Picking pockets to fuel antimicrobial drug discovery

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Abstract
The inhibition of essential enzymes in microbial pathogens offers a route to treatment of infectious diseases. However, although the biology of the organism dictates a need for a particular enzyme activity, this does not necessarily mean that the enzyme is a good drug target. The chemistry of the active site (size, shape and properties) determines the likelihood of finding a molecule with the right properties to influence drug discovery. Discriminating between good and less-good targets is important. Studies on enzymes involved in the regulation of oxidative stress and pterin/folate metabolism of trypanosomatid parasites and isoprenoid precursor biosynthesis in bacteria and apicomplexan parasites illustrates a range of active sites representing those that are challenging with respect to the discovery of potent inhibitors, to others that provide more promising opportunities in drug discovery.

Introduction
Today’s pharmaceutical industry, in part founded on antimicrobial drug discovery, is one of the most important successes of modern science. Yet, despite tremendous advances there remain some infectious diseases for which treatments remain unsatisfactory. Now, more so than at any other time, academics can contribute to the complex multistage process of antimicrobial drug discovery targeting these ‘neglected’ diseases [1]. Armed with genomic information, powerful genetic and chemical tools, combined with affordable technology, then researchers can generate understanding of microbial metabolism and pathogenesis, characterize novel drug targets and identify the first inhibitors or lead compounds.

The starting point is the target. This must be validated, that is, proven essential for growth, survival or infectious capability of the pathogen, and either absent from humans or sufficiently distinct that species-specific inhibition is possible [2]. The first inhibitors or hits for the target are then sought. Hits are found by HTS (high-throughput screening) of compound libraries if an assay is available, by VS (virtual screening) if the target structure is known or by SBLD (structure-based ligand design) if data are available in particular on chemical scaffolds that bind in an active site [3]. Hits, typically with IC_{50} values in the low micromolar range, are improved by chemical modification to create lead compounds (IC_{50} < 1 µM) and then lead series (incorporating structural variations). The IC_{50} values of a useful lead series are likely to be <100 nm. The lead series might supply candidates for preclinical assessment where ADMET (adsorption, distribution, metabolism, excretion and toxicity) is considered. An end-point would be a stable, bioavailable and potent inhibitor of an essential pathogen enzyme with minimal perturbation of human metabolism.

My laboratory studies the structural biology of trypanothione and pterin/folate metabolism in kinetoplastid parasites and the biosynthesis of isoprenoid precursors in a range of pathogens. We seek to understand fundamental aspects of pathogen biology but with the ambition to contribute to early stage drug discovery. Our crystal structures represent accurate models of the targets and help to delineate the chemical forces responsible for function or inhibition. Before outlining different systems being targeted, I will make some general comments on what makes a good target.

A good pocket or a bad pocket?
The first points to make are that there are often exceptions, and to this academic drug discovery appears to be all about probability and serendipity. It feels just like every other branch of science where a good idea and careful planning and data analysis optimize chances of success. When surprises occur or the original idea is perhaps not so good, then different models and ideas are brought forward and exploited. It also feels though that drug discovery is hard science with only limited opportunities for success. Learning the lessons of what works and what does not is one way in which our chances can be improved. The types of molecules likely to be bioavailable and the starting points for medicinal chemistry approaches dictate the composition of screening libraries and decisions for following up the first inhibitors. As an increasing number of structures are obtained, relevant to many different diseases, and more information is garnered on inhibition and on the types of molecules that make useful

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Abbreviations used: CDP-ME, 4-diphosphocytidyl-2C-methyl-0-erythritol; CDP-ME2P, 4-diphosphocytidyl-2C-methyl-0-erythritol-2-phosphate; DHFR, dihydrofolate reductase; DMR, dimethylallyl pyrophosphate; HDS, high-throughput screening; IPP, isopentenyl pyrophosphate; MTH, methotrexate; PFR1, pteridine reductase 1; PyR, trypanothione reductase; Ts, thymidylate synthase; VS, virtual screening.

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drugs [4,5], there are increasing possibilities that predictive methods might help prioritize targets. Efforts in this area may become increasingly important; the approaches are certainly becoming more sophisticated [6,7].

So, what makes for a good pocket to target in a rational manner? We first assume that the target has been validated, i.e. the biology of the system indicates that the target is essential and that inhibition will compromise the survival of the pathogen. Then the questions to address concern the physicochemical properties of the site of inhibition. Does the landscape of the target pocket, strongly influenced by thermodynamic properties, allow for efficient inhibition by the right type of chemical that constitutes a drug? In essence, is the target druggable [8]?

Part of my research is on targets for which the biology steer is clear. The chemistry of the systems is however very different and might help discriminate between good and less good targets and suggest where the priorities might lie and on how to proceed.

Trypanothione-dependent metabolism in kinetoplastids

Aerobic metabolism generates destructive reactive oxygen species that are eliminated by antioxidant enzymes. In eukaryotes, peroxide detoxification mainly depends on glutathione and involves glutathione peroxidase working with NADPH, and glutathione reductase. The medically important parasitic Kinetoplastida, genera *Leishmania* and *Trypanosoma*, are different and rely on a polyamine–peptide conjugate \( \text{N}^1,\text{N}^8\text{-bis(glutathionyl)spermidine} \) called trypanothione. This key metabolite regulates intracellular thiol balance, contributes in defence against oxidant stress and xenobiotics, and provides reducing equivalents for peroxide metabolizing systems and ribonucleotide reductase for nucleic acid synthesis [9,10].

Trypanothione regulates redox balance in concert with TryR (trypanothione reductase), a genetically validated drug target [11], tryparedoxin and tryparedoxin peroxidase. These proteins constitute the trypanothione peroxidase pathway, and we have determined the structure–function relationship of each component [12–15]. Our research led to understanding of TryR specificity (Figure 1A), the discovery of a natural product inhibitor scaffold by VS and modelling, and how synthetic inhibitors bind [12,16]. TryR is a target for HTS and medicinal chemistry at a number of institutes mainly because the biology of the problem suggests that it is an excellent target. However, the substrate is present in the parasites at high concentration, and structural studies of TryR reveal a water-filled open chasm of an active site. It is difficult to correlate inhibition with chemical structures and without such information it becomes problematic to apply medicinal chemistry approaches to derive potent lead compounds.

The structure of quinacrine mustard binding in the active site of *Trypanosoma cruzi* TryR [16] shows two molecules of the inhibitor in a single active site (Figure 1B) and each making covalent interactions with the enzyme. Covalent inhibition of TryR may offer the most appropriate route to progress this enzyme as a therapeutic target. The use of such inhibitors is discouraged in the pharmaceutical industry due to issues of reactivity and toxicity. However, remember: there are exceptions and with respect to covalent inhibition of enzymes in particular they abound. A survey of modes of action of enzyme inhibitors used in the clinic reveals that almost one-third of them function by covalent modification [17]. This may be an avenue worth some investment.

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**Figure 1** | The active site of *T. cruzi* TryR (A) in complex with the substrate and (B) in complex with quinacrine mustard

The enzyme is depicted as a van der Waals surface coloured according to atom type (C, white; N, blue; O, red, and S, yellow). Specific residues are labelled. The ligands are shown in stick mode with C positions black, N cyan, Cl green and O red. All images were prepared using the PyMOL Molecular Graphics System (DeLano Scientific; http://pymol.sourceforge.net).
Pteridine reductase

Trypanosomatids are auxotrophic for pterins required for cofactor, nucleic acid and amino acid biosyntheses. These parasites rely on a bifunctional DHFR (dihydrofolate reductase)–TS (thymidylate synthase) and PTR1 (pteridine reductase 1) to carry out reductions of acquired pterins [18]. Enzymes of folate metabolism are proven targets for the treatment of bacterial and some parasitic infections [19] and in theory antifolates, such as MTX (methotrexate), should be efficacious against trypanosomatids. However, resistance is mediated principally by amplification of a trypanosomatid specific PTR1 [20]. PTR1 catalyses the NADPH-dependent reduction of unconjugated and conjugated oxidized pterins to active tetrahydro forms, is the only enzyme known to reduce biopterin in trypanosomatids and is essential for parasite growth. PTR1 catalyses the same reaction as DHFR but is less susceptible to known antifolates, so provides a metabolic bypass to alleviate DHFR inhibition. Inhibition of PTR1 would facilitate the exploitation of DHFR-specific antifolates and provide an urgently required therapeutic approach. DHFR and PTR1 present distinct active sites and it would be difficult to obtain a single inhibitor with the necessary potency against both enzymes. Our aim is to discover a synergistic combination of PTR1/DHFR inhibitors to underpin drug development.

The structure, mechanism and inhibition of Leishmania major and Trypanosoma brucei PTR1 (LmPTR1) have been studied [21,22]. The protocols to analyse ligand complex crystal structures, enzyme assays and calorimetry to obtain accurate thermodynamic data are well established [23–25]. The active site of PTR1 is a narrow cleft formed between a phenylalanine and nicotinamide (Figure 2) and is divided into three zones. Zone I is near the catalytic tyrosine and aspartic acid residues. Zones II and III are cavities near hydrophobic residues. In T. brucei PTR1, these cavities involve residues Met163-Cys168 and Pro210-Trp221. The active site of PTR1 offers distinct features to assist development of highly potent inhibitors. The active site is relatively small and with a number of hydrophobic residues that provide exactly the right environment to support tight binding of inhibitors [25].

We have applied computational methods (VS, modelling and chemical similarity searches) to assist ligand identification. We are exploiting prior knowledge: using scaffolds derived from substrates/products or known inhibitors, the guidelines of Lipinski et al. [26], restrictions on the number of ring systems and the limitations of certain functional groups [4]. Since molecular complexity will develop as synthetic chemistry embroiders a scaffold during cycles of modification, we have concentrated on small, biologically relevant starting points [27,28]. At this point, we have identified distinct leads and are now entering rounds of ligand design to enhance binding affinity taking into account structural data on different systems. New PTR1 inhibitors must display selectivity over DHFR–TS, and not inhibit human DHFR. A potent generic PTR1 inhibitor may not be possible given active site differences and distinct molecules may be required to specifically target Leishmania or other Trypanosoma species.

Three targets in the non-mevalonate pathway of isoprenoid precursor biosynthesis

IPP (isopentenyl pyrophosphate) and DMAPP (dimethylallyl pyrophosphate) are the universal precursors of natural products called isoprenoids. These include compounds that participate in respiration, hormone-based signalling, transcription and post-translational processes that control lipid biosynthesis, meiosis, apoptosis, glycoprotein biosynthesis and protein degradation. Isoprenoids are also membrane components. Two distinct biosynthetic pathways make IPP/DMAPP. In mammals, the plant cytosol, certain bacteria and trypanosomatids, these compounds are products of the mevalonate pathway. In chloroplasts, most bacteria and apicomplexan parasites, the non-mevalonate pathway generates IPP/DMAPP [29].

The non-mevalonate pathway starts with condensation of pyruvate and D-glyceraldehyde 3-phosphate. Then the enzymes IspC and IspD convert DOXP (1-deoxy-D-xylulose 5-phosphate) into methylerythritol phosphate and transfer this on to CDP, producing CDP-ME (4-diphosphocytidyl-2C-methyl-D-erythritol-5-phosphate) into methylerythritol phosphate and transfer this onto CDP, producing CDP-ME (4-diphosphocytidyl-2C-methyl-D-erythritol). The ATP-dependent CDP-ME kinase, IspE, then produces CDP-ME2P (4-diphosphocytidyl-2C-methyl-D-erythritol-2-phosphate). Next, CDP-ME2P is converted into MECP (2C-methyl-D-erythritol-2,4-cyclodiphosphate) in a reaction catalysed by IspF. The
The active site of E. coli IspF in complex with CDP and cyclodiphosphate products

A similar scheme to that of Figure 1 is used with the addition that Zn$^{2+}$ is a purple sphere and P atoms are coloured orange.

cyclodiphosphate, in two enzyme-catalysed stages, undergoes a reduction and elimination to give IPP, and isomerization to DMAPP completes precursor biosynthesis [29].

The non-mevalonate pathway is absent from humans, occurs in serious human pathogens (e.g. Mycobacterium tuberculosis and Plasmodium falciparum) and has been validated, genetically and chemically, for broad-spectrum antimicrobial drug design [25]. IspD, IspE and IspF are all essential in Escherichia coli, Bacillus subtilis, Haemophilus influenzae and M. tuberculosis. Enzymes such as these are particularly valued for drug development since they constitute metabolic chokepoints, i.e. they uniquely consume a substrate or generate a specific product and their function cannot be compensated for by another enzyme [30,31].

Structure-reactivity relationships in IspD-E-F and a bifunctional IspD-F have been characterized [32–36]. The key residues for substrate or cofactor binding that contribute to catalysis in these enzymes are strictly conserved across species [32–34], strongly suggesting that broad-spectrum antimicrobial development might be feasible. With accurate structural data, we are applying computational methods to ligand discovery. Most progress has been made with IspF, where VS identified compounds for study, with four confirmed as novel ligands and complex structures determined [37,38]. Based on searches for molecules with antiviral agents, and structures have revealed alterations that are tolerated in particular areas of the active site [37]. However, despite using fairly large ligands and satisfying many of the hydrogen-bonding properties of the active site, the molecules are only modest inhibitors. In a similar fashion to TryR, the active site is open (Figure 3), solvent-filled, lined by many polar residues and at one end there is considerable flexibility in a substrate-binding loop. These properties suggest a poor pocket for ligand design. However, exceptions, remember! IspF is Zn$^{2+}$-dependent and functional groups capable of metal co-ordination are particularly valued for increasing binding affinity [39] and have been exploited in inhibitor development targeting other metalloenzymes [40].

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