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SLAS Discovery

Using fragment-based approaches to discover new antibiotics

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3 **Using fragment-based approaches to discover new antibiotics**
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Abstract

Fragment-based lead discovery has emerged over the past two decades as a successful approach to generate novel lead candidates in drug discovery programmes. The two main advantages over conventional HTS are more efficient sampling of chemical space and tighter control over the physicochemical properties of the lead candidates. Antibiotics are a class of drugs with particularly strict property requirements for efficacy and safety. The development of novel antibiotics has slowed down so much that resistance has now evolved against every available antibiotic drug. Here, we give an overview of fragment-based approaches in screening and lead discovery projects for new antibiotics. We discuss several successful hit-to-lead development examples. Finally, we highlight the current challenges and opportunities for fragment-based lead discovery towards new antibiotics.

Abbreviations List

AR – antibiotic resistance

CPMG – Carr-Purcell-Meiboom-Gill

FBLD – fragment-based lead discovery

HSQC – Heteronuclear single quantum coherence spectroscopy

HTS – high throughput screening

ITC – isothermal titration calorimetry

K_D – equilibrium constant

MST – microscale thermophoresis

NMR – nuclear magnetic resonance

SAR – structure activity relationships

SPR – surface plasmon resonance

STD – saturation transfer difference

TSA – thermal shift analysis

water-LOGSY - water ligand observed via gradient spectroscopy

Introduction

Antibiotic resistance (AR) has grown into a major global health problem over the past three to four decades. Over the same period many warnings about the dangers of emerging AR have been issued, but these have not yet resulted in improvement of the current treatment options for bacterial infections. Only in the last decade or two have national and international agencies (like ECDC, WHO)^{1, 2} proposed and implemented actual policy changes to address AR such as strengthening prudent use of antibiotics (especially in veterinary medicine), improving surveillance and diagnostics and increased awareness of the need for development of new antibiotic drugs.

The incidence of AR is increasing according to recent reports^{3, 4}. Of particular concern is resistance to last-resort drugs like carbapenems, cephalosporins, and polymyxins in hospitals. There are also hints of transferability of multiple resistance genes outside the clinic^{5, 6}. Lastly, incidence of multidrug-resistant *Mycobacterium tuberculosis* infections outside the clinic is also increasing⁷. Tuberculosis (TB) is often discussed separately because of its differing disease and treatment characteristics.

One of the biggest current problems is that the clinical pipelines do not contain innovative new compounds to address this rising AR^{8, 9}. This is often attributed to low incentives for development (any new antibiotic would be used as little as possible to avoid generation of resistance)¹⁰ and technical hurdles compared to other diseases (discussed later in this review)¹¹. The most recent new class of marketed broad-spectrum antibiotics (oxazolidinones) was discovered in the early 1980s¹² and the most recent first-in-class narrow-spectrum antibiotic to be marketed (anti-TB synthetic diarylquinoline) was discovered around the turn of the millennium¹³.

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5 This somewhat bleak picture of the current state of antibiotics development reveals the need
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7 for increased basic research efforts and antibiotic discovery programmes that can feed into
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9 the clinical pipeline. Current research does offer many promising new concepts and ideas to
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11 work with. Some examples are: systematic screening for inhibitors of apparently non-
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13 essential genes as antibiotic adjuvants (β -lactamase inhibitors are a classical example)
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15 (reviewed in ¹⁴); increased understanding of mechanisms of bacterial membrane influx and
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17 efflux^{15, 16}; and renewed interest in natural products using modern synthetic¹⁷⁻¹⁹ and
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19 bioinformatic tools²⁰⁻²².

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24 The last two decades have also seen technical advances in structure-based drug discovery²³,
25
26 in particular a variety of developments in biophysical techniques²⁴. These advances have
27
28 supported the evolution of the methods of fragment-based lead discovery (FBLD), for which
29
30 there have been many reviews²⁵⁻²⁷. There is a simple rationale behind the testing of fragment
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32 sized compounds, typically with molecular weights of 120 to 250 Da: fragments cover a large
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34 part of theoretically possible atom configurations per molecule because they contain fewer
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36 atoms and because of their small size are more likely to bind to a target. Thus, a well-
37
38 designed, diverse fragment library contains about a thousand-fold fewer compounds than the
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40 average high-throughput screening (HTS) library and covers a considerably larger chemical
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42 space. However, fragments bind with a much weaker affinity (useful fragments have a
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44 dissociation constant, K_D of up to 2-3 mM) than usual lead compounds and screening relies
45
46 on the high sensitivity, robustness and throughput of known and new techniques to both
47
48 identify and characterise fragment binding. Once the binding of a fragment is confirmed,
49
50 some initial structure activity relationships (SAR) can be explored by purchase of compounds
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52 which are similar to the fragment or which contain substructures of the fragment (sometimes
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3 called SAR by catalog). This can increase confidence in the validity of the fragment hit, but
4
5 in general, the process of growing the fragment to a lead compound is challenging without
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7 structural information generated either by crystallography or nuclear magnetic resonance
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9 (NMR) experiments. The structure of the fragment bound provides ideas about how to evolve
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11 the structure of the fragment, initially by systematic exploration of SAR making small
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13 changes to the core scaffold. For development of the fragment, there are three general
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15 strategies, all greatly benefiting from structural information and usually supported by
16
17 biophysical methods to confirm and characterise binding: a) fragment growing increases
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19 potency by optimally engaging the binding pocket, based on structural and/or SAR
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21 information, b) fragment linking and c) fragment merging in cases where there are two
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23 molecules binding close to each other.
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28 While the main goal of hit-to-lead optimization is to increase the affinity/activity of a series,
29
30 many researchers now try to optimize every single atom addition, guided by a metric called
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32 ligand efficiency (LE)²⁸, which is the average binding energy of the molecule per ligand non-
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34 hydrogen atom (i.e., $[-RT \ln(K_D)] / [\text{heavy atom count}]$). All in all, the two core concepts of
35
36 structure-guided and LE-optimized lead design leave relatively little room for serendipity in
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38 the FBLD process, but instead try to rationally optimize the chances and opportunities of
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40 finding lead compounds with novel scaffolds, favorable physicochemical properties, and
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42 target selectivity.
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46 In this mini-review, we first summarise the key features of the techniques which are used in
47
48 fragment-based discovery. The techniques are primarily considered for their use in screening
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50 to identify fragments, but the same methods can be used throughout the optimisation process.
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52 We then summarise some of the examples published where the methods have been used in
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54 antibiotic discovery projects.
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Screening Methods In FBLD

A wide range of biophysical, structural and biochemical assays have been used to identify fragments that bind to a protein target. Although some targets can have highly enclosed binding sites where fragment sized molecules bind with a K_D below 1 μM (such as G protein coupled receptors), most fragment screening campaigns need to identify compounds that bind with affinities in the 100s μM to low mM range. This places particular constraints on the screening approach – requiring high solubility for the ligands, high sensitivity of the detection method and for the assay not to be liable to interference from the high concentrations of fragment that are used. Most screening campaigns use biophysical methods, usually with an orthogonal method used for validation and characterisation of binding. There have been extensive reviews of the different fragment screening methods^{26, 29, 30}. The following is a brief summary of the main characteristics and considerations for each of the screening approaches. Key points for consideration of each method are also represented in Table 1.

TABLE 1 INSERTED AROUND HERE

NMR

There are a wide variety of NMR experiments where the spectra obtained are sensitive to the binding of a ligand to a protein. There are two main classes of experiments – protein-observed which detect changes in the spectrum of the protein and ligand-observed which detect changes in the spectrum of the ligand, both of which are briefly introduced below and in more detail described elsewhere³¹⁻³³.

Ligand-observed spectra for fragment screening are usually acquired with a large molar excess of ligand over the protein – typically with the protein at 10 μM and the ligand at 500

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3 μM . The three most widely used experiments are Saturation Transfer Difference (STD)³⁴,
4 water ligand observed via gradient spectroscopy (water-LOGSY)³⁵ and Carr-Purcell-
5 Meiboom-Gill (CPMG) experiments³⁶ in each of which binding is detected through a change
6 in the spectrum of the ligand. For STD, a series of pulses are applied at the chemical shift of
7 a core hydrophobic nucleus in the protein – this energy is transferred through the protein,
8 transferred to the ligand and persists when the ligand dissociates into solution. This results in
9 a difference in the spectra measured for the ligand with and without protein saturation. In
10 water-LOGSY, the energy is transmitted between water molecules and ligand molecules. The
11 efficiency of the transfer depends on the tumbling speed of the ligand molecules, giving
12 differential signals for free and bound ligands. The CPMG experiment more directly
13 measures the tumbling time of the ligand, which will be different when bound or free. These
14 ligand-observed experiments give no indication of the site of binding, and because of the high
15 concentrations used it can be possible to obtain false positive results from very weak non-
16 specific (and often superstoichiometric) binding. For this reason, a competitive step is
17 usually included to check for changes to the fragment binding signal when a known ligand
18 binds to the site of interest. In addition, the ligand-observed methods require exchange rates
19 (approaching the diffusion limit) that allow the excess ligand in a sample to bind at least once
20 to the protein within the timescale of the experiment, and so can miss high affinity
21 compounds. Also, the different physical bases of the NMR experiments can give rise to
22 artefacts and for this reason, it can be prudent to require positive signs of competitive binding
23 in all three NMR experiments³⁷. The advantages of these ligand-observed methods are that
24 the protein does not need isotopic labelling, there is no limit on the size of the protein and the
25 spectra that are obtained confirm that both the protein and the fragment are intact and in
26 solution. The main disadvantage is the large amount (typically 10s of mgs) of protein
27 required.

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5 The most widely used protein-observed NMR experiment is heteronuclear single-quantum
6 coherence (HSQC), which was used in the first published fragment-based discovery project
7 from Abbott³⁸. Transfer of signal between ¹H and ¹⁵N or ¹³C in the isotopically labelled
8 protein results in a spectrum where each amide or methyl group gives rise to a single peak,
9 where the position of each peak depends on the local chemical environment which can be
10 affected by ligand binding. The main limitations are the size of protein which can be studied
11 (typically 35 kDa), the need for isotopic labelling (which is difficult for proteins produced
12 through non-bacterial expression) and the need for higher protein solubility to give sufficient
13 signal (typically 20 – 100 μM). This requirement also increases the amount of protein
14 required for screening. However, the dynamic range of HSQC measurements is quite broad,
15 mainly limited by compound solubility, although there can be issues at low micromolar
16 affinity, where the exchange rates between free and bound populations lead to peak
17 broadening.. HSQC can also give additional information: the pattern of peaks that shift can
18 confirm that the fragments are binding to the same binding site and if the spectrum of the
19 protein is assigned, then where this binding site is. Also, as long as the ligand is soluble, it is
20 possible to titrate and obtain a K_D from the size of chemical shift on the protein.
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41 A more advanced use of NMR is to determine the binding mode of the ligand bound to the
42 protein. This requires more extensive NMR experiments that give an assignment of which
43 peak corresponds to which nucleus, and then collection of sets of distances between the
44 atoms from nuclear Overhauser effect (NOE) experiments. A full collection of NOEs can
45 give a complete structure for the protein with ligand bound. A variant is to collect limited
46 NOE data (using an isotope-filtered NOE experiment) to identify particular ligand – protein
47 NOEs which can be used to generate an NMR-guided model of how the ligand binds³⁹. Such
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3 protein-observed NMR experiments underpinned the fragment discovery work at Abbott^{38, 40,}
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5 41.

9 **Surface Plasmon Resonance (SPR)**

11 SPR is a method to measure the change in molecular mass when a ligand binds to a protein.
12
13 The most widely used equipment uses a surface to which either the ligand or the protein is
14 attached – the other component is then flowed over the surface. The refractive index of light
15 shone onto the surface is sensitive to the molecular mass of what is attached. If a protein is
16 immobilised, the increase in mass as the ligand is flowed over gives information about the
17 association rate; if the ligand is replaced with just buffer, then the dissociation rate can be
18 measured. The ratio of the dissociation and association rates is the equilibrium constant.
19
20 The current generation of instruments is sensitive enough to detect the binding of low
21 molecular mass compounds such as fragments. The main issues are in finding the conditions
22 and strategy to immobilise the protein to provide a homogenous surface where the protein
23 remains folded and having the reagents (and experience) to design suitable control
24 experiments to validate the system, as summarised in the excellent review from Giannetti⁴².
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40 **Thermal shift analysis (TSA)**

41 The principle behind TSA is that the temperature at which a protein unfolds will be changed
42 by binding of a ligand. In practice, a protein solution (+/- the ligand) is heated in the
43 presence of a fluorescent dye – the dye binds to the hydrophobic surface as the protein
44 unfolds. The advantage of the technique is that it uses relatively cheap equipment (a qPCR
45 machine is sufficient), uses small amounts of protein and is quite rapid. The method works
46 well to identify ligands that stabilise a protein for crystallisation⁴³ and for screening suitable
47 buffers to stabilise the protein⁴⁴ and has been used for screening libraries of larger
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3 compounds⁴⁵. TSA has been used for fragments, but there are issues with many false
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5 negatives⁴⁶; a weakly binding fragment does not necessarily stabilise the protein to a
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7 detectable level. However, it is a fast and economical way of screening, attractive to
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9 academic groups.

10 11 12 13 **Biochemical assay**

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15 A biochemical assay (such as a functional enzyme assay or a binding assay such as
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17 displacement of a fluorescently labelled probe) can be used to screen for fragments. The
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19 main requirement is that the assay is not compromised by the high concentration of ligand
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21 (and sometimes associated solvent) of ligand being used. The advantage of functional assays
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23 over biophysical techniques is that it only reports binders that modulate function (functional
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25 relevance of hits is often unclear, especially from crystallography⁴⁷) and can distinguish
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27 between different types of modulators if the assay is balanced properly⁴⁸. The review in⁴⁹
28
29 includes comparison of biochemical and biophysical assays.
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35 **Crystallography**

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37 There was a strong focus on high throughput X-ray crystallography for screening of
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39 fragments from some of the early adopters of the approach⁵⁰ and there have recently been
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41 significant improvements in streamlining data collection and structure determination⁵¹. There
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43 is an immediate advantage in providing a model for the binding of the fragment and the high
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45 concentrations required for soaking experiments means quite weak binding fragments can be
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47 identified. However, it does require the protein to crystallise with an accessible active site
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49 and a crystal packing resistant to moderate solvent and ligand concentrations. It is often the
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51 experience that it takes a number of attempts to obtain a crystal structure of a fragment
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53 binding to a protein even when it has been confirmed to bind by other techniques²⁹.
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Isothermal titration calorimetry (ITC)

An ITC experiment measures the heat (enthalpy or ΔH) which is released or taken up when a ligand binds to a protein and the titration gives the equilibrium binding constant from which the entropy component (ΔS) can also be determined. As long as there is some heat change on binding, the technique is extremely robust and can be used to detect and characterise binding for proteins which are difficult to assay in other ways. Importantly, it can confirm the stoichiometry of binding. However, it is quite expensive in protein and so is not used for screening (see the survey on screening methods²⁵). It also has high requirements for the solubility of the ligand (if titrated).

Other ideas and approaches

Many other approaches and technologies have been suggested and used for detecting low-affinity interactions. In general, no particular technique can be regarded as a ‘best-in-class’ solution, but it is useful to be aware of and to consider all available options. Some of these other methods are proprietary, such as the capillary electrophoresis method used by Selcia⁵², the target immobilized NMR screening (TINS) method of ZoBio^{52, 53} and weak affinity chromatography (WAC)⁵⁴. Mass spectrometry is limited to situations where the ligand binds with affinities better than approximately 100 μM , but has been applied successfully with covalently bound fragments⁵⁵, with the “tethered” fragments approach of Sunesis⁵⁶, and more generally by NovAliX⁵⁷. Another recently developed biophysical technique which has been successfully applied by some to FBLD is microscale thermophoresis (MST) (Nanotemper GmbH⁵⁸). In this approach, the mobility of molecules (observed from a fluorescent label) along thermal gradients (thermophoretic mobility) is used to identify changes in molecular hydration and thus molecular interactions.

General comments on fragment screening

There is much debate about the “best” technique to use for fragment screening (for example see the Practical Fragments blog²⁵). All the techniques have their pros and cons (see Table 1) – protein-observed NMR requires labelling and has a size limit, ligand-observed NMR requires large amounts of protein, X-ray crystallography requires a suitable crystal system, SPR (and MST) require effective labelling that does not affect function. Each of the techniques also has particular requirements in terms of the solubility of the protein, the solubility of the ligands screened and can robustly detect different dynamic ranges of binding affinity. There are occasionally studies which discuss the different hits obtained from fragment screening with different techniques (e.g. ⁵⁹). However, if the limitations of each technique are taken into account (sensitivity, buffers, solubility etc), then the same hits should be obtained ^{49, 54}. One should take caution if only taking the intersection of hits – this will identify the most robust binder, but means the diversity of hits is vulnerable to the least reliable method. As mentioned before, hit validation by orthogonal techniques will filter out some false positives when those emerged from limitations of the techniques (e.g. compound intrinsic fluorescence in some types of biochemical assay or direct saturation in STD NMR). There are still many possible ways in which false positives survive orthogonal validation, as drug discovery practitioners became aware of relatively recently^{60, 61}, but there seem to be no general rules applicable to all projects^{62, 63}. In the end a balance must be found between false positives and false negatives, depending on the goals of the project (taking into account scaffold diversity, chemical tractability, etc.).

Examples Of Fragment-based Discovery Of Antibiotics

Biotin Carboxylase

Bacterial proteins without human homologs have often been prioritised as antibacterial targets. However, unbiased whole-cell screening has yielded good targets, even when homologous to human targets, as in the case of the *E. coli* enzyme biotin carboxylase (BC)⁶⁴. BC is a subunit of the bacterial acetyl-CoA carboxylase that uses adenosine triphosphate (ATP) to catalyze the first step of the reaction. The ATP binding site of BC has similarities to human kinases. Mochalkin *et al.* followed up on this target by using FBLD to discover potent leads with new scaffolds⁶⁵. They employed a cascade of three screening experiments, summarised in Figure 1. First, a biochemical assay that was available from their earlier work⁶⁴ was used to screen ~5000 fragments in mixes of 10. The use of two different, moderate screening concentrations probably helped to recognize artefacts. About 20% of the fragment mixes showed inhibition of more than 25%. Then, to identify the active component(s) of each mix, their binding to BC was measured by STD NMR. This resulted in a set of 142 fragments (3% of the library). Finally, concentration-dependent inhibition was determined by titration of the single compounds in the original enzyme assay.

FIGURE 1 AROUND HERE

The fragment screen results were complemented by virtual screening. Out of a set of 2.2 million small organic in-house compounds (including fragments), a subset was selected for 3D similarity to previously identified HTS hit **1**⁶⁴. Visual inspection of subsequently generated binding poses at the BC ATP binding site yielded 525 hits. This hit set was tested in the enzyme assay and contained 48 compounds (9%) inhibiting BC at 50% inhibitory concentration (IC₅₀) values less than 10 μ M. Furthermore, it also contained several active fragments with novel scaffolds (Figure 1, such as fragment **3**).

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3 Several of the most potent hits (such as **2**) had pharmacophore features in common with the
4
5 earlier identified HTS hit **1** and the common binding mode was confirmed from co-crystal
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7 structures. This information was used to generate a number of lead series. For example,
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9 fragment **3**, a hit from both the fragment and virtual screen, could be optimized into a potent
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11 lead **4** with bactericidal activity. As with **1**, its specific inhibition of BC could be inferred
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13 from reduced activity against a spontaneous single amino acid substitution mutant of BC.
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15 Interestingly, the HTS- and fragment-derived leads showed differences in properties like
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17 efflux pump susceptibility, cell penetration, and activity against different Gram-negative
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19 pathogens. In addition, the lead series were at least 70-fold selective for *E. coli* BC over a
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21 panel of over 30 human kinases.
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24 25 26 **DNA gyrase**

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28 Bacterial DNA gyrase, a type II topoisomerase, is a tetrameric complex that acts on
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30 topological isomers of DNA during replication and transcription to relieve positive
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32 supercoiling of double-stranded DNA. This is an essential process and proceeds via similar
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34 mechanisms in all domains of life. There are several classes of successful bacterial DNA
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36 gyrase inhibitors (quinolones, aminocoumarins, novel bacterial type II topoisomerase
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38 inhibitors or NBTIs) both on the market and under development, targeting either the ATPase
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40 domain (GyrB) or the DNA cleavage domain (GyrA).
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44 There have been several fragment-based drug discovery campaigns against DNA gyrase. One
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46 of the earliest examples of FBLD⁶⁶ used a screening cascade similar to that described for BC
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48 (section 3.1). First, a selection of small fragments (then called ‘needles’) was made from a
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50 database of available compounds by pharmacophore fitting and docking into the GyrB ATP
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52 binding site. Then, this set of 3000 fragments was tested for biochemical activity at high (0.5
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54 mM) concentration. Finally, a set of 150 hits divided over 14 compound classes was taken to
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3 the validation stage which included a second biochemical assay, various biophysical binding
4 experiments (including SPR and NMR), and SAR elaboration. Further structure-guided
5 optimization yielded lead compounds (e.g. **6**) with up to ~30,000-fold improved inhibitory
6 activity *in vitro* (Figure 2A).
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11 FIGURE 2 AROUND HERE
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13 More recently, researchers at AstraZeneca reported the discovery⁶⁷ of pyrrolamide GyrB
14 inhibitors and their development⁶⁸ into clinical phase. A low affinity pyrrole carboxylate (**7**,
15 Figure 2B) binding in the adenine pocket was identified in a protein-observed NMR screen.
16 This fragment engages the conserved aspartate + water motif that is also exploited by **5** and
17 other GyrB inhibitors. Fragment **7** was part of a collection of fragments of known GyrB
18 inhibitors that was added to the generic diverse screening library. Fragment **7** was chosen as a
19 starting point for elaboration based on its favorable LE and growth vectors. Fragment hit **8**,
20 even though very weak and ligand inefficient, was interesting because it bound to the
21 aminocoumarin binding region of GyrB, presenting a way for **7** to gain affinity. Based on this
22 reasoning, a pyrrolamide library was prepared and screened in an *E. coli* DNA gyrase
23 ATPase assay. A 1-arylpiperidin-4-yl extension (**9**) proved to give the best increase in
24 activity, but was not yet potent enough for cellular activity. Guided by crystal structures of *S.*
25 *aureus* GyrB with bound pyrrolamides, the aromatic group was replaced by thiazole 4-
26 carboxylate for optimal interaction with the two arginine side chains close to the
27 aminocoumarin binding region. Together with a 100-fold increase in affinity, lead compound
28 **10** showed not only antibacterial but bactericidal activity against *S. aureus* as well as an *E.*
29 *coli* strain with impaired drug efflux. Further lead optimization eventually focused on the
30 stereochemistry of the piperidine ring, which influences protein-ligand interactions via the
31 orientation of the pyrrole and thiazole rings. The clinical candidate **11** (AZD5099) was
32 chosen for an optimal combination of pharmacokinetic and physical properties, in part
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3 conferred by an intramolecular hydrogen bond between the deprotonated carboxylic acid and
4 the secondary amide⁶⁸. Unfortunately, development of the pyrrolamide lead series was
5 stopped after the first phase I study with AZD5099⁶⁹.
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11 Currently, two new DNA gyrase inhibitors, both discovered during whole-cell screening, are
12 being evaluated by Entasis Therapeutics and GSK in late stage clinical trials for *N.*
13 *gonorrhoeae* infections⁸.
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18 19 20 **Cell division protein FtsZ**

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22 FtsZ plays an essential role in the separation of newly forming cells during bacterial
23 replication⁷⁰. It is homologous in structure to eukaryotic β -tubulin, a cancer target in humans.
24 Like tubulin, FtsZ binds guanosine triphosphate to polymerize into strands, but FtsZ is only
25 needed for cell division and not for chromosome separation. Inhibition of FtsZ does not
26 directly stop growth, but leads to formation of long filaments or large ‘balloons’ that
27 eventually lyse. Development of antibacterials targeting FtsZ has been ongoing for well over
28 a decade. Most of the reported compounds act by preventing FtsZ polymerization,
29 destabilizing polymers, or stabilizing polymers⁷¹.
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40 FIGURE 3 AROUND HERE

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42 Shortly after the discovery of the function of FtsZ⁷², the protein was reported as the primary
43 target for the fragment-like microbiological tool compound 3-methoxybenzamide (**12**)
44 (Figure 3A)⁷³. Seeing this as a starting point for fragment-based program, Czaplewski and
45 co-workers optimized the methoxybenzamide scaffold to a potent lead compound⁷⁴.
46 PC190723 (**13**) (Figure 3A) has exceptional antibacterial activity against several *S. aureus*
47 strains including multidrug-resistant ones⁷⁵. Unlike the usual fragment screening hits, **12** was
48 already known to be able to penetrate the bacterial cell membrane, and to cause filamentation
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3 by acting on FtsZ. In the absence of any structural information on how **12** binds to FtsZ,
4 optimization of the benzamide scaffold was done by thorough, systematic SAR only⁷⁴ (a
5 strategy not favored by many FBLD practitioners today). Optimization efforts were based on
6 minimum inhibitory concentration (MIC) and filamentation against *B. subtilis*. Derivatives
7 with variations on each possible position on the scaffold (Figure 3B) were either bought or
8 synthesized (less than five steps). This early SAR series clearly indicated that any
9 substituents larger than a single atom on any position except R³ eliminated growth inhibition
10 and filamentation *in vitro*. A hydrophobic group of medium size on R³ boosted potency up to
11 8000x. Unfortunately, the nonyl group of the most potent compound (**16**) (Figure 3B) is quite
12 un-drug-like. Further optimization eventually yielded **13** which is active against *B. subtilis*
13 and *S. aureus* but not other Gram-positive or -negative bacteria⁷⁵. Based on resistance
14 mutations profile and an apo structure of FtsZ, the researchers suggest that **12** and derived
15 inhibitors bind to an allosteric site next to the nucleotide binding site, homologous to the
16 Taxol binding site of tubulin. This hypothesis was later confirmed by crystallography⁷⁶
17 (Figure 3A) and this binding mode was shown to promote FtsZ polymerization and stabilize
18 FtsZ polymers⁷⁷.

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40 This compound class is one of the most promising candidate antibiotics, even though it has a
41 narrow spectrum (*S. aureus* including drug-resistant strains) and has proven difficult to
42 optimize in terms of pharmacokinetic properties⁷⁸. It is currently under active pre-clinical
43 development by Taxis Pharmaceuticals.

50 EthR

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52 Isoniazid and ethionamide are anti-TB drugs targeting the same component of the mycolic
53 acid synthesis pathway of mycobacteria. Because effective use of ethionamide requires high
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3 doses associated with liver toxicity, a way to lower the required dose would be an attractive
4 therapy. Isoniazid and ethionamide have different resistance profiles because they are pro-
5 drugs activated by different bacterial enzymes. Ethionamide is activated by EthA⁷⁹. The *ethA*
6 gene is regulated by the transcriptional repressor EthR⁸⁰. Therefore, several drug discovery
7 projects have searched for EthR inhibitors as adjuvants for ethionamide. EthR is a relatively
8 new anti-TB target with promising early inhibitors as will be described. It will be interesting
9 to see whether EthR inhibitors can be developed into therapies, as the role of EthA in
10 ethionamide activation is not yet fully understood, as suggested by recent reports of
11 redundant mechanisms⁸¹⁻⁸³.

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24 EthR has a deep, narrow, hydrophobic pocket that facilitates allosteric deactivation upon
25 compound binding. Researchers at the Pasteur Institute found several active hits among a
26 small set of compounds selected by pharmacophore modelling⁸⁴. After attempting to expand
27 the hits inside the binding pocket by *in situ* click chemistry⁸⁵, they took one of the click
28 reaction components, **17** (Figure 4A), as a starting point for fragment-based hit-to-lead
29 optimization⁸⁶. Although extension of the original hits with this structure did not increase the
30 potency, **17** does display weak potency on its own. The fragment was found to bind near the
31 bottom of the binding cavity, making the critical hydrogen bond to N179 with its sulfonamide
32 group. Based on this binding mode, a set of 61 x 16 combinations of commercially available
33 4-substituted benzenesulfonyl chlorides and amines (respectively) was generated and
34 screened *in silico*. The resulting SAR data were validated *in vitro* to yield **18** with 32-fold
35 improved inhibition of EthR DNA binding. Encouragingly, the *in vitro* activity of **18** was
36 matched by its *ex vivo* ability to boost the antibacterial effect of ethionamide at one tenth its
37 MIC. The binding mode of **18** was also confirmed by crystallography to match the predicted
38 one. Further optimizations included replacement of the sulfonamide group with an amide
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3 which picks up an additional hydrogen bond interaction with N176, leading to a lead
4 compound (**19**) with excellent LE and higher activity than its originator **20**. In this example
5 the fragmentation approach has worked well despite **17** adopting a different binding mode
6 (Figure 4B) than expected (from that of **20**, Figure 4C) and also lacking the previously
7 identified core scaffold⁸⁴.
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FIGURE 5 AROUND HERE

Fragment screening against EthR has produced another interesting series of compounds. Noting that the allosteric pocket of EthR is hydrophobic, Surade and colleagues used TSA to identify 86 hits from a library of 1250 fragments, some of which stabilize EthR by more than 5 °C⁸⁷. The hits were validated using orthogonal biophysical techniques. As mentioned earlier, it is not trivial to select the best primary and secondary screening techniques and to interpret their combined data, as each technique has its own false positive/negative rates and can be more suitable for certain screening subjects/libraries than others⁸⁸. In this case, SPR validation data agreed well with the primary screen, with only one out of 45 negative controls (non-hits from TSA) showing up as an inhibitor in SPR. Only slightly more than half of the 86 TSA hits were identified as inhibitors, perhaps because of the large difference in assay concentrations (10 mM in TSA vs. 0.5 mM in SPR). Several validated hits were soaked into EthR crystals to elucidate their binding modes. Interestingly, three hits contained similar arylsulfone scaffolds as discussed in the previous example, with their sulfone groups interacting with N179. A fourth hit **21** (Figure 5), however, was more interesting because of its ability to occupy a second subpocket in the crystals, as well as the hydrophobic cavity simultaneously. Linking the two slightly modified molecules together to form **22** dramatically decreased the IC₅₀. However, notably, the *in vitro* antibacterial potencies of these two compounds are identical, showing that straightforward optimization of binding strength and LE is not always an effective strategy. Indeed, further fragment merging efforts monitored

solely by biophysical techniques were unsuccessful⁸⁹. The authors suggest that decreased compound membrane permeability might explain the discrepancy. Instead, SAR exploration around hit **20** while staying within fragment size range, monitored by ITC as well as bacterial growth assays, yielded submicromolar ethionamide boosters such as **23**⁹⁰.

β -lactamase

Evolution of plasmid based β -lactamases to confer resistance to standard antibiotics was one of the first signs of a new era in healthcare. As the problem grew, inhibitors of β -lactamases (another example of adjuvants) were being used in hospital settings but not to great effect⁹¹. First generation inhibitors like the natural product clavulanic acid, themselves β -lactams, quickly lost effectiveness due to resistance and narrow activity spectrum. Another class of β -lactamase inhibitors are boronic acids. Although their effects were noted since the 1980s and gained interest from researchers, no boronic acids entered clinical use until very recently, with the FDA's approval of a combination therapy of vaborbactam with a carbapenem for urinary tract infections.

FIGURE 6 AROUND HERE

Sulfonamide boronic acids are another recently developed class of β -lactamase inhibitors. As with vaborbactam⁹², early discovery and optimization of these molecules were guided by docking and modelling^{93, 94}. Although several compounds in this series displayed high *in vitro* inhibitory activities, their effects on the antibacterial properties of β -lactamase-sensitive cephalosporins were only modest and did not follow the same trends. Reasoning that increasing β -lactamase affinities even further could solve the problem, the researchers made an in-depth analysis of possible enzyme-ligand interactions using results from virtual fragment screens and known inhibitor fragmentation approaches⁹⁵. Bound fragments can give great insight into the characteristics of the interactions of larger ligands, because fragment

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3 binding modes are unconstrained by distant binding pocket geometries. Thus, with
4 encouraging modelling results, lead inhibitor **24** could be modified to optimize the
5 conformation of its benzyl group and to pick up an extra hydrogen bond (compound **25**,
6 Figure 6A). Furthermore, fragment screens can efficiently offer insights into viable
7 alternative chemical space, as shown by the model-guided replacement of the carboxylate of
8 **24** by a tetrazole moiety, yielding **26** that retains similar affinity and interactions as **25**
9 (Figure 6B). Interestingly, while both **25** and **26** were active as β -lactamase inhibitors in
10 cellular assays, they showed slightly different profiles, probably due to their differing
11 physicochemical properties. Most pronounced was the difference in ability of these
12 compounds to also inhibit a class A β -lactamase, an effect to which no special effort was
13 made during the optimization process. A close derivative of **26** also promoted survival of
14 infected mice when treated with a β -lactamase-sensitive cephalosporin, although to a lesser
15 degree (65%) than could be reached with a β -lactamase-resistant carbapenem (90%).
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33 **Discussion**

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35 Not enough novel antibiotics have entered the market to mitigate the threat of antibiotic
36 resistance. It appears that the challenge of developing antibiotics is a particularly difficult
37 one, and there have been several explanations for this: biased screening libraries (both
38 synthetic and organism-derived natural products)⁹⁶, inadequate target selection, validation¹¹,
39 ⁹⁷ or screening methods⁹⁸. Apart from these, the fact remains that antibiotics development is
40 more difficult because of the extra barriers that bacteria present between a drug and its target,
41 such as additional and different (from human) cell membranes, drug efflux and metabolism
42 systems. Possible solutions to these problems have been proposed, though most of them too
43 recently to influence the antibiotics pipeline yet. For example, important aspects of drug
44 efflux mechanisms have been elucidated⁹⁹⁻¹⁰¹, allowing more rational design of inhibitors and
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3 other antibiotics. Similarly, past successful and failed antibiotics give insight into their
4 properties important for membrane permeation^{15, 102, 103}. However, all these aspects still put
5 extra constraints on a drug's properties. Additionally, to address the risk of rapid development
6 of resistance, antibiotics must have exceptional efficacy and low toxicity. A hit might be
7 modified so heavily to address all these issues that some of the original properties are lost, or
8 worse, that the hit properties conveying efficacy are incompatible with those needed for good
9 permeability, stability and safety. Though this might seem exacerbated in case of fragment-
10 based hits, FBLD could offer an advantage by having a higher chance of generating other
11 lead scaffolds (which is why we prefer the term fragment-based lead discovery). The process
12 from lead to candidate is much the same, by whichever method a lead was found.
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26 Recently, different approaches to screening for antibacterials have been explored, including
27 exploration of natural products from uncultured bacteria¹⁰⁴ and target-based whole cell
28 screening⁹⁸. Here, various FBLD approaches in antibiotic lead discovery have been
29 discussed. One of the principles of FBLD is that it is target-based. Given a target, FBLD can
30 be used to efficiently generate leads with control over desired properties such as lipophilicity
31 and selectivity. For example, for GyrB, fragment screening was a good choice because it
32 offered a way of finding new compounds for a validated mechanism of action. The fragment
33 hit **7** is a feature of clorobiocin, a member of the aminocoumarin class of gyrase inhibitors,
34 which are very potent but also have toxicity and solubility issues. The FBLD optimization
35 strategy, using structural information and concepts such as LE, yielded a potent lead that even
36 progressed to clinical candidate.
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52 In the case of GyrB leads **6** and **10**, and in the cases of EthR lead **18** and β -lactamase leads
53 **25/26**, it was fortunate that FLBD-guided increases in target affinity also resulted in whole-
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3 cell activity. Unfortunately, many other antibacterial discovery projects have shown that this
4 cannot always be achieved. Especially for FBLD antibacterial projects, most of which in the
5 early stages are carried out entirely *in vitro* with purified protein, it is important to keep an
6 eye on the potential to gain whole-cell activity, as soon as reasonable progress after hit
7 validation has been made. This was one of the key strategies for successful optimization of
8 EthR fragment screening hit **21** into **23** with cellular potency.
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18 In some cases, such as cell division protein target FtsZ, target-based whole cell screening can
19 even start from the fragment stage. The development of FtsZ inhibitor **13** is an unusual
20 example of fragment-based projects because no screening was involved, no structural
21 information was available, and the starting fragment **12** already showed specific activity in
22 cells. Rigorous medicinal chemistry efforts were made applying principles important for
23 every fragment-based project, especially the thorough characterization of the fragment
24 scaffold. Small changes can have great impact on the activity of scaffolds, as evident for
25 fragment **15** compared to **12** (as well as for EthR inhibitor **23** compared to **21**). However, the
26 useful metric of LE cannot be used when MICs are the primary measure of potency. Indeed,
27 it would not be easy to establish any SAR in these cases without a very specific target
28 inhibition phenotype, because changes in MIC could also come from off-target effects.
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44 There have recently been some exciting demonstrations of using fragments as probes for
45 target identification in phenotypic screening. Off-target effects are expected because of the
46 small size of the fragment, but it should have different affinities for the various targets
47 allowing the major targets to be identified. A recent example is where each of the fragments
48 in the library is attached to a photo-activatable group – this covalently links the fragment to a
49 target(s) which are then analysed by mass spectrometry together with methods of stable
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3 isotope labeling with amino acids in cells ¹⁰⁵. This could essentially be described as whole-
4 cell screening with direct detection of target binding, and has generated a lot of interest. It
5 will be interesting to see if this idea of functional screening of fragment libraries is successful
6 in identifying new targets for antibiotic drug discovery.
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11 12 13 14 15 16 **Acknowledgements**

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21 by the Wellcome Trust and EPSRC.
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28 29 **Figure Legends**

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31 **Figure 1. Screening against biotin carboxylase.** Fragment and virtual screens conducted in
32 parallel resulted in several highly ligand-efficient hits (**2**, **3**) with similar pharmacophore
33 features to HTS hit **1**, and novel scaffolds. A number of lead series (such as **4**) were
34 generated which showed antimicrobial activity. Red dashed circles indicate similar
35 pharmacophore features. MIC: minimum inhibitory concentration.
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44 **Figure 2. Hit-to-lead optimization of GyrB inhibitors.** **A)** Indazole (**5**), a validated hit from
45 a virtual screen, was optimized to lead compound **6**. **B)** Development of pyrrole hit **7** into
46 clinical candidate **11**. MNEC: minimum non-effective concentration. MIC₉₀: MIC value at
47 which 90% of tested strains were inhibited.
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3 **Figure 3. SAR exploration of the benzamide scaffold.** A) FtsZ inhibitor 3-
4 methoxybenzamide (**12**) was the starting point for optimization into lead compound
5 PC190723 (**13**), which was shown to bind in a hydrophobic cleft (PDB code 4DXD).
6 Residues that confer resistance to **13** upon mutation are shown as raspberry-colored sticks.
7 Hydrogen bonds are shown as dashed yellow lines. B) Several substituents were placed on
8 each R group, including combinations of 2 groups on different positions. Fil.: minimum
9 concentration at which filamentation was observed.
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20 **Figure 4. Fragmentation approach for EthR inhibitors.** A) Click reaction component **17**
21 can be seen as a fragment with weak activity. It was successfully grown via virtual (**18**) and
22 then actual medicinal chemistry to lead compound **19**. B) Crystal structure (PDB code
23 4M3B⁸⁶) showing the binding mode of **19** (green sticks) in the *M. tuberculosis* EthR
24 allosteric pocket (grey surface representation). The binding pose of **20** (cyan thin lines) in the
25 same pocket (PDB code 3O8H⁸⁵) is overlaid. C) Crystal structure (PDB code 3O8H) showing
26 the binding mode of **20** (cyan sticks) in its binding pocket (grey surface). The binding pose of
27 **19** (green thin lines) in the same pocket (PDB code 4M3B) is overlaid. Hydrogen bonds are
28 shown as dashed yellow lines. EC₅₀: concentration of EthR ligand at which *M. tuberculosis*
29 growth in macrophages is inhibited by 50% by ethionamide at one tenth of its MIC,
30 determined according to a standard procedure¹⁰⁶.
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46 **Figure 5. Fragment screening derived inhibitors of EthR.** EC₅₀: see description in Figure
47 4 legend. n.d.: not determined.
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52 **Figure 6. Fragments guide lead derivatization of β -lactamase inhibitors.** A) Lead
53 compound **24** (dark grey) was successfully modified to **25** (light grey) to pick up an extra
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3 hydrogen bond with residue G320 of *E. coli* AmpC, inspired by a fragment (orange) binding
4 pose (figures prepared using structures deposited with PDB codes 3O87⁹⁴, 2HDR¹⁰⁷, 4E3I⁹⁵,
5 for binding poses of **24**, the carboxylate fragment, and **25**, respectively). **B)** The same
6 interactions are made by **26** (light grey), modified to incorporate a tetrazole moiety as
7 suggested by another fragment (magenta) (figures prepared using structures deposited with
8 PDB codes 3O87, 3GR2¹⁰⁸, 4E3J⁹⁵, for binding poses of **24**, the tetrazole fragment, and **26**,
9 respectively). Hydrogen bonds are shown as dashed yellow lines. AmpC: fold reduction in
10 MIC of cefotaxime against a strain of *E. coli* overexpressing class C β -lactamase AmpC,
11 when the compound was added at a 1:4 cefotaxime to β -lactamase inhibitor ratio. CTX-M:
12 fold reduction in MIC of cefotaxime against a strain of *E. coli* overexpressing class A β -
13 lactamase CTX-M-14, when the compound was added at a 1:4 cefotaxime to β -lactamase
14 inhibitor ratio.
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<i>Technique</i>	<i>Throughput¹</i>	<i>Protein² consumption</i>	<i>Lower affinity limit (mM)³</i>	<i>Main limitation</i>	<i>Notes</i>
<i>Ligand-observed NMR</i>	High	High	10	No direct structural information	
<i>Protein-observed NMR</i>	Low	High	5	Usually limited to proteins < 35 kDa	
<i>SPR</i>	Moderate	Low	0.5	Requires protein immobilisation	Low false negative rate
<i>TSA</i>	High	Low	0.1	Insensitive	High false negative rate
<i>Biochemical assay</i>	High	Low	0.1	Many ways of interference	Direct functional information
<i>Crystallography</i>	Moderate	Moderate	No limit	Requires high quality crystals	Low false positive rate
<i>WAC</i>	Low	Low	1	Requires protein immobilisation	Low false negative rate
<i>MST</i>	Moderate	Low	0.5	Requires protein labelling	

Table 1. Summary of main features of most widely used fragment screening methods.

The comments are somewhat subjective and reflect the experience of the authors but summarise the comments made in the text. All techniques depend on the expertise of the user, particularly in recognising artefacts leading to false positive or false negative results. In addition, the limitations are affected not only by the sensitivity of the detection method but also by compound behaviour (solubility and aggregation). ¹Throughput – depends on the system and the instrumentation available but “high”, “moderate” and “low” are for many hundreds, tens or a few compounds per day. ²Protein consumption – “high”, “moderate” and “low” are where a screen of 1000 fragments would require many tens, single digit and below 1 mg of protein in most cases. ³ affinity limit – presuming the compounds have unlimited solubility, this is approximately the lowest detection limit for the technique. Note that ITC is

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3 not used for fragment screening – the protein consumption is too high, the experiment takes
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5 too long and the binding of some fragments is entropically driven, so they would be missed.
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For Peer Review

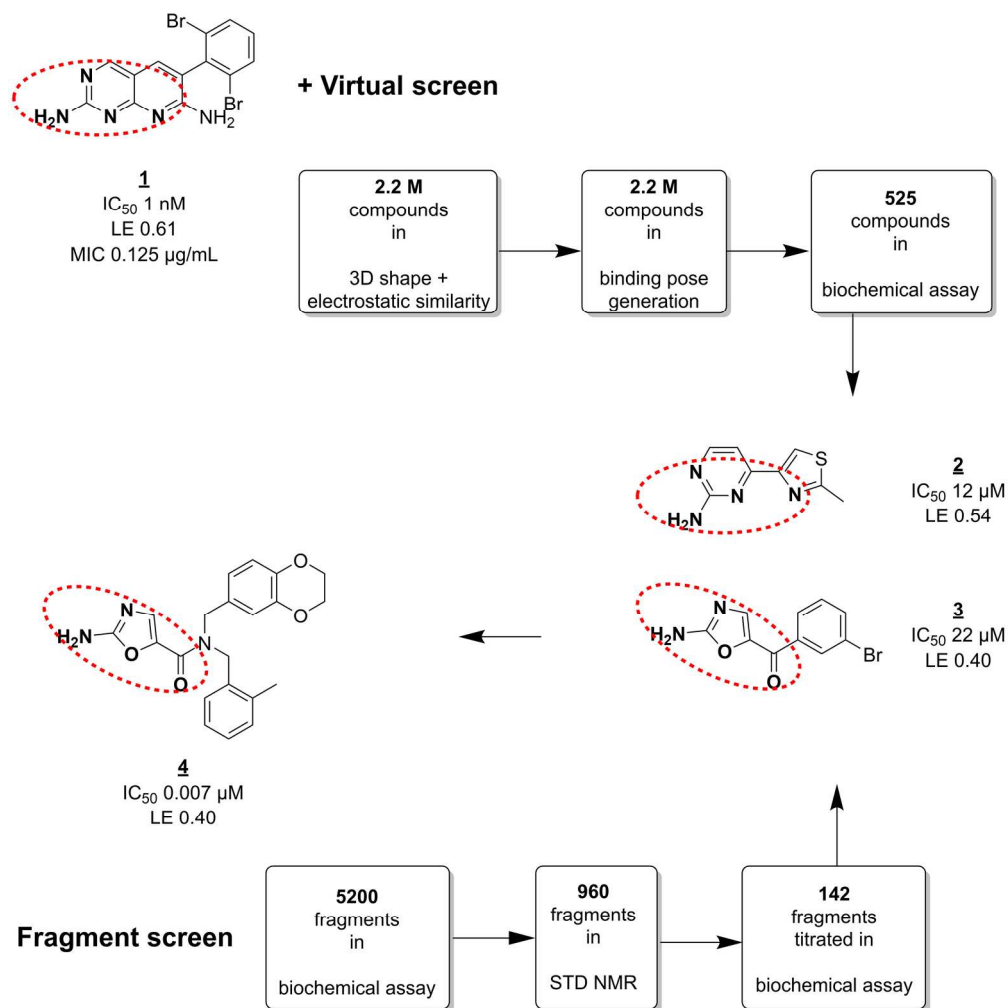


Figure 1

185x187mm (300 x 300 DPI)

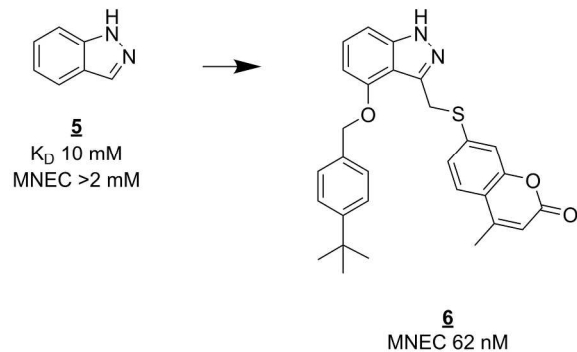
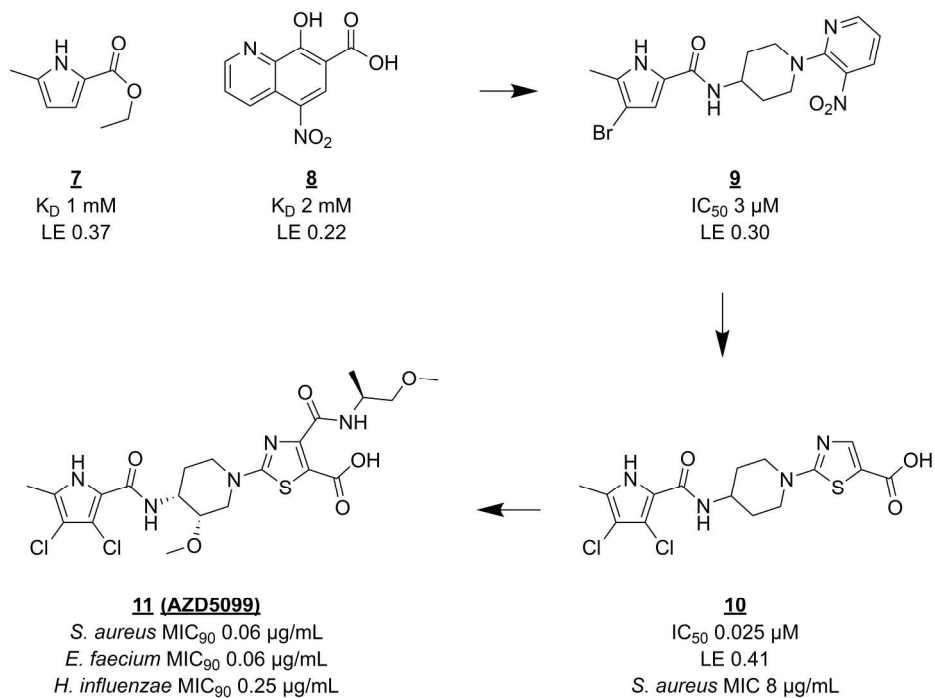
A**B**

Figure 2

209x269mm (300 x 300 DPI)

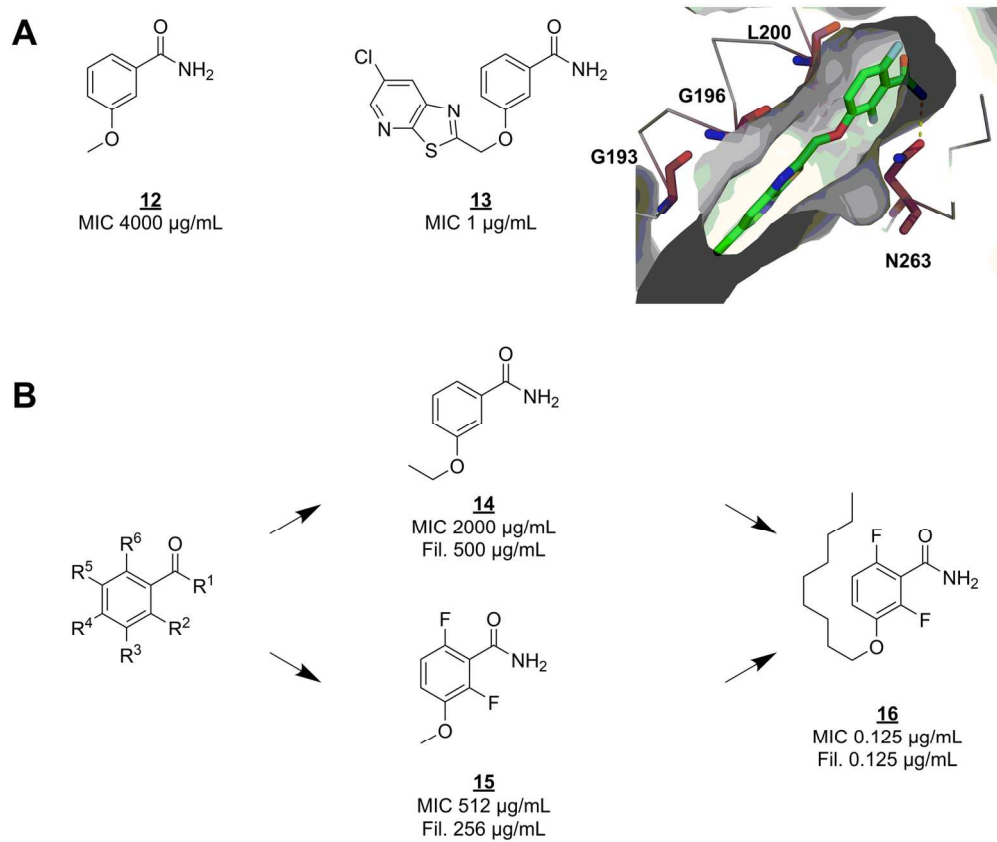


Figure 3

152x127mm (300 x 300 DPI)

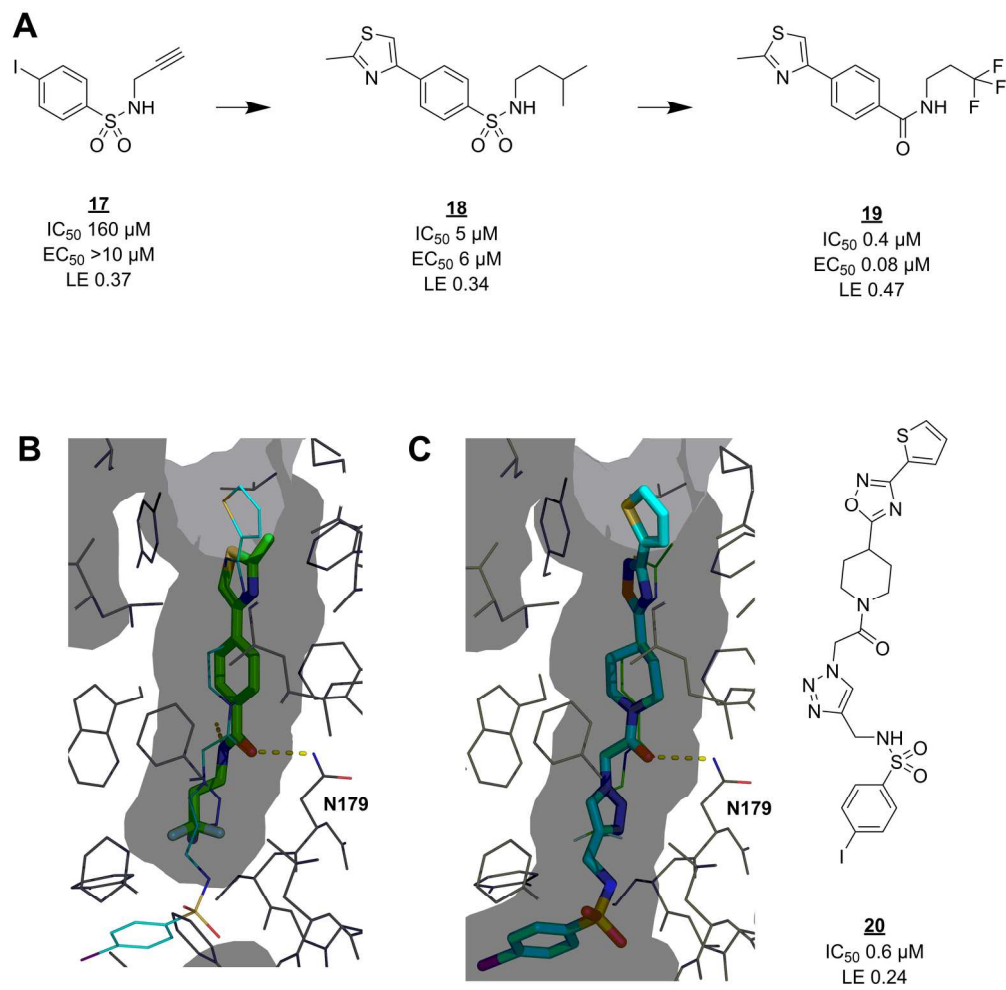


Figure 4

183x181mm (300 x 300 DPI)

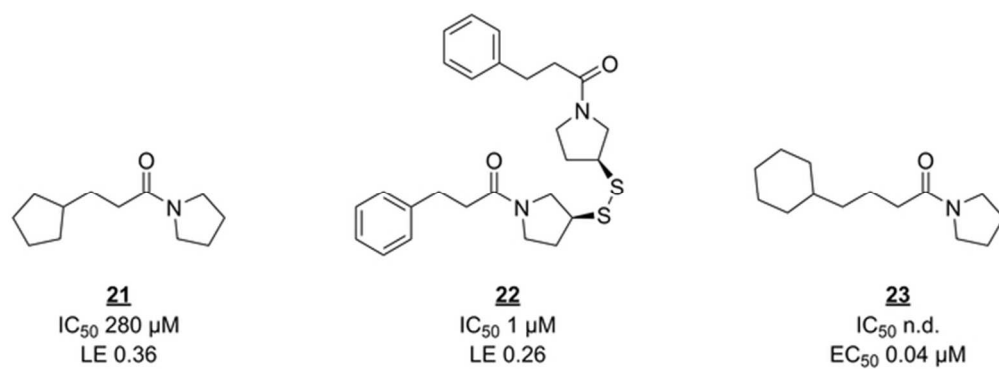
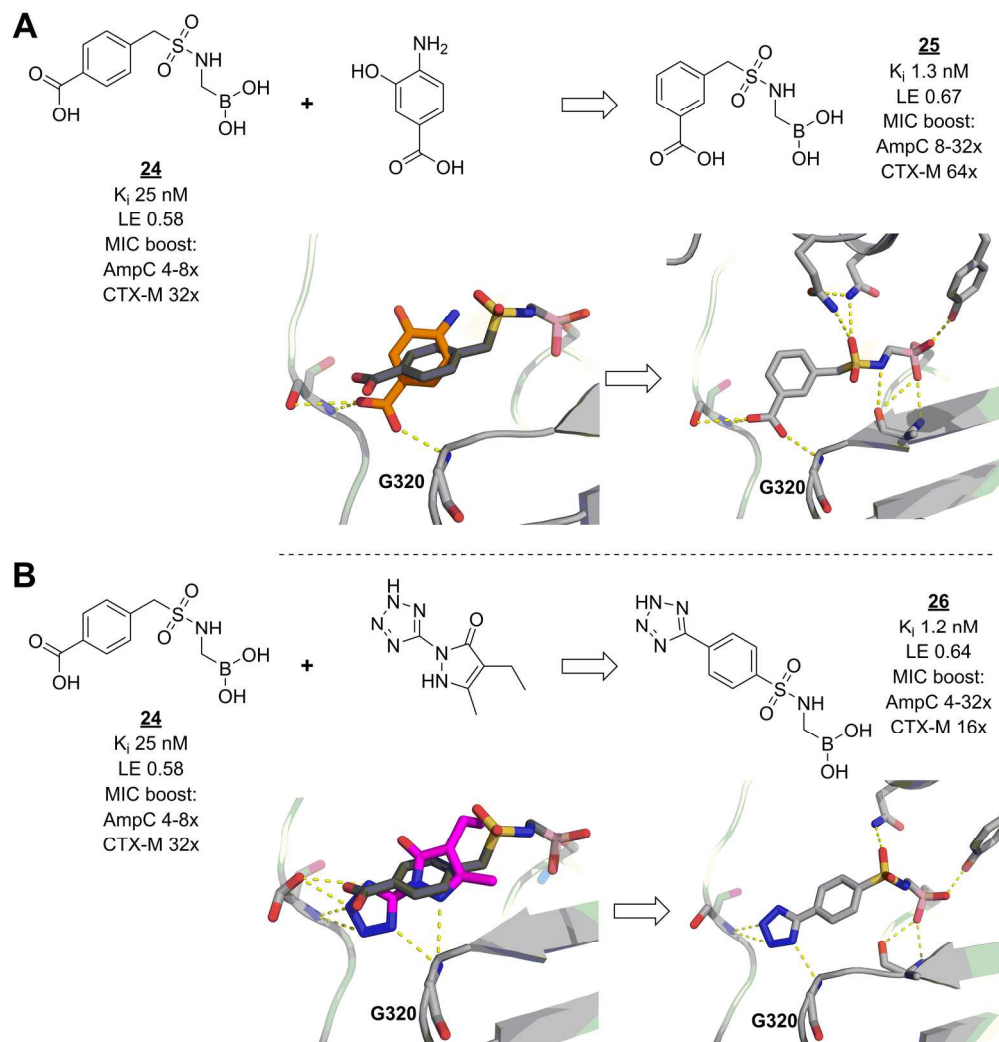


Figure 5

57x21mm (300 x 300 DPI)

Peer Review



192x200mm (300 x 300 DPI)