Structure-Based Optimization of a Non-β-lactam Lead Results in Inhibitors That Do Not Up-Regulate β-Lactamase Expression in Cell Culture

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Abstract.

Bacterial expression of *β*-lactamases is the most widespread resistance mechanism to *β* -lactam antibiotics. There is a pressing need for novel, non-*β*-lactam inhibitors of these enzymes.
Our lead, compound **1**, is chemically dissimilar to *β* -lactams and is a noncovalent, competitive inhibitor of the enzyme. However, at 26 μM its activity is modest (**Figure 1**).
Using the X-ray structure of the AmpC/**1** complex as a template, 14 analogues were designed and synthesized. Among these, compound **10**, had a Ki of 1 μM, 26-fold better than the lead.
The structures of AmpC in complex with compound **10** and an analogue, compound **11**, were determined by X-ray crystallography to 1.97 and 1.96 Å, respectively.
Compound **10** was active in cell culture, reversing resistance to the third generation cephalosporin ceftazidime in bacterial pathogens expressing AmpC. In contrast to β-lactam-based inhibitors compound **10** did not up-regulate *β*-lactamase expression in cell culture but simply inhibited the enzyme expressed by the resistant bacteria. Its escape from this resistance mechanism derives from its dissimilarity to *β* -lactam antibiotics.

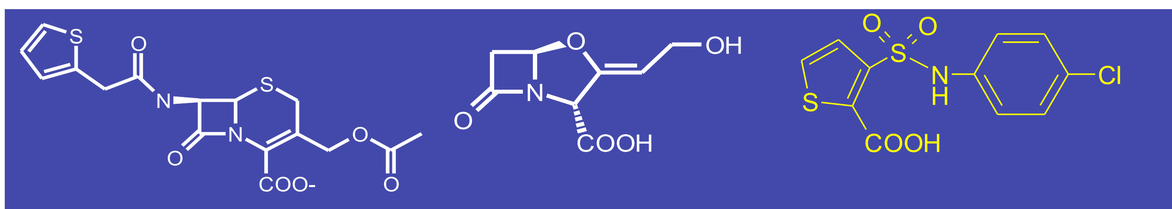


Figure 1. Confronting β-lactam and novel inhibitor of AmpC

Introduction.

β-lactamases catalyze the hydrolysis of β-lactam antibiotics and are the most widespread resistance mechanism to these drugs (**Figure 2**). β-lactam based inhibitors and "β-lactamase resistant" β-lactams have been introduced to overcome this problem, but they resemble the substrates, and bacteria have rapidly become resistant to these anti-resistance drugs.

Among several bacterial resistance mechanisms, the most serious adaptations is represented by β-lactamases themselves. Mutant variants β-lactamase have arisen that can evade β-lactam-based β-lactamase inhibitors (i.e. clavulanic acid). Enzymes that are naturally resistant to current β-lactamase inhibitors, including the class C β-Lactamases such as AmpC, have become increasingly prominent.

There is a pressing need for novel β-lactamase inhibitors. Such inhibitors would not be hydrolyzable by β-Lactamases and would not be recognized by the suite of bacterial resistance mechanisms mobilized against β-lactam-based β-lactamase inhibitors.

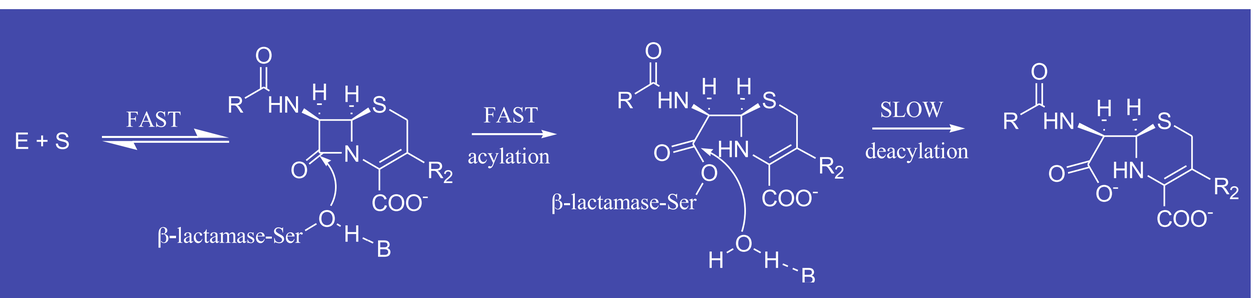
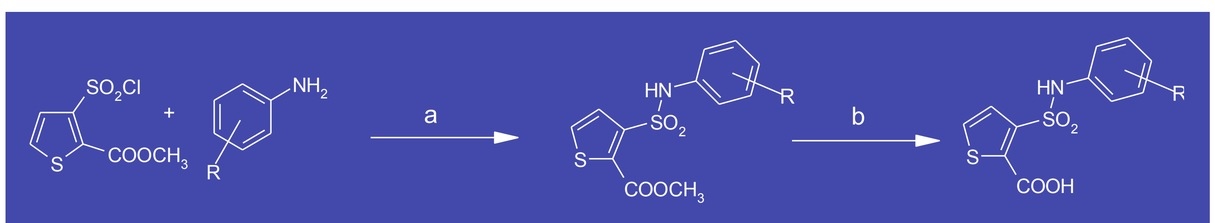


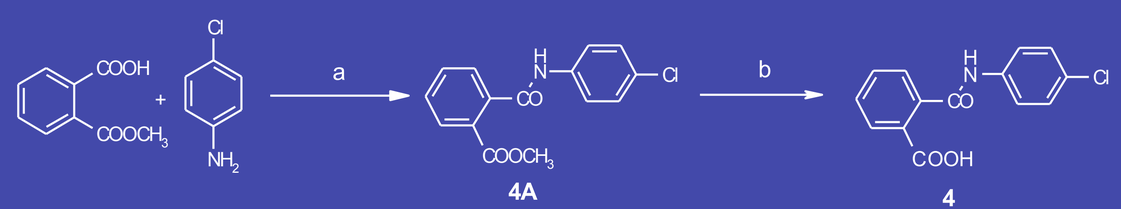
Figure 2. β-lactam hydrolysis by serine β-lactamases

Design and Synthesis.

We recently reported the discovery through virtual screening of a novel, non covalent inhibitor of AmpC, compound **1** (**Figure 1**). Though novel, the inhibitor was weak (Ki 26 μM) and its activity *in vivo* was poor. The crystallographic complex of AmpC-**1** revealed that compound **1** is highly complementary to the enzyme in the region where it bind, but leaves part of the binding site open. We wished to investigate which groups in the lead inhibitor **1** were critical for binding, and should be maintained, and where we could improve affinity through chemical elaboration.
In the design of 14 new derivatives, while exploring the structure-activity relationship pf the series, we considered both structural complementarity and bioavailability.



Scheme 1. Synthesis of 3-((4-chloroanilin)sulfonyl)thiophene-2-carboxylic acid derivatives
a: Py dry, 50°C, N_2. **b**: NaOH 2N, 80 °C, HCl dil.



Scheme 2. Synthesis of *N*-(4-Chloro-phenyl)-phthalamic acid derivative.
a: DMF, DIEA, PyBOP. 60°C, N_2. **b**: NaOH 2N, 80 °C, HCl dil.

X-ray crystallography.

Figure 3. A 2[*Fo*] – [*Fc*] electron density maps (blue) of the refined model of AmpC in complex with compound **10** contoured at 1σ. The simulated-annealing omit electron density maps for the inhibitors, is contoured at 3σ.
Co-crystals of AmpC with **10** were grown from 1.8M KPi, pH 8.7 in hanging drops. Diffraction data were collected at the Advance Light Source (ALS, Lawrence Berkeley Laboratory, CA). Space group: C2. Cell constants: a=117.75Å; b=76.77Å c=97.90Å β=116.63°. Unique reflections=55,165. Total reflections=105,106. R_{merge}=9.1% (25.8%). Completeness: 93.7% (94.6%). R=16.84%, R_{free}=21.5%.

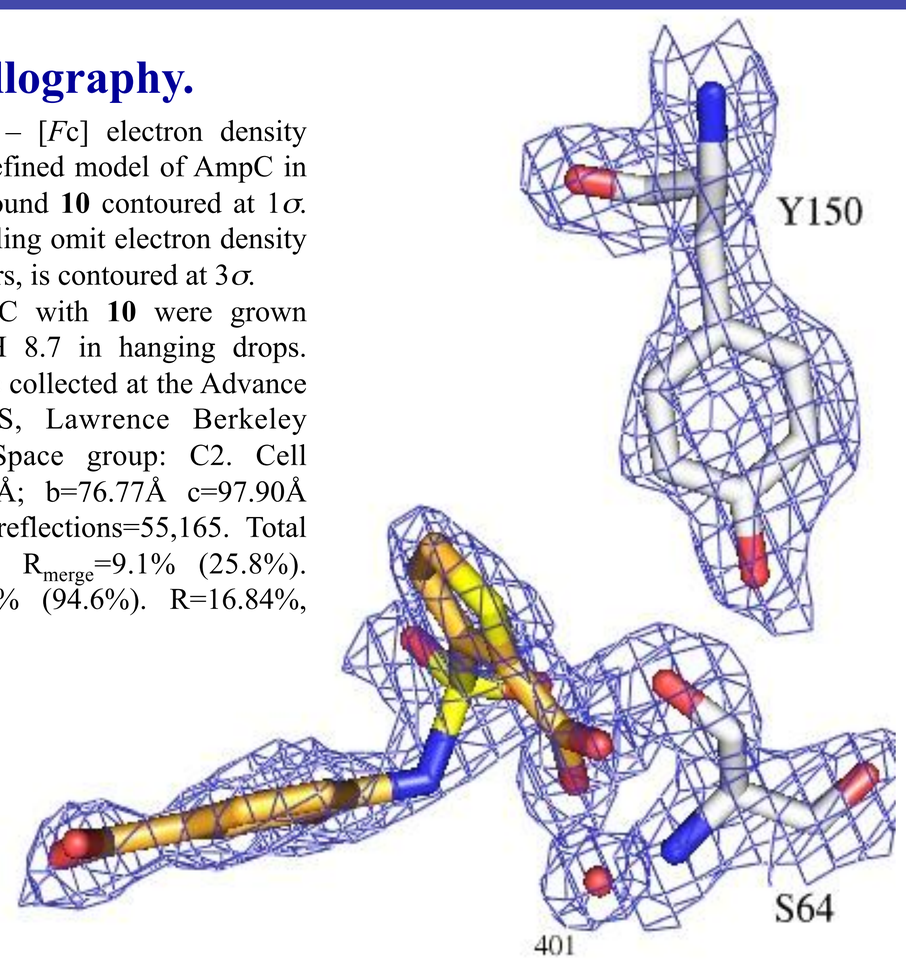


Figure 4. Active site of AmpC β-lactamase in complex with compound **10**. Dashed yellow lines represent key interactions. Red spheres represent water molecules.

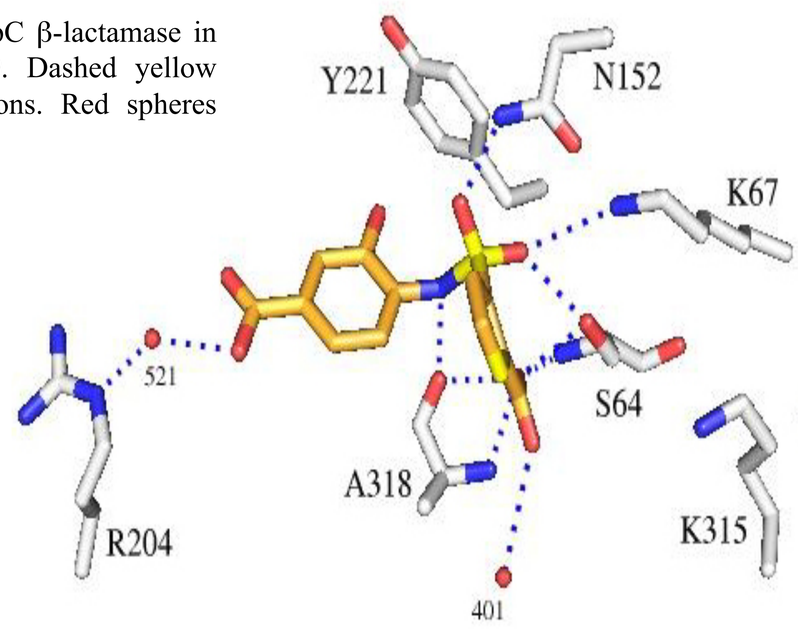


Table 1. Ki values of thiophene-carboxy derivatives vs AmpC

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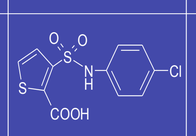
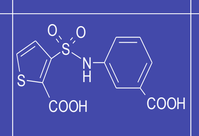
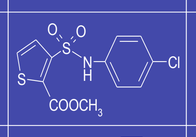
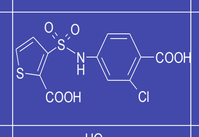
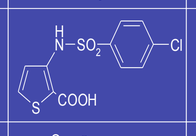
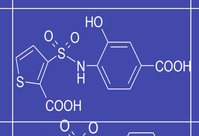
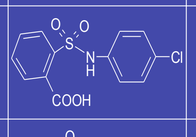
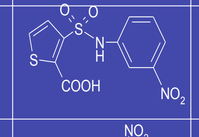
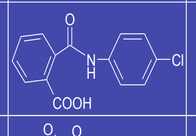
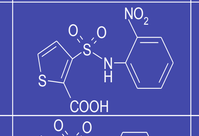
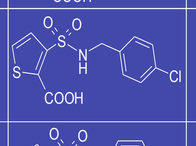
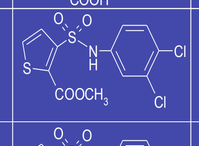
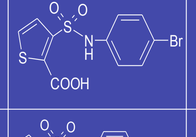
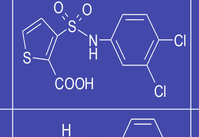
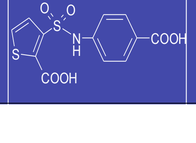
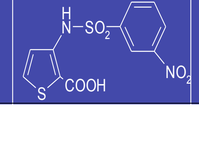
1		26	8		1.7
1A		>>100	9		1.9
2		310	10		1.0
3		45	11		14
4		340	12		10
5		26	13A		170
6		31	13		8.7
7		1.1	14		250

Enzymology and Binding Affinity.

Derivates that replaced the thiophene carboxylate with a methyl ester substantially reduced affinity, consistent with the importance of the ion-dipole interactions made by this carboxylate (e.g., compound **1A**, **Table 1**). Similarly, inverting the order of the sulfonyl-amino group reduced affinity 10-20-fold (compare compounds **1** and **2** or **11** and **14**, **Table 1**), and replacing the sulfonamide with an amide reduced affinity 8-fold, consistent with the importance of the hydrogen bond between this group and Asn152 observed crystallographically.

In an attempt to increase the affinity of compound **1**, we considered derivates that improved either the apolar or polar complementarity in the distal chloro-phenyl ring of the inhibitor. This functionality is located in a more open region of the active site, flanked by the conserved Tyr221 on the "floor" of the active site, bulk solvent at the "top" of the inhibitor, and polar and apolar residues (Arg204, Gly320, Thr319, and Val211) to its side. More polar derivates, such as carboxylate and hydroxyl groups, improved affinity more substantially, up to 26-fold in compounds **7**-**10** (**Table 1**).

Microbiology.

To investigate the potential of these compounds to reverse antibiotic resistance compounds **10** was tested in bacterial cell culture for synergy with the third generation cephalosporin ceftazidime (CAZ) against pathogenic bacteria from clinical isolates.
Compound **10** increased bacterial susceptibility to CAZ, improving its MIC values by 4- to 8-fold. Compound **10** synergized the activity of CAZ and the penicillin piperacillin (PIP) in disk diffusion assays but did not up-regulate its expression in cell culture, as do classic β-lactam-based inhibitors clavulanate and cefoxitin (**Figure 5**).

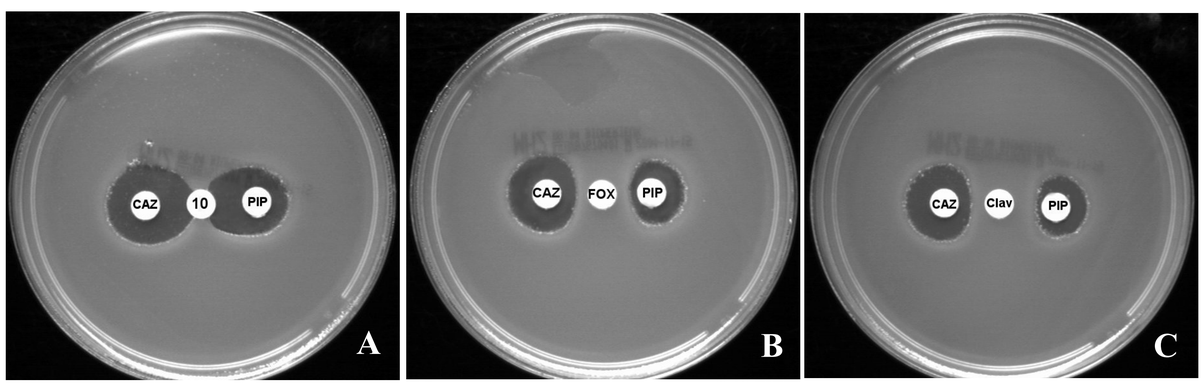


Figure 5.
The effects on up-regulation of a class C β-lactamase in *E. cloacae* by β-lactamase inhibitors*:*
A. The β-lactam based inhibitor clavulanate (center), with piperacillin (PIP) and ceftazidime (CAZ).
B. The β-lactam based inhibitor cefoxitin (FOX) with piperacillin and ceftazidime.
C. The non-β-lactam inhibitor **10** with piperacillin and ceftazidime.

Discussion.

A cycle of structure-based design and testing led to several novel low micromolar inhibitors whose improved affinities could be analyzed on the basis of the X-ray structures subsequently determined for their complexes.
The improved affinity is mirrored by the improved activity in cell culture: compound **10** inhibit β-lactamase and do not up-regulate its expression in cell culture, as do classic β-lactam-based inhibitors.
This antibiotic effect suggests that this series of inhibitors may be useful leads for further optimization. The X-ray crystal structures of compound **10** and **11** in complex with AmpC provide atomic resolution templates for future design efforts.

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