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'Tethering' fragment-based drug discovery to identify inhibitors of the essential respiratory membrane protein type II NADH dehydrogenase

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ABSTRACT

Energy generation is a promising area of drug discovery for both bacterial pathogens and parasites. Type II NADH dehydrogenase (NDH-2), a vital respiratory membrane protein, has attracted attention as a target for the development of new antitubercular and antimalarial agents. To date, however, no potent, specific inhibitors have been identified. Here, we performed a site-directed screening technique, tethering-fragment based drug discovery, against wild-type and mutant forms of NDH-2 containing engineered active-site cysteines. Inhibitory fragments displayed IC_{50} values between 3 and 110 μM against NDH-2 mutants. Possible binding poses were investigated by *in silico* modelling, providing a basis for optimisation of fragment binding and improved potency against NDH-2.

Antimicrobial resistance (AMR) is a rapidly evolving global emergency that threatens many of the achievements of modern medicine. The majority of our clinically-relevant antimicrobials were developed during the so-called 'golden era' of antimicrobial discovery.[1] These compounds target several cellular processes important for the growth and viability of microbial cells, including peptidoglycan biosynthesis, RNA and protein synthesis, DNA replication, and folic acid metabolism. Enzymes of central carbon metabolism and the generation of ATP are essential mediators of bacterial pathogen physiology, persistence and pathogenicity. However, these enzymes are often overlooked in drug discovery programmes due to a lack of sufficient species selectivity. Cellular bioenergetics is an area showing promise for the development of new antimicrobials, antimalarials and cancer therapy.[2–4]

The promise of respiration and ATP synthesis (oxidative phosphorylation) as a new target space for drug development in bacterial pathogens is highlighted by the discovery that modulating bacterial respiration influences the killing of *Escherichia coli* by common antimicrobials such as ampicillin, gentamicin and norfloxacin.[5] Several reports are emerging that in *Mycobacterium tuberculosis*, the causative agent of tuberculosis (TB), deleting particular respiratory complexes (e.g. the terminal oxidase cytochrome bd)[6,7]or activating respiration with redox cycling[8,9] or reducing agents[10,11] accelerates cell death in response to some TB chemotherapeutics under non-replicating conditions. Lewis and colleagues have recently reported that intracellular ATP depletion induces persister cell formation in both *Staphylococcus aureus* and *E. coli* implicating ATP generation in persistence.[12,13] These recent studies demonstrate the untapped opportunity to broaden the spectrum of our current antimicrobial armoury. An increased understanding of the function of different respiratory complexes and ATP synthase in pathogen biology will be key to advancing cellular bioenergetics as a new target space. A key respiratory enzyme in this context is type II NADH:menaquinone oxidoreductase (NDH-2). NDH-2 is a monotopic membrane protein and the primary entry point of electrons derived from NADH into the mycobacterial respiratory chain.[14,15] NDH-2 is essential for growth of mycobacteria,[16,17] absent from mammalian mitochondria[14] and is therefore a promising drug target candidate.

Membrane proteins are increasingly being identifying by high throughput screening (HTS) approaches as antibacterial targets.[18] In recent years, anti-TB phenotypic HTS has predominantly selected for inhibitors targeting membrane proteins.[18] However, many compounds in HTS libraries have subsequently proven unsuitable for further antibiotic discovery and development.[19] Fragment-based drug discovery (FBDD) accesses greater chemical space than traditional HTS libraries, improving scope for drug development and acting as an early indicator

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of lead discovery success likelihood.[20–22] Tethering-FBDD is a site-directed, hypothesis-driven screening approach which exploits the reversible covalent reaction of thiol-disulfide exchange to capture fragments *via* native or engineered cysteine residues on a protein.[23] As with many HTS technologies, FBDD has been used extensively with soluble, cytoplasmic proteins, but far less so with membrane proteins as they present significant challenges, such as difficulty obtaining sufficient material for screening and the presence of detergents.[24] Despite the promise of NDH-2 as an anti-TB drug target, very few potent, inhibitory drug-like molecules have been identified and none have progressed to further drug development. The phenothiazines, inhibitors of *Mycobacterium tuberculosis* NDH-2, remain problematic for use in anti-TB therapy, as the concentrations required are clinically un-achievable in patients.[25] Whilst the quinolinyl pyrimidines identified by Shirude et al. as part of a high throughput screening campaign are, compared to the phenothiazines, potent inhibitors of NDH-2 (IC_{50} = 0.0043 μM)[26] they have not yet progressed into the clinical development pipeline.[27,28] Most recently, 2-mercapto-quinazolinones were identified as inhibitors of NDH-2 with nanomolar potency.[29] However, compounds in this study were inactivated by glutathione-dependent adduct formation, as well as quinazolinone oxidation in microsomes. Pharmacokinetic studies also demonstrated modest bioavailability.[29] Whilst the 2-mercapto-quinazolinones compounds are an exciting step towards development of potent NDH-2 inhibitors, there remain serious barriers likely to restrict their clinical development. Recently, we determined both the high resolution crystal structure of bacterial NDH-2[15] and the detailed mechanism of catalysis and substrate binding,[30] providing a molecular framework for a tethering-FBDD approach to identifying inhibitory fragments. We therefore decided to exploit the unique ability to specifically direct chemical discovery to a site of interest (in this case, the quinone-binding pocket of NDH-2), without a known competitive ligand in-hand, that tethering-FBDD approach affords, in order to identify inhibitory fragments. Here we describe the application of this technique to the essential, respiratory membrane protein, bacterial NDH-2.

Examination of the quinone-binding pocket of bacterial NDH-2 revealed no native cysteine residues suitable for a tethering-FBDD screen.[15] We therefore selected two residues, R347 and R382, located on either side of the quinone-binding site (within 5–10 Å), but which were not predicted to be directly involved in quinone-binding, for site directed mutagenesis (SDM) to cysteines. Prior to introducing cysteines in the quinone-binding site the three native cysteines found in NDH-2, none of which were predicted to have a structural or catalytic role, were each mutated to alanine, producing a cysteine-free enzyme, and thereby minimising the risk of non-specific interactions during fragment screening. This resulted in C-terminally His-tagged NDH-2 mutants, R347CNDH-2 and R382CNDH-2 for use in screening alongside the wild-type (WT) NDH-2 protein. The Michaelis-Menten parameters of the purified mutant NDH-2 enzymes for menadione were comparable to the WT, demonstrating quinone-binding was unaffected by the SDM (Supplementary Fig. 1). Size exclusion chromatography demonstrated that the oligomeric states of the mutants were unchanged from that of WT, with no aggregation observed following the introduction of the surface-exposed cysteine residues.

Previously, Tethering-FBDD has been successfully performed on a soluble protein target by employing intact mass spectrometry screening to detect increased molecular weight corresponding to fragment mass adducts.[31] We therefore assessed the performance of purified, detergent (octyl-glucoside) soluble WT and R347CNDH-2 for a positive mode, intact mass tethering-FBDD approach. Unfortunately, R347CNDH-2 performed poorly and could only be resolved at high target loadings (≥1 pmol) with poor signal-to-background ratio. A β-mercaptoethanol (BMe) titration, employed to identify a thiolate concentration suitable for initial screening, successfully resolved a +78 Da mass shift consistent with mercaptoethanol labelling (50% estimated at 1 mM BMe) at an exchangeable thiol. Following buffer exchange into a mass spectrometry compatible buffer (20 mM ammonium bicarbonate, 20 mM NaCl pH 8, supplemented with 0.05% w/v acid labile MS-compatible surfactant), R347CNDH-2 (2 μM) was screened by intact mass spectrometry against a library of 1920 disulphide-containing fragments (100 μM fragment, 30 min room temperature incubation). Unfortunately, neither automated deconvolution nor the majority of manual deconvolutions around the target mass (44,200 Da) were successful, converging only into a noisy baseline spectrum, demonstrating that NDH-2 was not a suitable target for intact mass spectrometry screening.

To overcome these difficulties, an alternative functional tethering screen was undertaken, exploiting the NADH-dehydrogenase activity of NDH-2. Purified NDH-2 mutants were screened against the same disulphide-containing library of 1920 fragments (100 μM) as used for intact mass spectrometry screening. Following incubation (1h at 20 °C) of fragments, enzyme and menadione (100 μM) the reaction was initiated by addition of NADH (200 μM). Menadione was used as a more soluble electron acceptor than the native menaquinone.[15] NADH dehydrogenase activity was followed at 340 nm over 8 min. WT NDH-2 was counter-screened against the same library. Counter-screening was performed to discern between those fragments displaying inherent NDH-2 inhibition and those which displayed greater, specific inhibition of the cysteine-containing mutants, suggestive of tethering. Z′ values for all assays were ≥0.7 and Z-factors ranged from approximately 0.4–0.7 demonstrating that the assay performance was well optimised for fragment screening.[32] Actives for each enzyme isoform were defined as fragments displaying inhibition of NADH oxidation greater than 3σ away from the mean signal. R347CNDH-2 displayed lower inhibition than R382CNDH-2 with 3σ at 47% inhibition. So as not to exclude R347CNDH-2 inhibiting fragments, hits were selected from 22 actives displaying greater than or equal to 47% inhibition of R347C-NDH-2. After exclusion of fragments which contained pan-assay interference compounds (PAINS) substructures,[33] displayed very low or negative inhibition for WT NDH-2 or for which a very large difference between mutant isoforms existed, 10 hit fragments were identified (Table 1). Follow up concentration-dependent inhibition assays were performed on hits to establish their relative IC_{50}s against each mutant and an 'inhibition ratio' was calculated (WT IC_{50}/mean $IC_{50}$of both mutants) (Table 1). The inhibition ratio provided a single, simple value by which to assess the ability of a given hit to selectively inhibit the cysteine-modified mutant NDH-2 active site over the WT site, suggesting tethering of fragments was responsible for inhibition rather than an inherent, non-specific ability to occlude to the active site. Fragment 917534 displayed the largest differential between inhibition of the WT (42.2 μM) and mutant isoforms (5.9 and 3.2 μM for R347CNDH-2 and R382CNDH-2, respectively) and was also the most potent inhibitor of either mutant (Table 1).

Hits from FBDD are typically 'grown' via medicinal chemistry into drug-like molecules with higher affinity for the target[34], a process greatly facilitated high resolution X-ray crystal structures containing the bound fragment. We therefore, attempted co-crystallisation of fragments 916595, 917534, 95775 and 966531 with mutant NDH-2. This selection was based on both the inhibition ratios (Table 1), whereby preferential inhibition of either mutant over the WT suggested a tethering reaction, and the anticipated ease of synthesis for downstream applications. Unfortunately, we were unable to generate crystals of either mutant in the presence of fragments. Despite this, however, our previously determined high resolution (2.5 Å) structure[15] provided a framework for *in silico* docking experiments to give some insight into possible fragment binding poses.

To investigate likely binding poses for tethered fragments we performed covalent docking calculations using CovDock[35] (details can be found in Supplementary Experimental Materials). These calculations comprised two parts. The first determined the affinity of the ligands to the binding site without the influence of covalent bond formation. This was performed by docking the ligand non-covalently but with