PDB codes 2V58 and 2V59, respectively) and ADP (in black) (PDB code 2J9G). (b) Schematic representation of interactions between **14** and BC active site residues (PDB code 2V58) as obtained with PoseViewWeb.

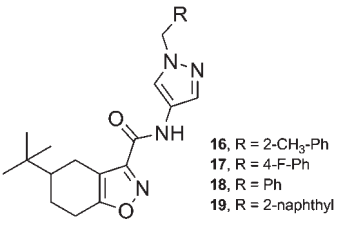

Figure 9. Pyrazole-based PS inhibitors.

the ATP-binding sites of Ddl (Figure S7 in the Supporting Information) and protein and lipid kinases, 27 different ATP-competitive kinase inhibitors were selected and screened for Ddl inhibition. Compounds **12** (K_i = 290 μM) and **13** (K_i = 185 μM) (Figure 6) exhibited modest ATP-competitive inhibition of Ddl, suggesting that a broader screen of ATP-competitive kinase inhibitor cores could identify additional inhibitors with improved activity.[56]

Another successful example is the study from Pfizer, in which nanomolar selective BC inhibitors were developed based on a protein kinase inhibitor pharmacophore. BC is a part of the multifunctional biotin-dependent enzyme acetyl-CoA carboxylase (ACCase) that catalyzes the first committed and rate-controlling step in the biosynthesis of long chain fatty acids.[59,60] Pyridopyrimidines **14** and **15** (Figure 7) were identified using whole-bacterial cell screening of the company compound library. By use of genetic and biochemical tools, BC was identified as the bacterial target of these compounds. The crystal structures (PDB codes 2V58 and 2V59) reveal that BC inhibitors **14** and **15** bind to the ATP-binding site of BC[61] (Figure S6 in the Supporting Information and Figure 8). The pyridopyrimidine moiety of the inhibitor interacts with the adenine-binding region, whereas the bisubstituted phenyl group occupies the hydrophobic

pocket neighboring the ribose-binding site. Compounds **14** and **15** inhibit *E. coli* ACCase with IC_{50} values less than 5 and 28 nM, respectively, and possess in vitro and in vivo antibacterial activity against Gram-negative pathogens, including *Haemophilus influenzae*, with a minimal inhibitory concentration (MIC) of 0.125 μg/mL.[9]

2.3. High-Throughput Screening (HTS) and Virtual Screening (VS) Combined with Structure-Based Drug Design (SBDD). Many pharmaceutical companies have sought to identify novel antibacterial agents from HTS campaigns using purified enzyme targets that were shown by genomic approaches to be essential for the organism. However, random screening provided very few suitable lead structures for novel inhibitors of the bacterial enzymes.

The HTS screening campaign was successful in discovering inhibitors of pantothenate synthetase (PS) from *Mycobacterium tuberculosis*. PS is an ATP-dependent enzyme, which catalyzes the formation of pantothenate from D-pantoate and β-alanine in the final step of the pantothenate biosynthetic pathway.[62,63] Compounds **16** and **17** (Figure 9) were identified as *M. tuberculosis* PS inhibitors by HTS of more than 10 000 compounds. Docking poses predicted by the FRED docking program show that the *tert*-butyl group occupies the hydrophobic pocket while the tetrahydrobenzoisoxazole ring binds into the adenine-binding pocket of PS (Figure S8 in the Supporting Information). The SAR was further investigated by the synthesis of new analogues bearing different substituents on the pyrazole ring. Improved inhibitory activity, compared to **16** (IC_{50} = 120 nM) and **17** (IC_{50} = 150 nM), was achieved by the synthesis of compounds **18** (IC_{50} = 97 nM) and **19** (IC_{50} = 90 nM) (Figure 9). Unfortunately, compounds **16**−**19** did not inhibit the growth of *M. tuberculosis* (MIC > 128 μg/mL).[64]

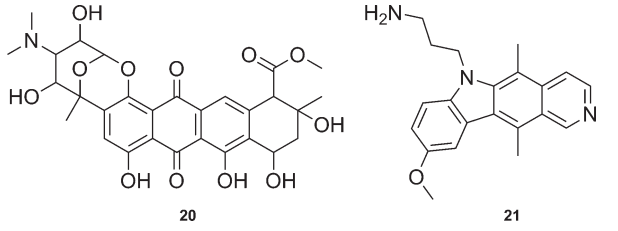

Figure 10. ATP-competitive inhibitors of DdlB.

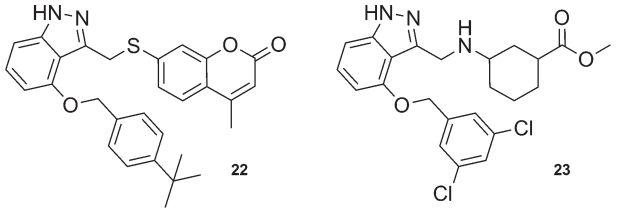

Figure 11. Indazole-based inhibitors of DNA gyrase.

"Virtual HTS" was also found to be a successful approach in which databases containing structures of small molecules are docked into a region of interest in silico and scored according to their predicted interactions in the target site. A recent successful example of the use of this method is the structure-based virtual screening of the UK National Cancer Institute diversity set of 2000 compounds using a crystal structure of Ddl.[65,66] The top 130 compounds from the list of compounds, ranked according to their estimated binding affinity to the protein, were tested in an in vitro assay. Three of the tested compounds inhibited DdlB, and of these, compounds **20** (K_i = 42 μM) and **21** (K_i = 11 μM) (Figure 10) showed ATP-competitive inhibition. Moreover, compound **21** showed promising antibacterial activity with MIC values of 8 and 32 μg/mL against *E. coli* SM1411 and *S. aureus* 8325-4, respectively.[66]

In the search for ATP-competitive DNA gyrase inhibitors, an alternative approach has been developed that combines an in silico needle screening and a biased high-throughput DNA gyrase screening with validation of the screening hits by biophysical methods and a 3D guided optimization process. From the obtained structural classes, the indazole scaffold was chosen for optimization by the use of crystal structure information. The indazole ring of the designed DNA gyrase inhibitors, such as compound **22** (Figure 11), occupies the adenine-binding pocket. However, compound **22** (MNEC = 0.03 μg/mL), which is 10 times more potent than novobiocin, is too lipophilic for penetration into the bacterial cell and showed no inhibition of bacterial growth. To reduce the lipophilicity of **22**, the coumarin ring was replaced by various amines, which led to the discovery of **23** (IC_{50} = 0.25 μg/mL) (Figure 11) with good antibacterial activity against resistant strains of staphylococci and enterococci (MIC = 4 μg/mL).[67]

In a Schering-Plough Research Institute a series of benzimidazole-based inhibitors of BC targeting ATP-binding site were identified from a mixture-based combinatorial library, using an affinity selection mass spectrometry (AS-MS) platform. Initial hit **24** (Figure 12), which inhibited BC with IC_{50} value of 5 μM, was cocrystallized with the enzyme (PDB code 3JZF). The results of subsequent structure-based optimization and computer-aided drug design were compounds **25**−**29** (Figure 12 and Figure 13) with improved inhibitory activity (IC_{50} values between 0.01 and 0.7 μM). The most

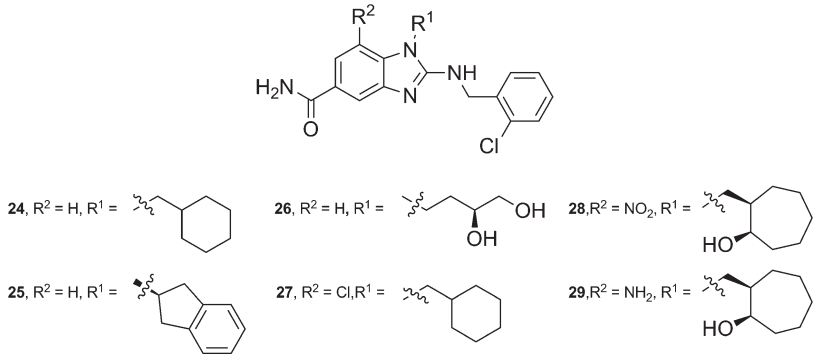

Figure 12. Benzimidazole and 4-aza-benzimidazole inhibitors of biotin carboxylase.

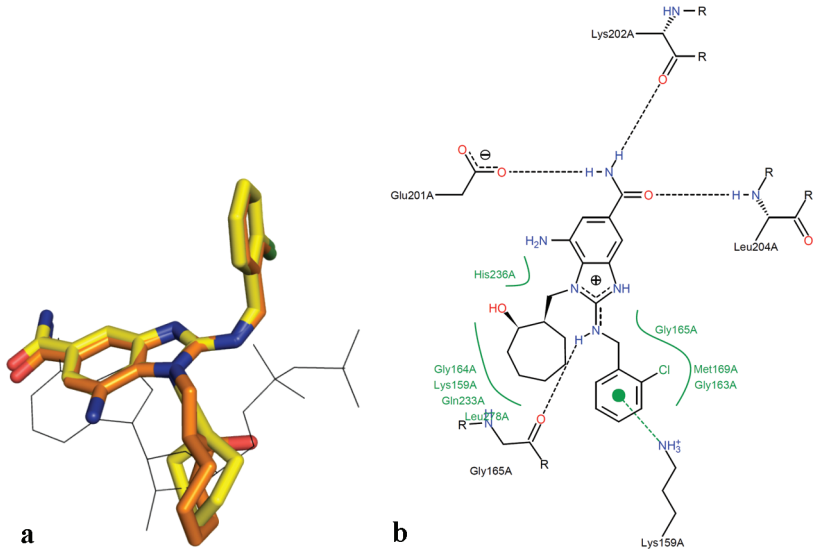

Figure 13. (a) Superposition of inhibitors **24** (in yellow) and **29** (in orange) (PDB codes 3JZF and 3JZI, respectively) and ADP (in white) (PDB code 2J9G). (b) Schematic representation of interactions between **29** and BC active site residues (PDB code 3JZI) as obtained by PoseViewWeb.

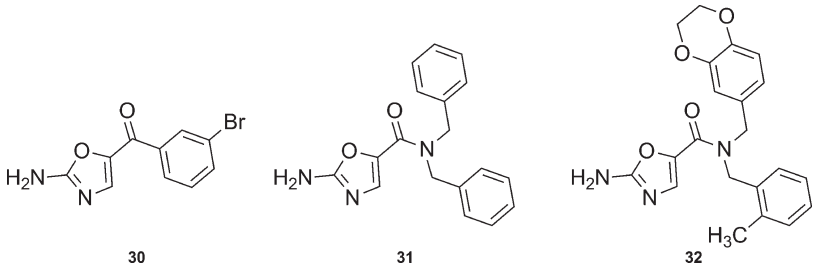

Figure 14. Aminooxazole-based inhibitors of biotin carboxylase.

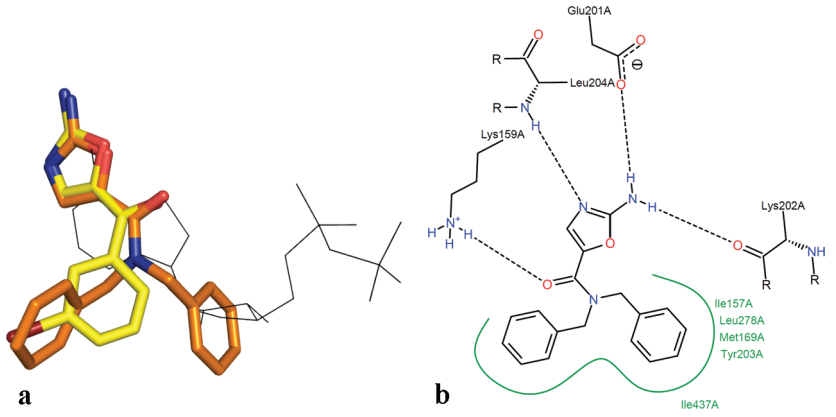

Figure 15. (a) Superposition of inhibitors **31** (in yellow) and **32** (in orange) (PDB codes 2W6M and 2W6N, respectively) and ADP (in black) (PDB code 2J9G). (b) Schematic representation of interactions between **32** and BC active site residues (PDB code 2W6N) as obtained with PoseViewWeb.

potent BC inhibitor **28** also showed very promising antibacterial activity against *E. coli* HS294 with a MIC value of 0.2 μg/mL and was selective for BC against eukaryotic carboxylases and human kinases.[68]

2.4. Fragment-Based Design of ATP-Competitive Inhibitors. One of the hypotheses explaining the lack of success in antibacterial drug discovery is a lack of chemical diversity compatible with novel antibacterial drug targets. In addition, it is believed that the physicochemical rules describing antibacterial compounds that can penetrate into the bacterial cytoplasm are considerably different from those used for eukaryotic target drug discovery.[7] One possible approach to address these issues is fragment-based drug design. The advantage of this strategy is that smaller compound fragments can feasibly probe a greater proportion of the chemical space in the active site of the target protein, and furthermore, through a process of structure-based fragment elaboration and growing, it is possible to design and build in desirable physicochemical properties.[69]

Researchers from Pfizer employed a combination of two lead discovery strategies: de novo fragment-based drug discovery and virtual screening in a search of BC inhibitors.

They screened a collection of unbiased low-molecular-weight molecules and identified a structurally diverse collection of weak-binding but ligand-efficient fragments as potential building blocks for the design of BC ATP-competitive inhibitors. The binding modes of identified fragments were characterized using X-ray crystallography, which revealed that all fragments interact with the adenine-binding region of the ATP-binding site. The potency of the initial hits was improved by up to 3000-fold by iterative cycles of structure-based drug design while maintaining their ligand efficiency and desirable physicochemical properties. Optimization of aminooxazole fragment **30** (IC_{50} = 21.5 μM) by fragment growing resulted in the most potent BC inhibitors **31** and **32** (Figure 14 and Figure 15) that inhibited the enzyme with IC_{50} values of 125 and 7 nM, respectively. Moreover, both inhibitors possessed antibacterial activity against Gram-negative pathogens (MIC values between 0.125 and 64 μg/mL), which was caused by specific inhibition of fatty acid biosynthesis. Compound **31** also exhibited significant selectivity against a panel of over 40 different human protein kinases (IC_{50} > 10 μM).[60]

Hung et al. used a fragment growing and fragment linking technique to discover new inhibitors of *M. tuberculosis* PS. Initially, 5-methoxyindole (**33**) (Figure 16) was identified as an ATP-competitive inhibitor of PS by WaterLOGSY NMR spectroscopy. Isothermal titration calorimetry (ITC) confirmed the binding of the fragment with a K_d value of 1.1 mM, and its precise binding mode was determined by X-ray crystallography to a resolution of 1.6 Å. The fragment was found to occupy the adenine pocket of the ATP-binding site and was selected to initiate a fragment-growing design, which finally led to compounds **34** (K_d = 29 μM) and **35** (K_d = 1.5 μM) (Figure 16 and Figure 17) with improved inhibitory activity.[62]

Benzofuran-2-carboxylic acid (**36**) (Figure 18) was identified as a PS inhibitor by a thermal-shift screen and confirmed by WaterLOGSY NMR spectroscopy and ITC (K_d = 1 mM). The X-ray structure showed that **36** binds across the pantoate-binding pocket, 3.1 Å away from the binding site of the indole **33**. The crystal structure of the PS·**36** complex (PDB code 3IME) suggests that **36** may be able to bind to the active site simultaneously with fragment **33** and could be used in a linking strategy. Various linkers between C2 atoms of the two fragments were designed to enable both fragments to adopt their original binding modes (compounds **37**−**39**, Figure 18), and the sulfamoyl linker in compound **39** (K_d = 1.8 μM) has proved to be the most effective (Figure 19).[62] Furthermore, fragment linking of **36** and **40** (K_d = 800 μM) to give a potent PS inhibitor **41** (K_d = 860 nM) (Figure 18) was guided by optimization of the interligand Overhauser effect (ILOE) observed between starting fragments **33** and **36**.[70]

2.5. Multitarget Inhibitors. Using network models of antimicrobial drugs, Csermely et al. showed that multitarget attacks perturb complex systems more effectively than focused attacks, even if the number of targeted interactions is the same.[71] Targeting multiple bacterial enzymes that are structurally related or recognize common structural motifs and the possibility of finding a small molecule that binds to more than one bacterial enzyme family member is therefore preferred. An interesting rationale for the design of dual-target inhibitors, with the potential to reduce the development of target-based resistance, offers the similarity of crystal structures of bacterial GyrB and ParE. Indeed,

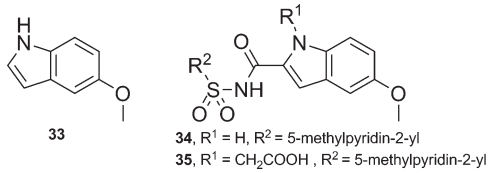

Figure 16. 5-Methoxyindole-based PS inhibitors discovered by fragment-based design.

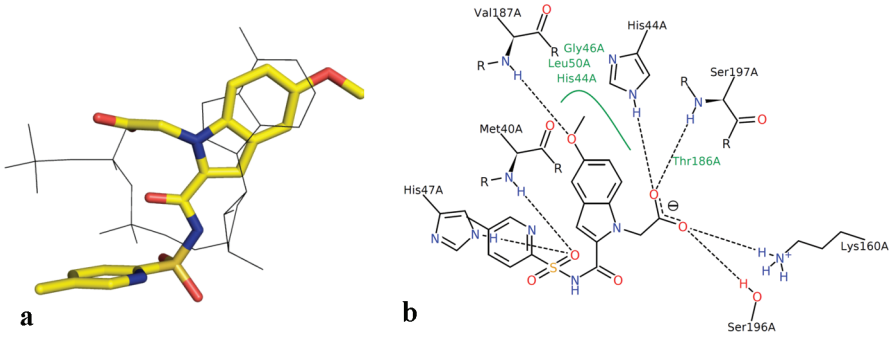

Figure 17. (a) Superposition of inhibitor **35** (in yellow) (PDB code 3IUE) and adenosine 5′-(α,β-methyleno)triphosphate (AMPCPP) (in black) (PDB code 1N2B). (b) Schematic representation of interactions between **35** and PS active site residues (PDB code 3IUE) as obtained with PoseViewWeb.

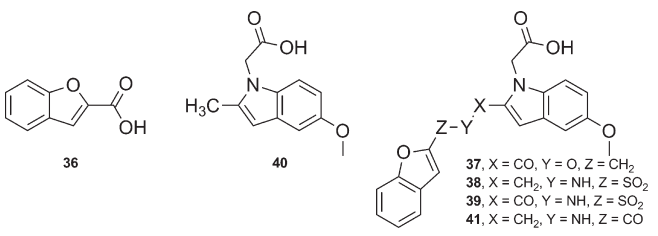

Figure 18. Benzofuran-based PS inhibitors designed by the fragment linking strategy.

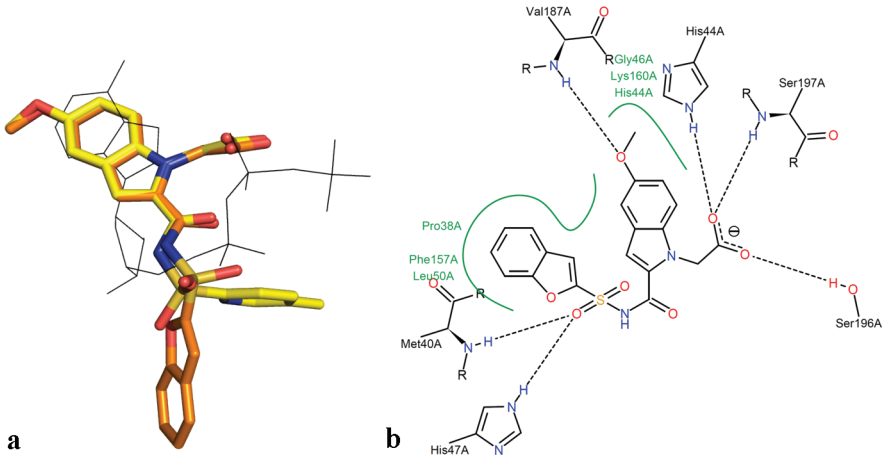

Figure 19. Superposition of inhibitors **35** (in yellow), **39** (in orange) (PDB codes 3IUE and 3IVX, respectively) and AMPCPP (in black) (PDB code 1N2B). (b) Schematic representation of interactions between **39** and PS active site residues (PDB code 3IVX) as obtained with PoseViewWeb.

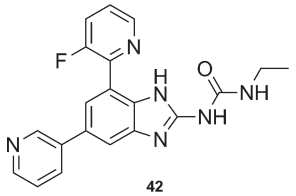

Figure 20. Dual-targeting DNA gyrase and TopoIV benzimidazole urea inhibitor.

dual-targeting benzimidazole urea inhibitors of DNA gyrase and TopoIV with potent antibacterial activity against a wide spectrum of relevant pathogens were reported recently. A representative compound **42** (Figure 20) inhibits DNA gyrase from *S. aureus* and *E. coli* with K_i values of 14 nM and less than 4 nM, respectively, and TopoIV from *E. coli* with a K_i value of 23 nM. Compound **42** also possessed potent antibacterial activity against *S. aureus, Streptococcus pneumoniae*, and *H. influenzae* with MIC values below 1 μg/mL.[72]

3. Selectivity Challenge

As mentioned above, the conserved nature of ATP-binding sites among different eukaryotic and prokaryotic protein classes makes a design of an inhibitor that targets selectively the bacterial enzyme ATP-binding site a great challenge. One of the characteristic examples of the target for which the selectivity achievement can be a difficult task are members of GHKL superfamily, such as PhoQ. Superposition of PhoQ and human pyruvate dehydrogenase kinase reveals high structural similarity between the enzymes (Figure 21a).[46] Both enzymes belong to the GHKL superfamily and share a conserved Bergerat fold in the ATP-binding domain. This fact raises the possibility that the histidine kinase inhibitors targeting this domain will also affect the activity of other mammalian enzymes having the homologous domain and may not exhibit sufficient selectivity for sensor kinases of pathogenic microorganisms.[30] The prime example is ATP-competitive inhibitor **6** (Figure 4), which inhibits the in vitro activity of the mammalian GHKL protein pyruvate dehydrogenase kinase in a manner similar to that in which it inhibits PhoQ (Figure 21b).[47]

Sometimes the important structural differences between bacterial and eukaryotic enzymes make the achievement of selectivity possible. For example, the three-dimensional structure of APH(3′)-IIIa reveals a striking structural similarity to eukaryotic protein kinases, such as the catalytic subunit of cAMP-dependent protein kinase (cAPK) and the catalytic domains of casein kinase-1 and MAP kinase ERK2, despite the lack of sequence homology (< 5%). The superposition of the nucleotides from crystal structures of APH(3′)-IIIa and cAPK reveals important differences between the two enzymes, which offers possibilities for structure-based drug design of APH(3′)-IIIa inhibitors selective against structurally related protein kinases. In particular, the adenine ring in APH(3′)-IIIa forms a $\pi-\pi$ stacking interaction with the aromatic side chain of Tyr42, a residue highly conserved in APH(3′) enzymes (Figure 22). Its corresponding residue in protein kinases is alanine, which excludes the possibility of forming such a stacking interaction.[73] Importantly, thermodynamic analyses have demonstrated that the $\pi-\pi$ stacking interaction between APH(3′)-IIIa and the adenine ring can supply up to one-third of the total nucleotide binding energy.[74] Moreover, the polar nature of the adenine ring−Met90 contact in the case of APH(3′)-IIIa differs from the role of Met residues in eukaryotic protein kinases, which often act as gatekeeper residues and usually reside in a more hydrophobic pocket.[75]

Miller et al. showed that it is possible to achieve selectivity, despite the structural similarity between the ATP-binding sites of eukaryotic protein kinases and BC. The distinctively different binding modes of pyridopyrimidine-based inhibitors observed in the BC and FGFR2 kinase domain suggested that BC selectivity could be achieved despite the structural similarity of the ATP-binding sites (Figure 23). It was observed that inhibitors **14** and **15** (Figure 7) display excellent selectivity for bacterial BC over FGFR2, VEGFR1, and 28 other eukaryotic protein kinases. The only eukaryotic protein kinase that was inhibited by **14** was Src, but the IC_{50} value of this inhibitor was still 70-fold weaker than that against *E. coli* BC.[9]

Another way to attain selectivity is to design inhibitors that mimic the reaction intermediate of specific enzyme-catalyzed reaction. This approach was successfully used on PS, which belongs to the cytidylyltransferase superfamily of enzymes that catalyze the formation of an acyl adenylate (or acyl cytidylate) via attack at the α-phosphate of ATP (or CTP) and following release of pyrophosphate.[76] Tight binding of

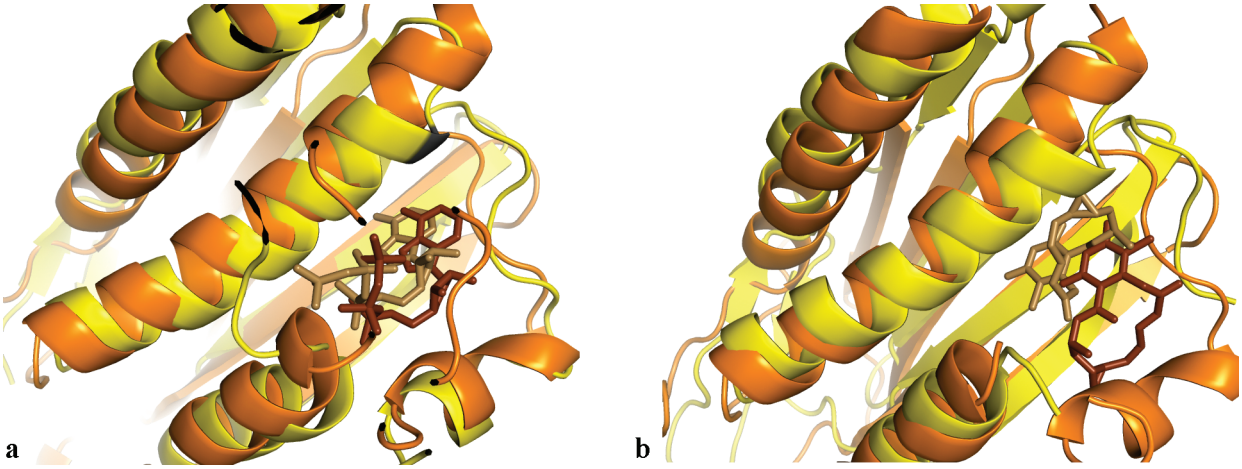

Figure 21. (a) Superposition of PhoQ (in yellow)·AMPPNP (in brown) and human pyruvate dehydrogenase kinase (in orange)·ATP (in light brown) complexes (PDB codes 1ID0 and 1Y8P, respectively). (b) Superposition of PhoQ (in yellow)·**6** (in brown) and human pyruvate dehydrogenase kinase (in orange)·**6** (in light brown) complexes (PDB codes 3CGY and 2Q8I, respectively).

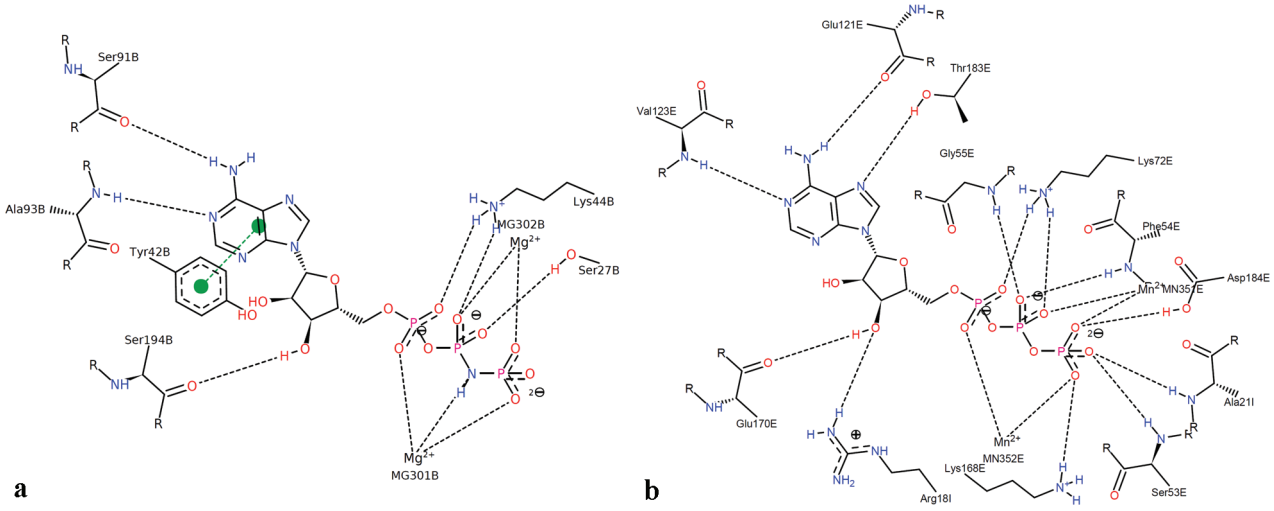

Figure 22. (a) Schematic representation of interactions between AMPPNP and APH(3′)-IIIa ATP-binding site residues (PDB code 1J7U). (b) Schematic representation of interactions between ATP and cAMP-dependent protein kinase from *Mus musculus* ATP-binding site residues (PDB code 1ATP) as obtained with PoseViewWeb.

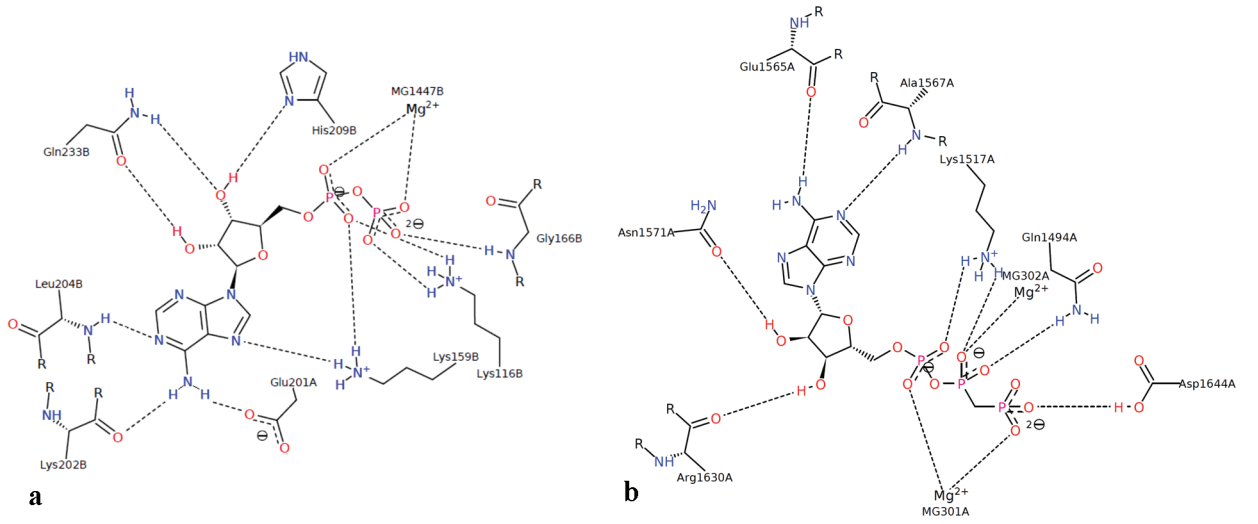

Figure 23. (a) Schematic representation of interactions between ADP and BC active site residues (PDB code 2J9G). (b) Schematic representation of interactions between AMPPNP and FGFR2 active site residues (PDB code 3CLY) as obtained with PoseViewWeb.

pantoyl adenylate to PS provided an excellent starting point for the design of its inhibitors by mimicking this reaction intermediate (Figure 24).[77]

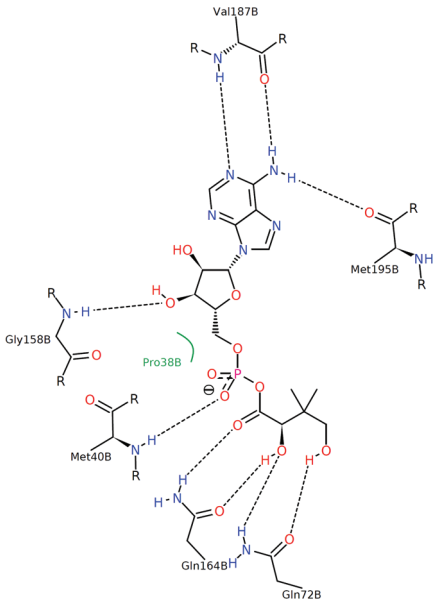

Figure 24. Schematic representation of interactions between pantoyl adenylate and PS active site residues (PDB code 1N2H) as obtained with PoseViewWeb.

Ten pantoyl adenylate analogues were synthesized, in which the labile phosphodiester moiety was replaced by either an ester or a sulfamoyl group, and the pantoyl functionality was modified. The sulfamoyl analogues **43**−**47** (K_i = 0.3−65 μM) (Figure 25) were approximately 100-fold more potent inhibitors than their ester counterparts (K_i values in the low millimolar range), which reflects their closer structural similarity with pantoyl adenylate.[78] Compound **47** (Figure 26), which is structurally the most similar to the reaction intermediate, was the most potent inhibitor in this series with K_i = 0.3 μM.[79]

To continue with the successful design of PS inhibitors by mimicking pantoyl adenylate, Scott et al. used dynamic combinatorial chemistry coupled with structure-based design, in which 5′-deoxy-5′-thioadenosine **48** (K_d = 380 μM) (Figure 27) was used as an anchor building block to find thiols that would form disulfides templated by PS. A WaterLOGSY NMR experiment was used to show binding of thioadenosine **48** to *M. tuberculosis* PS. This and ITC both clearly indicated a competition between compound **48** and ATP for the same binding site, which was later confirmed by the X-ray crystal structure of PS·**48** complex (Figure 28). The most potent disulfides designed were compounds **49** and **50** (Figure 27) with K_d values of 210 μM and 80 μM, respectively.[80]

To estimate the probability that the chosen bacterial enzymes (Table 1) represent promising targets for the design of selective ATP-competitive bacterial enzyme inhibitors, the

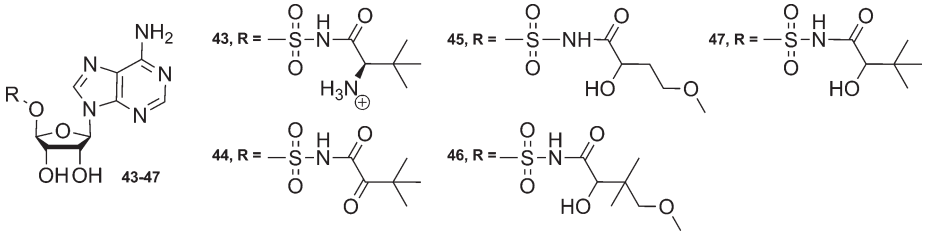

Figure 25. Structures of pantoyl adenylate analogue inhibitors of *E.coli* PS.

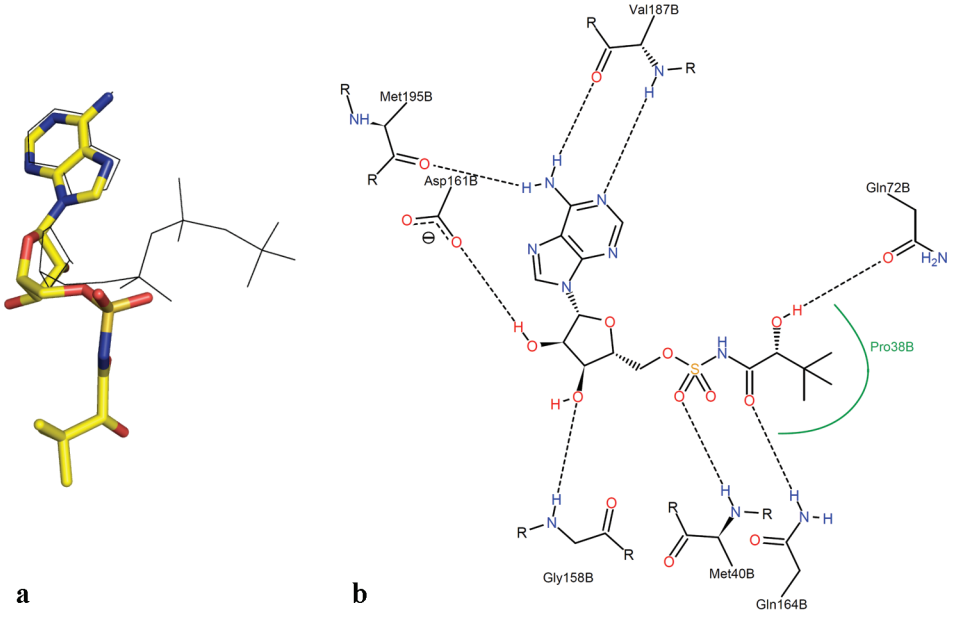

Figure 26. (a) Superposition of inhibitor **47** (in yellow) (PDB code 3COW) and AMPCPP (in black) (PDB code 1N2B). (b) Schematic representation of interactions between **47** and PS active site residues (PDB code 3COW) as obtained with PoseViewWeb.

similarity of the ATP-binding sites of bacterial enzymes was studied using ProBiS,[81,82] a Web server for detecting protein binding sites based on local structural alignments. The structure of the query enzyme is compared to those of members of a database of protein 3D structures in order to identify proteins that share local structural similarities with the query enzyme. The ProBiS results (Table S2 in the Supporting Information) for representative enzymes of the four studied superfamilies are presented in Tables S4−S7 in the Supporting Information. For example, the ATP-binding site of aminoglycoside-modifying enzyme APH(3′)-IIIa (Table S4 in the Supporting Information) is structurally similar to the ATP-binding sites of 188 enzymes in the RCSB Protein Data Bank.[20] Enzymes with a large number of aligned residues belong to other members of aminoglycoside-modifying enzymes and to several human protein kinases. Moreover, most of the identified protein structures similar to APH(3′)-IIIa are of human origin. Another example is bacterial histidine kinase PhoQ of the GHKL superfamily (Table S5 in the Supporting Information), whose ATP-binding site was found to be similar to those of 77 protein structures. Top ranked similar structures are those of other members of the GHKL superfamily, such as DNA gyrase, TopoIV, histidine kinases EnvZ and CheA, and human pyruvate dehydrogenase kinase. On the other hand, the number of structures similar to those of bacterial BC, Ddl, and PS (Tables S6 and S7 in the Supporting Information) is smaller and they also show less similarity to human enzymes. Analysis of the results obtained with ProBiS indicates that the ATP-binding sites of aminoglycoside-modifying enzymes and of members of the GHKL superfamily possess higher structural similarity to the ATP-binding sites of human enzymes, particularly of protein kinases. This suggests that selectivity toward bacterial enzymes will be harder to achieve than for the members of the ATP-grasp fold and cytidylyltransferase superfamilies, which share more structural similarities with enzymes within each superfamily.

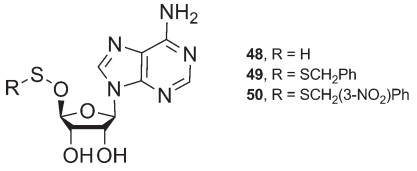

Figure 27. Thioadenosine-based inhibitors of *M. tuberculosis* PS.

4. Defining Target Selectivity by K_i Values

Another issue concerning inhibitor selectivity that needs to be addressed is how to measure and evaluate selectivity between bacterial and human targets. To show in vivo activity, ATP-competitive inhibitors must be able to compete with the high ATP concentrations present in the bacterial cell (0.6−18 mM)[10−12] and must also be highly selective because of the similarity of the ATP-binding site in bacterial and human enzymes. The potency of an inhibitor for its target is typically expressed by the IC_{50} value minus the concentration of drug at which 50% of the enzyme activity is inhibited. For reversible and ATP-competitive inhibitors, the IC_{50} depends on the intrinsic activity of the inhibitor (the dissociation constant K_i) as well as the competition from ATP under the specific assay conditions (the [ATP] and the $K_{m(ATP)}$). These variables are related by the Cheng−Prusoff equation:[83]

$$K_i = \frac{IC_{50}}{1 + \frac{[ATP]}{K_{m(ATP)}}}$$

This equation expresses the fact that at low ATP concentrations, there is no significant competition from substrate and $IC_{50} \approx K_i$. As the ATP concentration exceeds the $K_{m(ATP)}$, the IC_{50} increases at approximately the same rate. Importantly, the IC_{50} does not plateau at a maximum value at high concentrations of ATP. For this reason, the potency of an inhibitor in cells depends critically on the $K_{m(ATP)}$ of its target. Consequently, an inhibitor that has similar K_i values against multiple targets will inhibit more potently those targets that have higher $K_{m(ATP)}$. Additionally, an important warning concerning the use of $K_{m(ATP)}$ values is that they are sensitive to the specific assay conditions (such as choice of protein substrate or counterion). Hence, any meaningful comparison of inhibitor affinity for the enzymes must be based on measurements of dissociation constants, and this should be taken into account when selectivity data of the inhibitor for bacterial and human enzymes are given.[84]

5. Concluding Remarks and Perspectives

In the field of antibacterial drug discovery, researchers were skeptical about the possibility of making any selective ATP-competitive inhibitors of bacterial enzymes. Clearly one of the major problems in the field is to achieve selectivity for

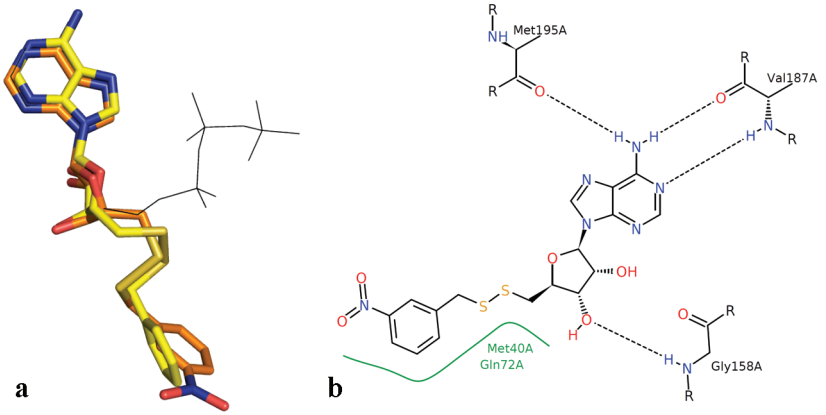

Figure 28. Superposition of inhibitors **49** (in yellow) and **50** (in orange) (PDB codes 3IOC and 3IOD, respectively) and AMPCPP (in black) (PDB code 1N2B). (b) Schematic representation of interactions between **50** and PS active site residues (PDB code 3IOD) as obtained with PoseViewWeb.

bacterial targets over human enzymes. Consequently, approaches to identify novel classes of antibacterials have tended to focus on genomic targets that share little or no similarity to human enzymes. For this reason, the successful use of HTS in other fields of drug discovery has here been limited because available compound libraries are often biased toward compound classes targeted at major human target families, such as protein kinases, G-protein-coupled receptors, proteases, etc. However, by use of structure-based and computer-aided drug design methods, recent more successful work supports the possibility of finding a selective inhibitor targeting the ATP-binding site of bacterial enzyme using eukaryotic biased compound libraries. It is suggested that for targets with high sequence and structure homology to known human targets, a potent inhibitor is more likely to be found within the chemical space covered by synthetic compound libraries. Such inhibitors may offer good starting points for the design of compounds with improved activity and selectivity profiles. On the other hand, fragment-based drug design enables the synthesis of target specific, chemically diverse molecules, which has already proved successful in the discovery of novel BC and PS inhibitors with good selectivity profiles.

While the similarity of ATP-binding sites in bacterial and human enzymes makes the design of selective ATP-competitive inhibitors difficult, inhibition of multiple bacterial enzymes with similar ATP-binding sites should be beneficial in combating the development of target-based resistance to antibacterial agents and should result in potent antibacterial activity in whole-cell assays. An excellent example is the reported dual-target DNA gyrase and topoisomerase IV benzimidazole urea inhibitor that has potent activity against Gram-positive and Gram-negative bacteria.

Another promising approach to designing inhibitors targeting the ATP-binding site can be the design of compounds that occupy not only the ATP-binding site but at the same time exploit other substrate-binding sites or allosteric pockets. Here the use of SBDD provides direct knowledge of critical interactions of an inhibitor with the amino acids of the target protein, allowing optimization of binding affinity and selectivity by exploiting interactions with amino acid residues adjacent to the ATP-binding domain but unique to the target enzyme.

Finally, we wanted to show that the ATP-binding sites of bacterial enzymes represent viable targets for design of novel antibacterial agents. The design of bacterial target-selective inhibitors is very challenging but can be successfully overcome with the knowledge of the target structure and important dissimilarities between the target and other similar human ATP-binding enzymes.

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Biographies

Veronika Škedelj graduated from the University of Ljubljana, Faculty of Pharmacy, Slovenia in 2008. She is presently working as a Ph.D. student at the Department of Medicinal Chemistry, Faculty of Pharmacy, in the field of design, synthesis, and biological evaluation of novel antibiotics targeting the peptidoglycan biosynthesis. Her research interests include medicinal chemistry, heterocyclic chemistry, and enzyme kinetics.

Tihomir Tomašić received his Master of Pharmacy degree (2006) from the Faculty of Pharmacy, University of Ljubljana, Slovenia. Currently, he is a Ph.D. student of Biomedicine at the Department of Medicinal Chemistry, Faculty of Pharmacy, University of Ljubljana. His Ph.D. thesis research work is focused on computer-aided drug design and synthesis of novel antibacterials targeting intracellular steps of peptidoglycan biosynthesis. His research interests include medicinal chemistry, heterocyclic chemistry, and molecular modeling.

Lucija Peterlin Mašič completed her doctoral studies at the University of Ljubljana, Faculty of Pharmacy, Slovenia (2003). She did postdoctoral research in AstraZeneca, Sweden, at the Department for Computational Toxicology (Safety Assessment). Currently, she is Assistant Professor for Medicinal Chemistry at the Faculty of Pharmacy, and she continued her interest in toxicology, computational methods for the site of metabolism and reactive metabolites formation predictions, toxicity testing in the early phases of drug discovery process, and study of mechanisms of toxicity, structure-based drug design, discovery, and development of new chemical entities in cardiovascular and anti-infective areas.

Anamarija Zega received her Ph.D in Medicinal Chemistry from the University of Ljubljana, Faculty of Pharmacy, Slovenia, in 2002. She did her postdoctoral research in Dr. Michel Arthur's group at Laboratoire de Recherche Moléculaire sur les Antibiotiques, Inserm, in Paris. She is currently Assistant Professor for Medicinal Chemistry at the Faculty of Pharmacy in Ljubljana, and she continues her interest in structure-based drug design with a particular emphasis on antimicrobials.

Supporting Information Available: Figures of ATP-binding sites of the studied enzymes in complex with ATP or its analogue and ProBiS results. This material is available free of charge via the Internet at http://pubs.acs.org.

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