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Fragment-based inhibitor discovery against β-lactamase

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Abstract

The production of β-lactamase is one of the primary resistance mechanisms used by Gram-negative bacterial pathogens to counter β-lactam antibiotics, such as penicillins, cephalosporins and carbapenems. There is an urgent need to develop novel β-lactamase inhibitors in response to ever evolving β-lactamases possessing an expanded spectrum of β-lactam hydrolyzing activity. Whereas traditional high-throughput screening has proven ineffective against serine β-lactamases, fragment-based approaches have been successfully employed to identify novel chemical matter, which in turn has revealed much about the specific molecular interactions possible in the active site of serine and metallo β-lactamases. In this review, we summarize recent progress in the field, particularly: the identification of novel inhibitor chemotypes through fragment-based screening; the use of fragment-protein structures to understand key features of binding hot spots and inform the design of improved leads; lessons learned and new prospects for β-lactamase inhibitor development using fragment-based approaches.

The β-lactams are the most widely used antibiotics due to their effective inhibition of the penicillin binding proteins, which are essential enzymes in bacterial cell wall synthesis (Figure 1) [1,2]. The primary mediators of bacterial resistance to these antibiotics are β-lactamases, an evolutionarily ancient family of hydrolases that catalyze β-lactam hydrolysis and inactivation [3,4]. β-lactamases can be classified under the Ambler system by sequence similarity and catalytic mechanism, or under the Bush–Jacoby system by their substrate preferences [5,6]. The Ambler system recognizes three families of serine β-lactamases (Class A, C and D) which employ a catalytic serine to mediate the hydrolysis reaction, and a fourth family (Class B) of metallo enzymes.

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Since the discovery of penicillins, many new β-lactam drugs, such as cephalosporins and carbapenems, have been developed that can evade and/or inhibit β-lactamase hydrolysis, with many acting as mechanism-based suicide substrates for penicillin binding proteins and/or β-lactamases (Figure 1) [1]. Almost invariably however, mutations or new enzymes have emerged that enable the recognition and hydrolysis of the latest generation of β-lactam antibiotics. The CTX-M Class A β-lactamases, the most common of the **extended-spectrum β-lactamases** (ESBLs), continue to evolve enhanced activity against the third generation cephalosporins [7–11]. Other Class A ESBLs are also of increasing concern clinically [11,12]. In addition, in recent years, carbapenemase activity has been observed in many Class A, B and D enzymes, rendering even the venerable carbapenems ineffective for treating bacterial infections in life threatening situations [13–15].

The use of a β-lactamase inhibitor in combination with a β-lactam antibiotic is a well-established strategy to counter resistance [16]. Classical β-lactamase inhibitors such as clavulanate (Figure 1), tazobactam and sulbactam have been of significant clinical value in this regard, but these inhibitors are primarily effective against Class A enzymes and therefore are not broadly useful for countering the diversity of β-lactamase enzymes present today. These classical inhibitors also contain a β-lactam ring and, as a result, they are susceptible to resistance resulting from upregulation of β-lactamase expression, new β-lactamase acquisition and other mechanisms that have evolved over millions of years of competition between bacteria and β-lactam-producing microorganisms [17–20]. These limitations, as well as the widespread presence of ESBLs and carbapenemases in multidrug-resistant Gram-negative pathogens, have motivated the search for new classes of β-lactamase inhibitors, as reviewed recently by Drawz and Bonomo [16]. Two notable examples include avibactam (previously known as NXL104) [21–23], and RPX7009 [24,25] (Figure 1). These new β-lactamase inhibitors can form a stable covalent bond with the catalytic serine in a wide range of β-lactamases, and thus possess broad-spectrum activity. Avibactam is currently in late-stage clinical trials as cephalosporin or carbapenem combinations. RPX7009 is undergoing Phase I trials in combination with a carbapenem, biapenem. These recent successes demonstrate that novel, non-β-lactam compounds can be employed clinically to inhibit β-lactamases in resistant bacteria [26–29].

Developing novel antimicrobial chemotypes is crucial for addressing bacterial resistance, yet traditional **high-throughput screening** (HTS) against isolated drug targets has proven largely ineffective [30,31]. For example, 51 out of 67 HTS campaigns on antibacterial targets at GlaxoSmithKline (GSK) failed to yield any hits, and only five from the remaining 16 generated leads suitable for further optimization [30]. HTS against whole bacteria, however, has produced at least one new class of drugs, the oxazolidinones [32,33]. A problem with existing HTS screening libraries is their limited size and biased chemical composition favoring G-protein coupled receptor (GPCR)- and kinase-type ligands [34–37]. Fragment-based approaches instead focus on lower molecular weight (MW <250 Da) compounds that target sub-pockets of a binding site [38], usually with higher ‘ligand efficiency’ (binding energy per heavy atom) than typical HTS hits. Fragments, because of their smaller size, provide a more complete representation of chemical space [39–45]. This drug-discovery paradigm often offers an efficient lead optimization strategy through

growing or merging of structurally characterized fragments into high-affinity compounds [46], and has led to several drug candidates in Phase II/III trials and one US FDA approved drug [47].

HTS versus fragment-based methods in inhibitor design against β-lactamases

HTS has produced mixed results against serine and metallo β-lactamases. For serine β-lactamases, HTS has consistently failed to yield meaningful lead compounds. The HTS campaign by GSK against P99 Class C β-lactamase did not uncover any hits out of half a million compounds screened [30]. Another screen of ~70,000 compounds from the NIH Chemical Genomics Center against AmpC Class C β-lactamase led to 12 promiscuous covalent inhibitors but no specific reversible inhibitors [31]. Computational docking methods were slightly more successful against AmpC, producing two inhibitors from 16 compounds chosen from the same NIH Chemical Genomics Center library [31], and three inhibitors from an earlier effort using 56 molecules selected from 200,000 compounds of Available Chemical Database [48]. Similar efforts using the Available Chemical Database against TEM Class A β-lactamases led to two high μM allosteric inhibitors [49].

In contrast to this poor track record against serine β-lactamases, HTS has been applied successfully to identify novel inhibitors against metallo β-lactamases. Although screening of the GSK library against CfiA metallo β-lactamase did not lead to any hits, HTS at Merck [50,51], Meiji Seika Kaisha Ltd [52], and academic groups [53,54] all identified novel chemotypes using large libraries containing hundreds of thousands of compounds. One reason for this success may be the presence in the screening compounds of functional groups such as thiol, carboxylate and tetrazole that can form strong interactions with the zinc center in these metallo-enzymes. This has led to the screening of more focused libraries containing some of these chemotypes [55–57]. The relatively shallow and open active sites of metallo β-lactamases also reduce the chances of steric clashes with potential inhibitors [58–60]. By comparison, there is no single feature in serine β-lactamases that can by itself attribute binding affinity comparable to the metal–ligand interaction in Class B enzymes. It thus becomes less likely that one will find HTS hits of reasonable affinity for serine β-lactamases, since multiple binding interactions would need to be satisfied. However, these very features of serine β-lactamases (multiple binding sub-sites) make them especially attractive targets for fragment-based approaches.

Fragment-based lead discovery (FBLD) has proven in the past several years to be a very promising method to develop new inhibitors against both serine and metallo β-lactamases, including CTX-M Class A [61,62], AmpC Class C [28,63] and IMP-1 Class B enzymes [64,65]. It has been successfully applied in both lead identification to uncover novel chemotypes for non-covalent inhibitors, and in lead optimization to probe additional binding hot spots for covalent inhibitors. Several trends have emerged from these studies.

First, the availability of high quality structural data has played a crucial role in this area [66]. The majority of β-lactamase crystals can diffract to very high resolutions in x-ray crystallographic analysis. Some extraordinary examples include Class A enzymes TEM-1

(0.85 Å) [67], CTX-M-9 (0.88 Å) [68], SHV-2 (0.9 Å) [69], KPC-2 (1.23 Å) [70], Class C enzymes AmpC (1.07 Å) [71], Class D enzymes OXA-10 (1.35 Å, PDB 2X02), and Class B enzymes NDM-1 (1.05 Å, PDB 4HL2). Accordingly, this group of enzymes is ideally suited to the FBLD approach, with the high resolution structures not only providing better templates for computational modeling, but also allowing more accurate determination of fragment binding modes. The low binding affinity of fragment inhibitors often results in their low occupancy in complex crystals and weak signals in electron density maps. For lower resolution crystal structures including those approximately 2.0 Å, it usually means significant ambiguities in the binding conformations of fragment inhibitors. The high quality of β-lactamase crystal structures overcomes this difficulty and the improved accuracy is particularly useful in elaborating fragments into leads via growing or merging fragments [28,61–63].

Second, computational docking technology has matured to the point that it is now very useful for prioritizing fragments for experimental testing [72,73]. The low binding affinity of fragment inhibitors means that they have to be tested at very high concentrations, usually in the high μM or low mM range. Traditional biochemical assays are often plagued by artifacts when performed in these concentration ranges and so biophysical methods such as NMR, x-ray crystallography, and surface plasmon resonance have more often been deployed to screen fragment compounds in a low-throughput fashion, usually testing up to only a few thousand molecules in each screening [66,74–76]. Computational docking allows a rapid initial prioritization of compounds from the approximately half a million commercially available fragment compounds [77], significantly expanding the overall chemical space being sampled in a fragment screening campaign. Despite initial skepticism, computational screens have uncovered novel fragment inhibitors against both Class A and Class C β-lactamases at a hit rate higher than the average of experimental fragment screening, demonstrating the potential of this technique in FBLD against β-lactamases. In addition, molecular docking performed well in predicting fragment binding poses when compared with subsequently determined crystal structures.

Finally, *in vitro* biochemical assays are available for β-lactamases that allow for the biochemical characterization (K_i) of fragments with very weak affinities. This is in contrast to many other classes of targets, where functional biochemical activity is not easily measured for weak affinity fragments. Many β-lactam substrates have been developed over the years to study β-lactamase activities in the context of bacterial resistance, and these reagents now provide convenient UV absorption-based assays for testing fragments [78]. More substrates are being developed, such as those for fluorescence-based activity assays [79], and this allows the selection of a suitable substrate for a particular β-lactamase and/or the identification of false positive hits by using substrates with different fluorophores. The use of molecular docking also reduces by orders of magnitude the number of compounds that need to be experimentally tested. This enables biochemical assays to be used for primary experimental testing alongside, or in place of, other techniques such as surface plasmon resonance [63].

Class A β-lactamases

Clinically, the Class A β-lactamase CTX-M is the most commonly encountered ESBLs [8]. This very diverse group of enzymes has evolved enhanced activities against third generation cephalosporins such as cefotaxime and ceftazidime. There are five subgroups of CTX-M β-lactamases, CTX-1, CTX-M-2, CTX-M-8, CTX-M-9 and CTX-M-25, based on sequence similarities. The active sites of all these subgroups share high sequence and structural conservation, with a slightly enlarged binding site (e.g., Asp240 vs Glu240 in TEM-1) to accommodate the bulky side chains of extended spectrum β-lactam antibiotics, as well as particular protein residues to enhance substrate binding (e.g., Ser237). Given the importance of this family of enzymes, it is not surprising that CTX-M has been the focus of the most significant FBLD efforts to date on β-lactamase.

Using CTX-M-9 as a model system, a series of fragment screening and optimization experiments have led to the first nM-affinity noncovalent inhibitor against any serine β-lactamase (Figures 2 &3) [61,62]. The program DOCK was employed to screen both the fragment and lead-like subset of the **ZINC** small molecule database [77,80,81]. Out of 70,000 fragment compounds, 69 were selected for experimental testing and ten were shown to be inhibitors (Figure 2A). In contrast, no inhibitors were found among the 34 candidates chosen from 1,000,000 lead-like compounds. The failure with screening lead-like compounds is likely due to a combination of the inaccuracy of docking methods in general and the more sporadic coverage of chemical space by the larger lead-like compounds. Inadequate sampling of conformational space and errors in scoring functions may pose a larger challenge for docking lead-like compounds than fragments because such problems can be amplified due to the larger number of conformations and interactions that need to be calculated for lead-like compounds. Also, as discussed below in studies using AmpC as a model system, many inhibitor chemotypes may be available at much lower percentages in commercial lead-like databases in comparison to fragment databases, increasing the difficulty of identifying these inhibitors in screening lead-like compounds.

Five crystal structures of CTX-M-9 were determined in complex with fragment inhibitors to resolutions of 1.5 Å or better. Several inhibitors only appeared in the active site with partial occupancy, and they overlapped with a phosphate and water molecules usually observed in the apo crystal. The value of high-resolution structures for FBLD against β-lactamases is demonstrated by details of the ligand electron density permitting the differentiation of the apo and complex states of the active site. These structures also demonstrated the ability of molecular docking to successfully predict ligand binding conformations, which played a significant role in subsequent lead optimization efforts.

The crystal structures with fragment inhibitors revealed several key binding hot spots (Figure 2B):

- The subpocket (Ser130, Thr235) that normally harbors the C3(4)′-carboxylate group of the β-lactam substrates and that is well complemented by the tetrazole ring present in several fragments;

- The polar side chain of Asn132, which also hydrogen bonds with the amide bond in β-lactam compounds;
- An aromatic/hydrophobic site at the base of the active site formed by Tyr105;
- The residues Gly236, Ser237 and Gly238 in the β3 strand.

A subsequent search for commercial tetrazole analogs uncovered a 21 μM inhibitor that served as the template for ensuing fragment-based lead optimization efforts (Figure 3). Interestingly, the 21 μM inhibitor was present in the lead-like subset of the ZINC database but did not rank especially high in the original docking screen, and therefore was not among the 34 docking hits initially analyzed. At this stage, chemical synthesis was employed to explore additional functional groups targeting either of two binding hot spots; a hydrophobic site formed by Pro167, and the side chain of Asp240. Compounds targeting each site were identified, and these exhibited binding affinities of 1–2 μM [62]. Combining these features in new molecules targeting both sites then yielded compounds with sub-μM affinities, including an 89 nM inhibitor that reduced the MIC of cefotaxime by 32–64-fold in clinical *Escherichia coli* isolates expressing CTX-M-9 (Figure 3). This early effort with CTX-M suggests FBLD can be a powerful platform for probing hot spots in the active sites of other Class A β-lactamases.

Due to the high structural conservation among CTX-M enzymes, the new tetrazole-based scaffolds are expected to inhibit other CTX-M β-lactamases, and some recent unpublished results have confirmed this hypothesis. The tetrazole functional group may also be useful in targeting other serine β-lactamases because the corresponding protein subpocket is mostly conserved in these enzymes. Interestingly, as discussed below, a tetrazole functional group has also been shown to be an effective chelator of the zinc ion in metallo β-lactamases.

Class C β-lactamases

For many years, AmpC Class C β-lactamase has been the subject of novel drug discovery efforts and a model system for investigating fragment-based methods. Overproduction of AmpC confers resistance to cefotaxime and other broad-spectrum β-lactam antibiotics [82], yet unlike CTX-M and Class A ESBLs, AmpC is resistant to classical β-lactamase inhibitors such as clavulanate [83].

A recent virtual screening campaign docked a library of 137,639 fragment compounds against *E. coli* AmpC [63]. A total of 48 molecules were selected for experimental testing from the top-ranking list and 23 were shown to be inhibitors (Figure 4A). The complex structures of these fragments, together with those from a previous study, highlighted several binding hot spots similar to those revealed by the fragments identified for CTX-M (Figure 4B):

- The subpocket (Tyr150, Thr316) for the C3(4)′-carboxylate group of the β-lactam substrates;
- The polar side chain of Asn152, which serves as a hydrogen bond donor;
- A hydrophobic shelf formed by Tyr150, Leu119, or in some cases Ile291;

- The residues Gly317, Ala318 and Thr319 in the β3 strand, including the oxyanion hole formed by the backbone amide groups of the catalytic Ser64 and Ala318.

The fragment inhibitors also revealed some novel binding hot spots such as a distal sub-pocket surrounding Ser212 and Gly320, and suggested the merging of these fragment scaffolds with earlier boronic-acid based covalent inhibitors (Figure 5) [84–87]. These fragment-derived modifications were found to increase binding affinity of the covalent inhibitor by more than 20-fold [28]. Subsequent optimization efforts improved the K_i by an additional 25-fold to 50 pM. The development of this sub nanomolar inhibitor demonstrates the value of FBLD in modifying and improving existing lead compounds, including those identified through traditional screening and medicinal chemistry. The virtual screening study also highlighted molecular docking's ability to accurately predict ligand binding poses. Four out of the eight structures determined showed close resemblance to the docking predictions, with root-mean-square deviations (RMSDs) ranging from 1.2 to 1.6 Å. Two additional complex structures showed RMSDs of approximately 2.0 Å. The remaining two fragments bound to the protein in conformations dramatically different from the docking predictions, due to protein flexibility not captured by the computational method.

Studies using AmpC as a model system have also produced important insights into the challenges and pitfalls of FBLD methods in general. Babaoglu *et al.* deconstructed a known lead-like AmpC inhibitor into three fragments and found that the fragments did not adopt the same binding conformation as they did as part of the lead compound [88]. In fact, the fragments interacted with two new sites in the active site. However, as the fragments were combined and elaborated into larger molecules, the new molecules did assume the same binding mode as the original lead-like molecule. These studies underscore the ability of fragments, particularly those with very few functional groups, to assume multiple binding poses. This work thus highlights the value of fragment-based approaches to define multiple binding sub-sites, while pointing to potential pitfalls for fragment optimization when structural information about binding poses is not available.

Several fragments identified from docking to AmpC were also among the high-scoring fragment hits in the screen against CTX-M. Moreover, biochemical testing of fragment hits from the CTX-M studies revealed that they also inhibit AmpC (Figure 2). These results demonstrate that the high hit rate associated with fragment screening is attributable to the ability of fragments to form a diversity of binding orientations and interactions. Despite some similarities between the active sites of the two enzymes, the cross-inhibiting fragments did not necessarily bind to the two proteins in the same orientation. Crystal structures of one compound bound by CTX-M and AmpC showed that the ligand occupies entirely different subpockets in the two enzymes. However, as the crystal structures in both cases capture only the most favorable binding conformation, it is possible that there are other alternative conformations that could be similar between the two proteins. Accordingly, it is likely that a shared fragment binding orientation could be exploited in the design of elaborated molecules that bind both sites with improved affinities.

The virtual screening experiments against AmpC were also used to investigate the chemical space covered by fragments in comparison to lead-like molecules [63]. The commercially

available fragment databases have approximately 1% of all possible chemical scaffolds (<17 non-hydrogen atoms) containing validated fragment inhibitors as substructures. In comparison, the available lead-like databases have just 0.0000001% of all compounds (<25 non-hydrogen atoms) containing these fragments. Thus, the significantly better chemical space coverage by fragments is due both to the far fewer number of chemically possible compounds at the fragment level, and to the low specificity of fragment inhibitors as illustrated in these studies.

Class D β-lactamases

Although Class D enzymes include several problematic carbapenemases, FBLD against this group of enzymes has been scarcely investigated [89]. However, some fragment-size molecules, particularly polycarboxylic acids, have been found to bind these proteins and may serve as starting points for future development of novel inhibitors. Interestingly, similar fragments that have previously bound to the Class A enzyme BS3 were used to develop lead-like inhibitors against the Class D enzyme OXA-10. Specifically, citrate, isocitrate and aminocitrate had been previously discovered with modest affinity against BS3, with K_i values of 490 μM, 2200 μM and 250 μM, respectively [90]. Using these fragments as starting points, Beck *et al.* were able to develop a series of lead-like derivatives (Figure 6A). When tested against a wide range of β-lactamases, several of these derivatives showed binding affinity against OXA-10 in the low μM range. The close similarities between the various polycarboxylate chemotypes suggest their ability to target both Class A and D enzymes (Figure 6B) [91,92].

Class B β-lactamases

Metallo β-lactamases have recently received much attention due to the emergence of NDM-1 and several other enzymes in Gram-negative pathogens [93]. The broad substrate spectrum of these β-lactamases is of great concern in the medical community, and consequently metallo β-lactamases have been the subject of many drug discovery efforts in recent years [78,94]. Although HTS has proved more effective for metallo β-lactamases than serine β-lactamases, FBLD remains a viable and underexploited alternative approach to uncover novel chemotypes for metallo-enzymes.

Using a chromogenic substrate CENTA, a 500-compound fragment library from May-bridge was screened against *Pseudomonas aeruginosa* IMP-1 β-lactamase [64]. Ten inhibitors were identified (Figure 7A). Most of these compounds exhibited mixed competitive and uncompetitive inhibition, and molecular docking was used to understand both inhibition mechanisms. One of these compounds functioned mainly as a competitive inhibitor and served as the starting point of a lead-optimization effort (Figure 8) [65]. This resulted in a modest improvement of approximately tenfold in the IC_{50} of inhibitors of the same chemotype. Interestingly, many precursors for chemical synthesis also showed activity against IMP-1 and they were subjected to further derivatization. These experiments led to an inhibitor with a K_i of 11 μM.

A docking screen of the fragment set in ZINC against a metallo β-lactamase from *Bacteroides fragilis* uncovered five validated inhibitors among the 15 compounds selected

for testing from the 50 highest-scoring hits (Figure 7B) [95]. In addition to the very impressive enrichment rate of true inhibitors in the high-ranking molecules, these inhibitors displayed K_i - values ranging from 120 to 2 μM, significantly better than the more typical high μM to low mM affinities observed for the fragment inhibitors against serine β-lactamases. This result highlights the general ability of metal centers in these enzymes to form strong interactions with specific functional groups (e.g., sulfhydryl) as well as the relative abundance of such moieties in existing small molecule databases. The selectivity exhibited by such inhibitors for metallo β-lactamases over other metallo-enzymes is likely to be an important factor in the further development of such inhibitors if previous experience with other drug targets (e.g., histone deacetylases, matrix metalloproteinases) is any guide.

Many additional fragment-sized inhibitors have been identified from other screening assays not necessarily involving fragment-based approaches (Figure 7C, compounds: **1** [96], **2** [97], **3** [98], **4** [99,100], **5** [101], **6** [65], **7** [102], **8** [103], **9** [104], **10** [52], **11** [105,106], **12** [107], **13** [108,109], **14** [110], **15** [64], **16** [104], **17** [111], **18** [112]). They cover a diverse range of chemotypes such as compounds containing thiol [99] (including mercaptoacetic acids [96,98,113] and mercaptophosphonates [103]) and dicarboxylate groups (including those with heterocyclic rings [104]). Many of these small compounds were identified by traditional screening at much lower concentrations than normally employed for fragment testing. This again reflects the intrinsic affinity of metal centers for metal-chelating functionality. In addition to the zinc ions, these fragments and previously-identified larger inhibitors have revealed other binding hot spots such as the hydrophobic residues on a flexible loop that closes down upon substrate binding as well as a lysine/serine residue that anchors carboxylate groups from substrates/inhibitors. Some of the fragment inhibitors have already been incorporated into larger lead-like molecules, providing good starting scaffolds for future optimization efforts. The different binding modes of D-captopril (Figure 7C, 2) in complex with BlaB subclass B1 [97], CphA subclass B2 [99], and FEZ-1 subclass B3 metallo β-lactamase [114] again demonstrate the ability of fragment inhibitors to adapt to the particular binding environment of a target protein (Figure 9A & B) [97,115]. Comparing the D-captopril-BlaB structure with another complex structure of IMP-1 also suggests that functional groups from D-captopril can be fused with the mercaptocarboxylate inhibitor of IMP-1, underscoring the utility of fragment compounds in probing binding hot spots (Figure 9C) [58,97].

Perspective

In the past several years fragment-based approaches have been successfully applied to identify novel inhibitors for both serine and metallo β-lactamases. The strength of FBLD lies in its ability to effectively probe protein binding hot spots and efficiently identify fragment compounds complementing these subpockets. When structural information is also obtained, the elaboration of weak binding fragments into more potent lead compounds can occur rapidly, with modest synthetic chemistry resources. As still more β-lactamase fragments are identified and characterized, the specific molecular recognition properties and ‘druggability’ of the various sub-sites will become better understood. New fragments for β-lactamases are likely to originate both from *denovo* screening as detailed herein, and from the deconstruction of existing inhibitors identified through traditional non-fragment based

approaches. Most importantly, testing these various fragments and leads across classes of β-lactamases and their mutants will reveal which hot spots are best conserved across enzymes, and might therefore be employed in developing expanded spectrum inhibitors. From the early efforts detailed herein, it is already clear that viable inhibitors of β-lactamases can be drawn from a much wider swath of chemical space than has been previously appreciated. Of course, much more work will be required to advance these new leads towards molecules that can be deployed clinically to counter bacterial resistance in Gram-negative pathogens.

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Key Terms

Extended-spectrumβ-lactamases	Group of enzymes that are able to hydrolyze not only penicillins and early-generation cephalosporins, but also third-generation extended-spectrum cephalosporins (e.g., ceftazidime) and the monobactam aztreonam
High-throughput screening	Experimental procedure that uses robotic systems and software to quickly test and analyze the effects of individual compounds from a large library on target proteins or organisms. These experiments usually focus on lead-like and drug-like molecules and can screen millions of compounds
Fragment-based lead discovery	Experimental approach to lead discovery that seeks to first identify small organic fragments (typically <250 Da) that bind to a drug target of interest. These fragments can then be linked or grown into larger inhibitors with higher affinity and specificity
ZINC	Free online database containing >20 million commercially available compounds grouped into various subsets, including fragment-like and lead-like databases

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▪▪ of considerable interest

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Executive summary

Introduction

- β-lactamases hydrolyze β-lactam antibiotics including penicillins, cephalosporins and carbapenems, representing one of the primary resistance mechanisms used by Gram-negative bacterial pathogens against these antibiotics.
- Four classes of β-lactamases are commonly found in resistant bacteria. Class A, C and D use a catalytic serine to catalyze the reaction, whereas Class B are metallo enzymes.

High-throughput screening versus fragment-based lead discovery against β-lactamases

- High-throughput screening has been ineffective in identifying novel inhibitor chemotypes against serine β-lactamases, but has been used with some success in inhibitor discovery against metallo β-lactamases.
- Fragment-based approaches use low-molecular-weight compounds (<250 Da) to probe small molecule binding sites in proteins and enzymes. Their small size allows for better coverage of chemical space than larger drug-like molecules. Fragments can be converted into more potent lead compounds by employing fragment linking or growing strategies, which are facilitated when fragments are structurally characterized.
- Fragment-based lead discovery (FBLD) against β-lactamases have benefitted from the high quality crystal structures of these enzymes, many of which can be determined in the range of ~1–1.5 Å.
- Molecular docking has played an important role in prioritizing fragments for experimental testing.
- Biochemical inhibition assays have been used as primary assays for fragment testing in inhibitor discovery against β-lactamases.

FBLD case studies against four classes of β-lactamases

- FBLD has been applied to identify a novel tetrazole-based inhibitor scaffold against CTX-M Class A β-lactamase and to develop the first nM-affinity non-covalent inhibitor of a serine β-lactamase.
- Fragment inhibitors were used to probe protein binding hot spots of AmpC Class C β-lactamase, and to uncover new functional groups that were merged with an existing boronic acid covalent inhibitor scaffold. This led to a sub-nM inhibitor.
- Fragment binders were also identified against Class B and D β-lactamases. Lead optimization resulted in μM-affinity inhibitors.

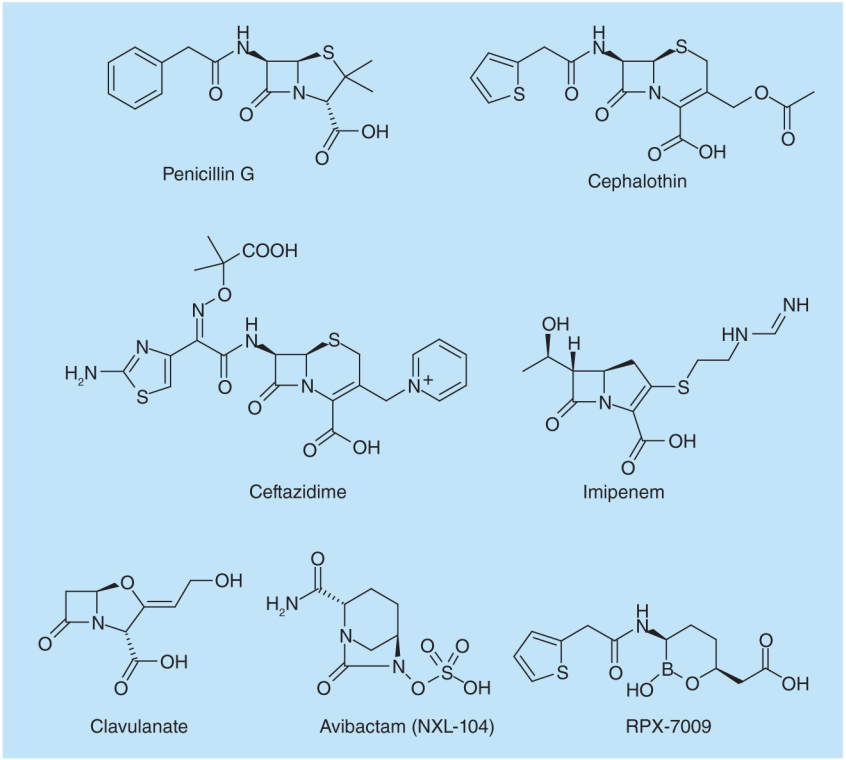

Figure 1. β-lactam antibiotics and β-lactamase inhibitors

Compounds represent the main classes of β-lactam antibiotics in clinical use such as penicillins, cephalosporins, including third-generation such as ceftazidime, and carbapenems. Clavulanate is a Class A β-lactamase inhibitor while avibactam and RPX7009 are broad-spectrum covalent inhibitors in clinical trials.

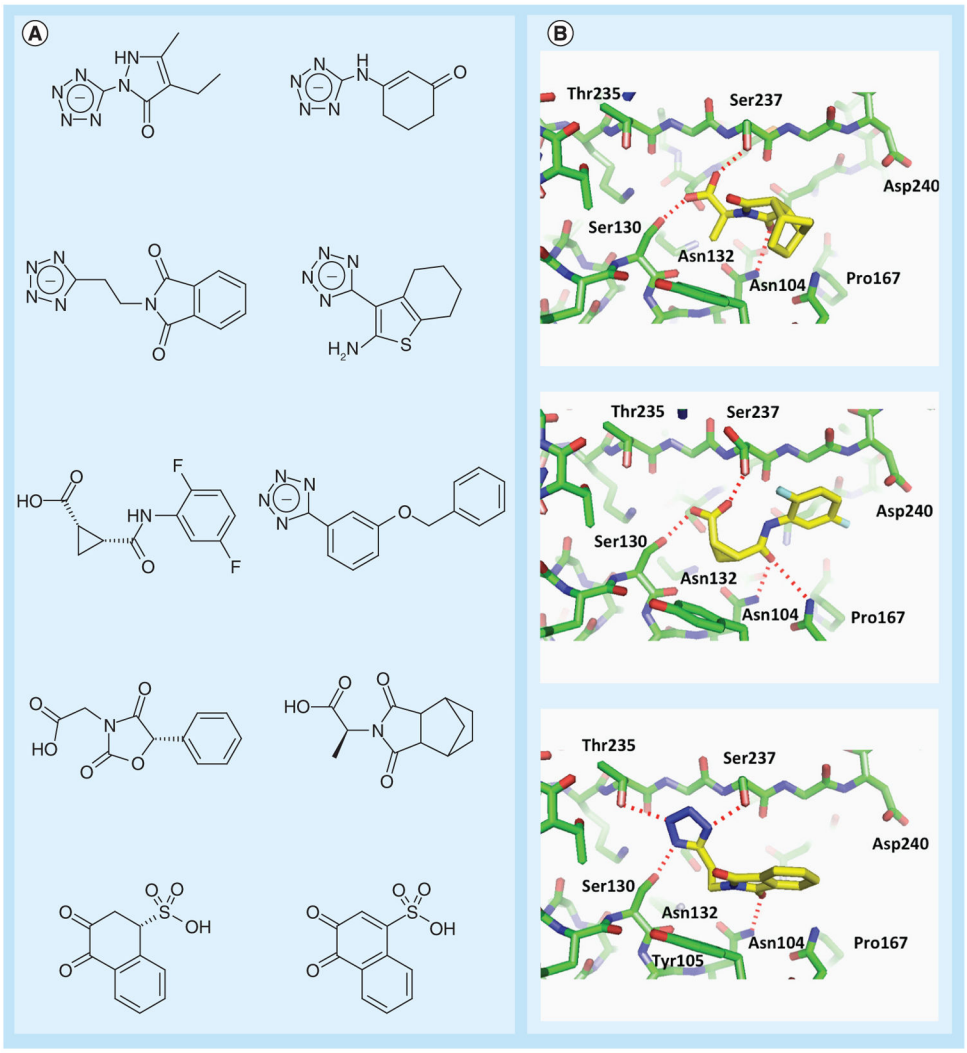

Figure 2. Fragments targeting Class A β-lactamase

(A) Anionic fragments identified by virtual screening against CTX-M Class A β-lactamase [61]. Many of these fragments also demonstrated activity against the Class C enzyme AmpC. **(B)** x-ray crystallographic structures of fragments in complex with CTX-M β-lactamase.

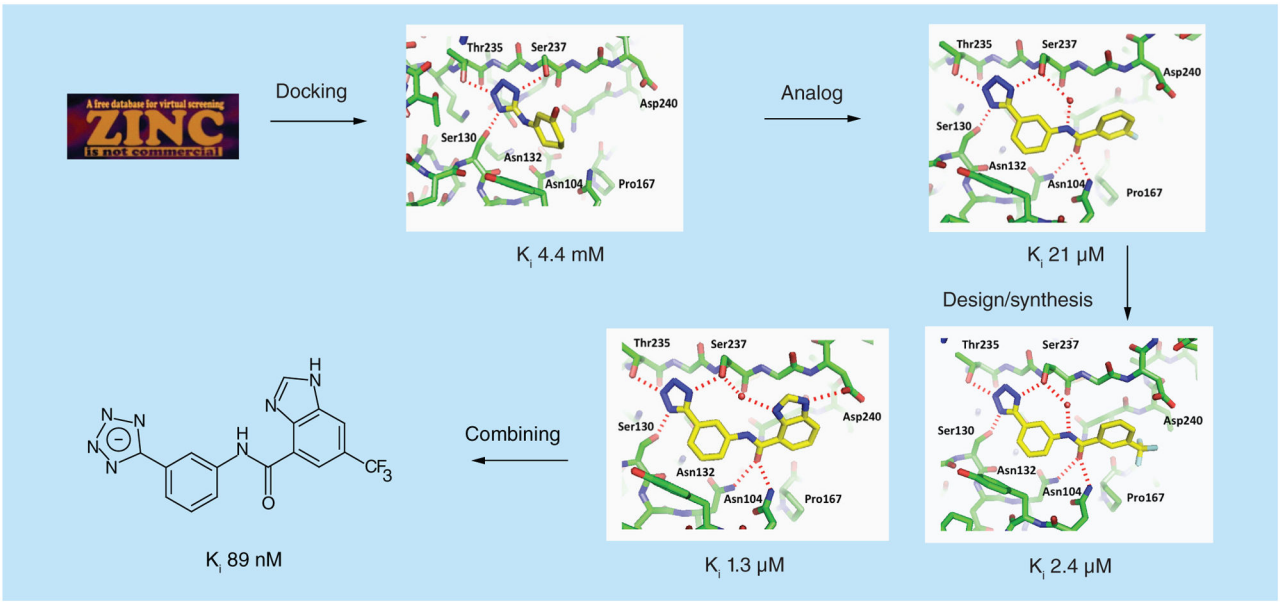

Figure 3. Fragment-based drug design against Class A β-lactamase

Fragment-based approach to develop a lead-like inhibitor against CTX-M-9 β-lactamase.

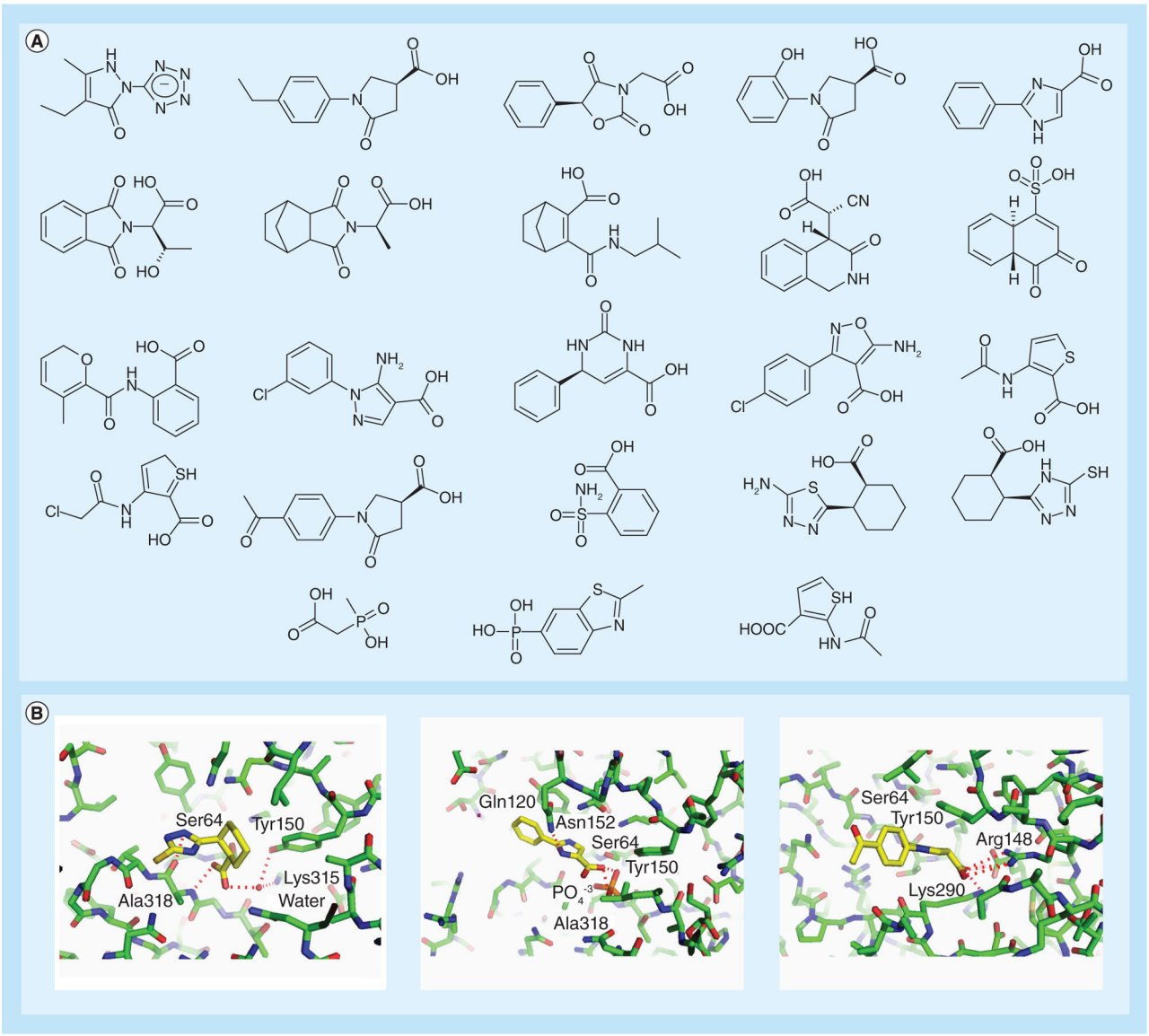

Figure 4. Representative fragments identified for the Class C β-lactamase AmpC

(A) Many fragments possess similar features, including anionic groups and pendant hydrophobic functionality. **(B)** x-ray crystallographic structures of fragments in complex with AmpC β-lactamase.

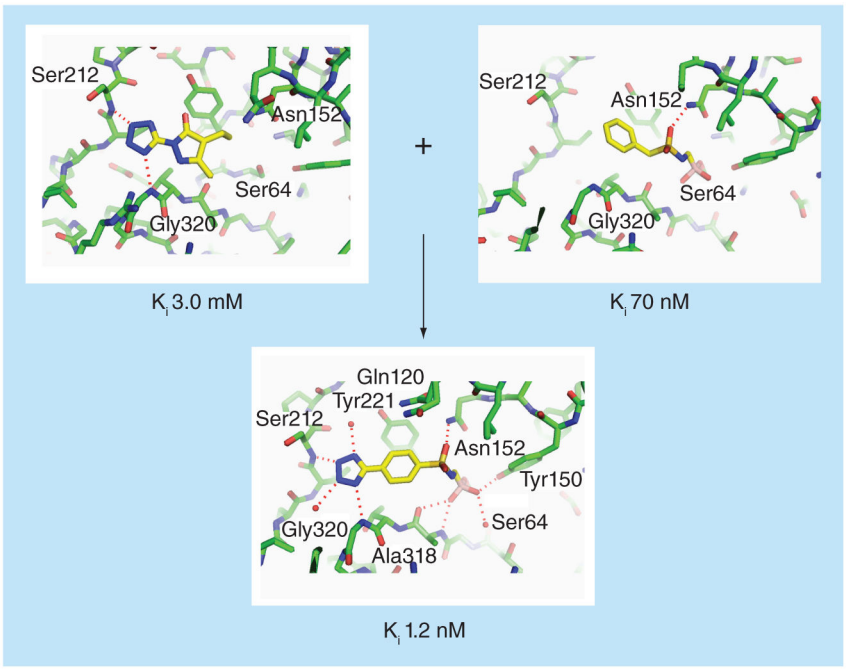

Figure 5. Fragment-based drug design against Class C β-lactamase

Fragment-merging approach to develop a lead-like inhibitor against AmpC β-lactamase [28,63].

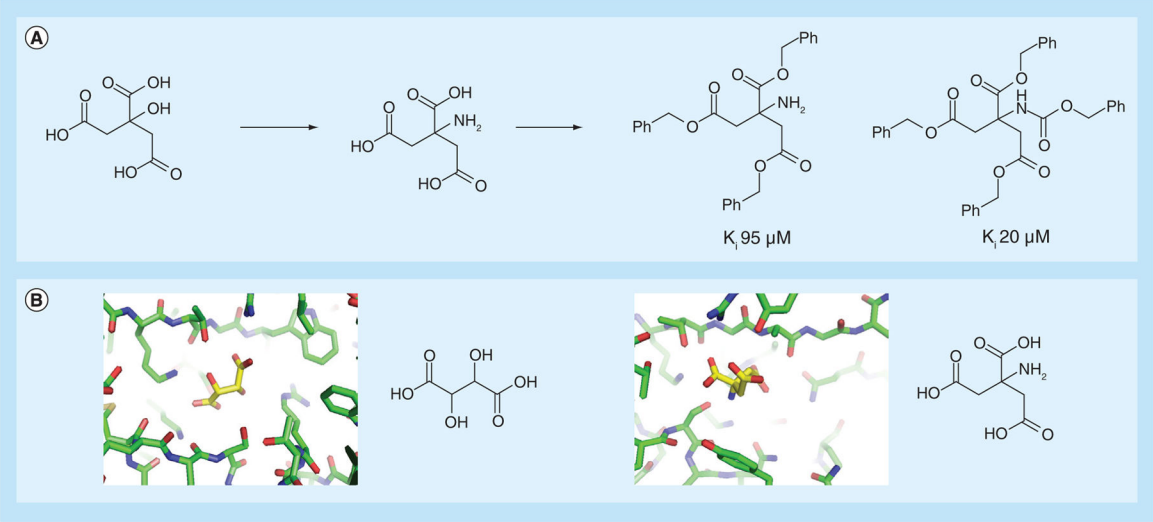

Figure 6. Fragment-based drug design against classes D β-lactamase

(A) Fragment-based approach to develop a lead-like inhibitor against OXA-10, a Class D β-lactamase. Initially, the fragments were identified inhibiting the Class A β-lactamase BS3. Lead-like optimization of these fragments allowed for the identification of compounds inhibiting OXA-10 [90]. **(B)** x-ray crystal structures of Class D, OXA-46 in complex with tartaric acid (PDB ID 3IF6), and Class A BS3 in complex with aminocitrate (PDB ID 3B3X), showing polycarboxylic acid molecules binding to the active sites of both proteins.

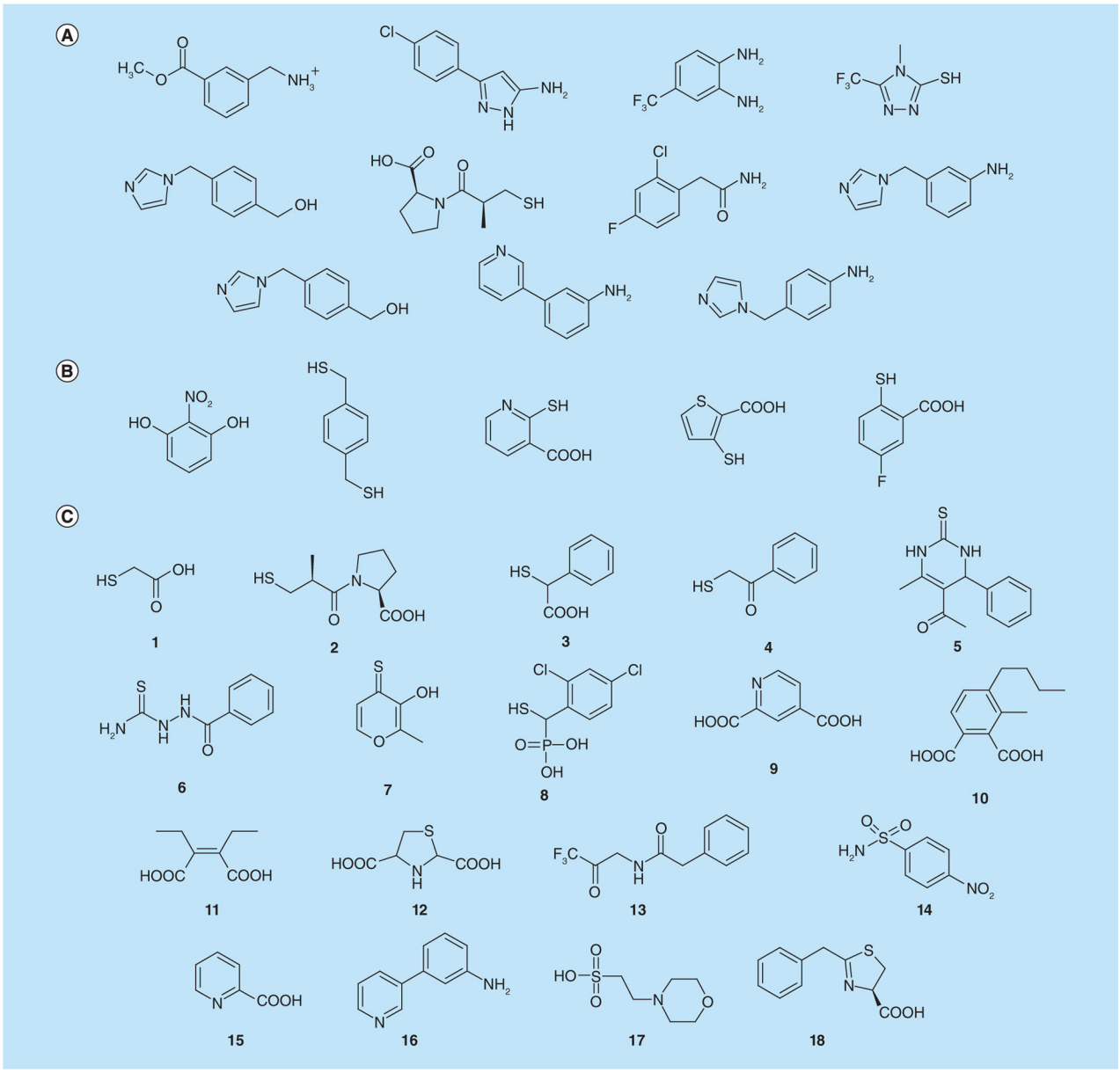

Figure 7. Fragments targeting Class B β-lactamase

(A) Fragment inhibitors discovered using a fragment-based experimental screening approach against IMP-1 β-lactamase. **(B)** Fragment inhibitors discovered by virtual screening against a metallo β-lactamase from *Bacteroides fragilis*. **(C)** Various fragment inhibitors including thiol derivatives and dicarboxylates.

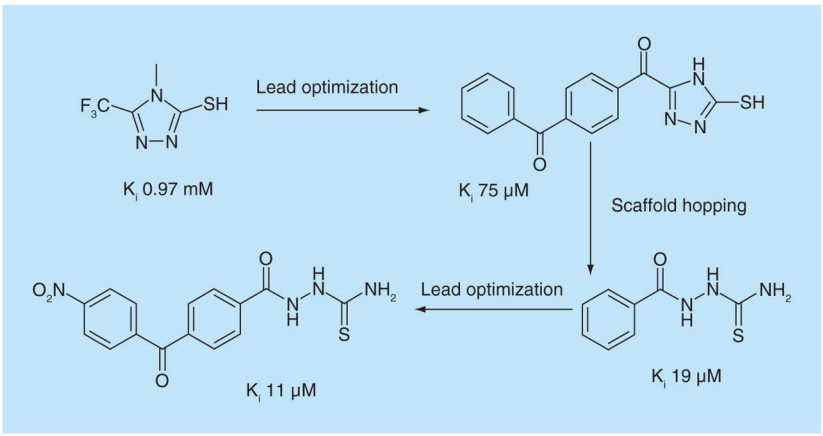

Figure 8. Fragment-based lead discovery for a Class B β-lactamase

Fragment-based approach to develop a lead-like inhibitor against IMP-1, a metallo β-Lactamase [64,65].

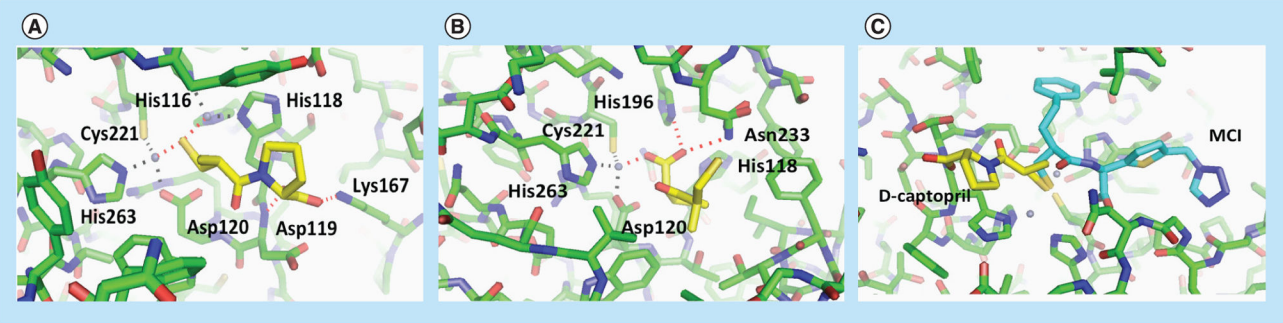

Figure 9. Fragment binding hot spots for Class B β-lactamases

(A) x-ray crystallographic structure of the inhibitor D-captopril in complex with BlaB metallo β-lactamase (PDB ID 1M2X). **(B)** x-ray crystallographic structure of D-captopril in complex with CphA metallo β-lactamase (PDB ID 2QDS). **(C)** The binding pose of D-captopril (yellow) from the BlaB complex structure (enzyme structure not shown, PDB ID 1M2X) is superimposed on the complex structure of IMP-1 with MCI (cyan), a mercaptocarboxylate inhibitor (PDB ID 1DD6). The IMP-1 residues are in green. The zinc ions are in grey.