Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com

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

Adam Heikal[a, b, *], Yoshio Nakatani[a, b], Wanting Jiao[b, c], Chris Wilson[d], David Rennison[e], Marion R. Weimar[a], Emily J. Parker[b, c], Margaret A. Brimble[b, e], Gregory M. Cook[a, b, *]

[a] Department of Microbiology and Immunology, School of Biomedical Sciences, University of Otago, Dunedin, New Zealand
[b] Maurice Wilkins Centre for Molecular Biodiscovery, The University of Auckland, Private Bag 92019, Auckland 1142, New Zealand
[c] Ferrier Research Institute, Victoria University of Wellington, Wellington, New Zealand
[d] Small Molecule Discovery Center, University of California, San Francisco, San Francisco, CA 94143, United States
[e] School of Chemical Sciences, University of Auckland, Auckland, New Zealand

ARTICLE INFO

Keywords:
Pathogens
Antimicrobial resistance
Fragment-based drug discovery
Membrane protein
Respiration
NDH-2

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

* Corresponding authors at: Department of Microbiology and Immunology, School of Biomedical Sciences, University of Otago, Dunedin, New Zealand.
Email addresses: adam.heikal@farmasi.uio.no (A. Heikal); greg.cook@otago.ac.nz (G.M. Cook)

https://doi.org/10.1016/j.bmcl.2018.05.048
Received 22 January 2018; Received in revised form 22 May 2018; Accepted 25 May 2018
Available online xxx
0960-894/

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 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 ($IC_{50} = 0.0043\,\mu M$)[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.

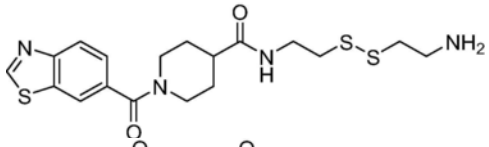
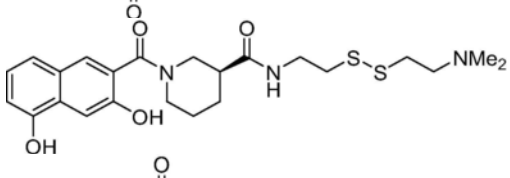
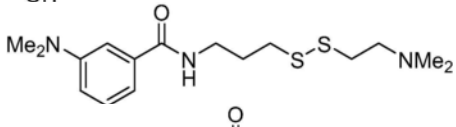
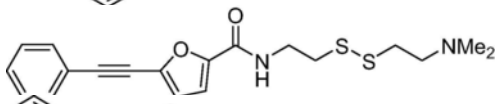
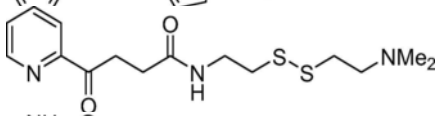
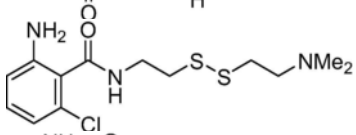
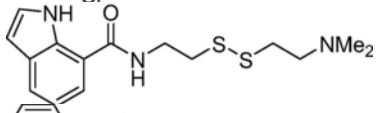
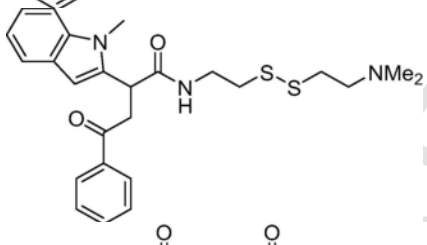
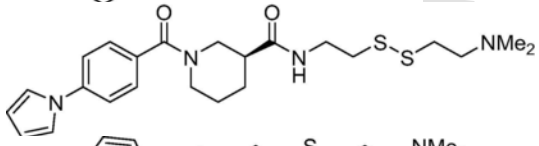
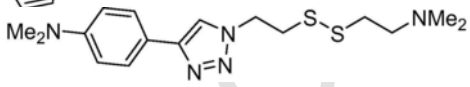
Examination of the quinone-binding pocket of bacterial NDH-2 revealed no native cysteine residues suitable for a tethering-FBDD screen.[15] We therefore selected two residues, R347 and R382, located on either side of the quinone-binding site (within 5–10 Å), but which were not predicted to be directly 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 R347C-NDH-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 previ-

Table 1
Structures and IC_{50}s of fragment screening hits.

Fragment	Structure	R382C[a]	R347C[a]	WT[a]	Inhibition ratio[b]
916959		18.6	9.9	52.0	3.7
916976		13.0	21.9	11.3	0.6
917163		11.5	14.5	36.0	2.8
917530		6.3	5.9	5.6	0.9
917534		3.2	5.9	42.2	9.3
917535		33.5	60.8	38.1	0.8
917546		32.6	110	61.9	0.9
917559		7.0	12.9	12.8	1.3
957775		26.9	26.5	80.3	3.0
966531		24.0	56.8	147.0	3.6
TPZ[c]		n.d.	n.d.	50.0	n/a

n.d. – not determined, n/a – not applicable.
[a] IC50s reported as μM.
[b] WT/Mutant mean.
[c] Trifluoroperazine.

ously 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 constraints applied to atoms involved in bond formation. Once a suitable pose was found, the second part of the calculation explored conformations around the bond formation site, to determine the best pose for reaction. We selected two compounds for *in silico* binding analysis namely, 917534 which was the most potent inhibitor of NADH oxidation, and 966531, which showed the next greatest differential between mutant and WT enzyme inhibition (6-fold difference in IC_{50}), strongly suggestive of a tethered inhibitor. An atomic level understanding of the basis for such a large differential between WT and mutant enzyme inhibition would likely be beneficial to down-stream medicinal chemistry. The receptors for covalent docking were generated by mutating R382 to a cysteine residue *in silico*. When modelled in R382C-NDH-2 the aromatic ring of fragment 917534, the fragment with the lowest IC_{50}

value, was predicted to bind within a 'hydrophobic clamp' formed by Q317 and I379, deep in the quinone-binding pocket (Fig. 1A). The formation of three predicted hydrogen bonds, between the carbonyl oxygen of 917534 to both the side chain of T349 and to FAD as well as between the backbone carbonyl of I379 to the nitrogen atom in the fragment tail, are potentially major contributors to the high binding affinity shown by this fragment. In contrast, when fragment 966531 was modelled in R382C-NDH-2 (Fig. 1B), the predicted binding pose showed more limited molecular interactions in the quinone-binding pocket. The triazole group is clamped, similarly to the aromatic group of 917534, but other parts, including the six-membered ring, are exposed to solvent. This might explain the higher a IC_{50} value of 966531 compared to that of 917534. Given this limited interaction, inhibitory activity of this compound against WT i.e. in the absence of a tethering reaction, would not be expected, consistent with the IC_{50} value observed for the WT.

For each pose predicted by covalent docking, an apparent affinity score was calculated, with a more negative score predicting greater fragment potency. The difference between apparent affinity scores for 917534 (−5.1 kcal/mol) and 966531 (−4.8 kcal/mol) was not large (0.3 kcal/mol). The small difference in affinity scores, despite the lack of polar interactions in 966531, is due to a higher number of hydrophobic residues interacting with this fragment, as a more extended binding conformation was predicted. However, 917534 was predicted to have greater affinity for the quinone-binding site and this was also reflected in[24] the experimentally determined IC_{50} value (Table 1).

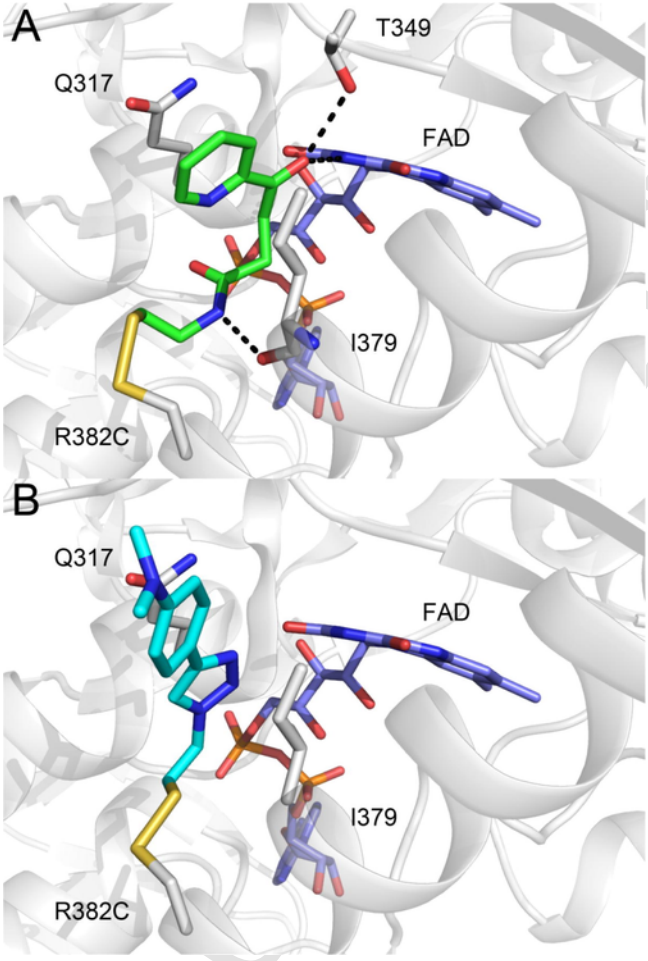


Fig. 1. Best scored poses from covalent docking calculations, for (A) 917534 (green) and (B) 966531 (cyan) in R382C-NDH-2. Modelling was performed on chain B of NDH-2 (PDB: 4NWZ), rendered in grey ribbon. Fragments are depicted in sticks, with disulfide bonds in dark yellow. Hydrogen bonds are shown in black-dashed lines.

Despite the lack of potent and specific inhibitors identified to date, NDH-2 remains a highly attractive antimicrobial drug target, chiefly by virtue of its essentiality and lack of a mammalian homologue. Here we attempted to advance NDH-2 as an anti-bacterial (e.g. *M. tuberculosis*) drug target via a hypothesis-driven, tethering-FBDD screening approach not previously attempted with membrane protein targets from bacteria. Membrane proteins are important drug targets but are notoriously challenging to work with and often not amenable to biophysical techniques. This proved to be at least partially true in this instance, with NDH-2 unsuitable for intact mass spectrometry screening. However, functional screening based on inhibition NADH dehydrogenase activity successfully identified inhibitory fragments which appeared to demonstrate tethering-mediated inhibition of NDH-2. Modelling of these fragments in the NDH-2 quinone-binding pocket provided insight into possible binding poses. Taken together, the experimental IC_{50} values and the modelled poses now serve to inform possible synthetic chemistry approaches to grow fragments into larger, more potent and specific inhibitors of NDH-2, though this was outside the scope of this investigation. Tethering-FBDD presents an opportunity to advance previously intractable antimicrobial targets, such as NDH-2 and eventually deliver new hit compounds into the clinical development pipeline.

Funding sources

This work was supported by the Maurice Wilkins Centre for Molecular Biodiscovery Flexible Research Seeding Programme. AH, YN, WJ were supported by the Maurice Wilkins Centre for Molecular Biodiscovery.

Acknowledgments

The authors wish to thank Dr. Torsten Kleffmann of the Centre for Protein Research, Biochemistry Department, University of Otago, New Zealand for technical assistance.

A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.bmcl.2018.05.048.

References

1. C.T. Walsh, T.A. Wencewicz, Prospects for new antibiotics: a molecule-centered perspective, J Antibiot 67 (2014) 7–22.
2. G.M. Cook, C. Greening, K. Hards, M. Berney, Energetics of pathogenic bacteria and opportunities for drug development, Adv Microb Physiol 65 (2014) 1–62.
3. G.A. Biagini, N. Fisher, A.E. Shone, et al., Generation of quinolone antimalarials targeting the Plasmodium falciparum mitochondrial respiratory chain for the treatment and prophylaxis of malaria, Proc Natl Acad Sci USA 109 (2012) 8298–8303.
4. S.K. Parks, J. Chiche, J. Pouyssegur, Disrupting proton dynamics and energy metabolism for cancer therapy, Nat Rev Cancer 13 (2013) 611–623.
5. M.A. Lobritz, P. Belenky, C.B.M. Porter, et al., Antibiotic efficacy is linked to bacterial cellular respiration, Proc Natl Acad Sci 112 (2015) 8173–8180.
6. M. Berney, T.E. Hartman, W.R. Jacobs Jr., A Mycobacterium tuberculosis cytochrome bd oxidase mutant is hypersensitive to bedaquiline, mBio 5 (2014) e01275–01214.
7. N.P. Kalia, E.J. Hasenoehrl, N.B. Ab Rahman, et al., Exploiting the synthetic lethality between terminal respiratory oxidases to kill Mycobacterium tuberculosis and clear host infection, Proc Natl Acad Sci USA 114 (2017) 7426–7431.
8. A. Heikal, K. Hards, C.Y. Cheung, et al., Activation of type II NADH dehydrogenase by quinolinequinones mediates antitubercular cell death, J Antimicrob Chemother 71 (2016) 2840–2847.
9. T. Yano, S. Kassovska-Bratinova, J.-S. Teh, et al., Reduction of clofazimine by mycobacterial type 2 NADH: quinone oxidoreductase: a pathway for the generation of bactericidal levels of reactive oxygen species, J Biol Chem (2010).
10. C. Vilcheze, T. Hartman, B. Weinrick, et al., Enhanced respiration prevents drug tolerance and drug resistance in Mycobacterium tuberculosis, Proc Natl Acad Sci USA 114 (2017) 4495–4500.

11. G.B. Coulson, B.K. Johnson, H. Zheng, et al., Targeting mycobacterium tuberculosis sensitivity to thiol stress at acidic pH kills the bacterium and potentiates antibiotics, Cell Chem Biol 24 (8) (2017) 993–1004, e1004.
12. B.P. Conlon, S.E. Rowe, A.B. Gandt, et al., Persister formation in Staphylococcus aureus is associated with ATP depletion, Nat Microbiol 1 (2016) 16051.
13. Y. Shan, A. Brown Gandt, S.E. Rowe, J.P. Deisinger, B.P. Conlon, K. Lewis, ATP-dependent persister formation in escherichia coli, mBio 8 (2017).
14. A.M.P. Melo, T.M. Bandeiras, M. Teixeira, New insights into Type II NAD(P)H : quinone oxidoreductases, Microbiol Mol Biol Rev 68 (2004) 603.
15. A. Heikal, Y. Nakatani, E. Dunn, et al., Structure of the bacterial type II NADH dehydrogenase: a monotopic membrane protein with an essential role in energy generation, Mol Microbiol 91 (2014) 950–964.
16. C.M. Sassetti, D.H. Boyd, E.J. Rubin, Genes required for mycobacterial growth defined by high density mutagenesis, Mol Microbiol 48 (2003) 77–84.
17. R.A. McAdam, S. Quan, D.A. Smith, et al., Characterization of a Mycobacterium tuberculosis H37Rv transposon library reveals insertions in 351 ORFs and mutants with altered virulence, Microbiology-Sgm 148 (2002) 2975–2986.
18. R.C. Goldman, Why are membrane targets discovered by phenotypic screens and genome sequencing in Mycobacterium tuberculosis?, Tuberculosis (Edinburgh, Scotland) 93 (2013) 569–588.
19. D.J. Payne, M.N. Gwynn, D.J. Holmes, D.L. Pompliano, Drugs for bad bugs: confronting the challenges of antibacterial discovery, Nat Rev Drug Discov 6 (2007) 29–40.
20. M.M. Hann, A.R. Leach, G. Harper, Molecular complexity and its impact on the probability of finding leads for drug discovery, J Chem Inf Comput Sci 41 (2001) 856–864.
21. A.L. Hopkins, C.R. Groom, A. Alex, Ligand efficiency: a useful metric for lead selection, Drug Discovery Today 9 (2004) 430–431.
22. F.N.B. Edfeldt, R.H.A. Folmer, A.L. Breeze, Fragment screening to predict druggability (ligandability) and lead discovery success, Drug Discovery Today 16 (2011) 284–287.
23. D.A. Erlanson, J.A. Wells, A.C. Braisted, Tethering: fragment-based drug discovery, Annu Rev Biophys Biomol Struct 33 (2004) 199–223.
24. V. Früh, Y. Zhou, D. Chen, et al., Application of fragment based drug discovery to membrane proteins: biophysical identification of ligands of the integral membrane enzyme DsbB, Chem Biol 17 (2010) 881–891.
25. M.V. Bettencourt, S. Bosne-David, L. Amaral, Comparative in vitro activity of phenothiazines against multidrug-resistant Mycobacterium tuberculosis, Int J Antimicrob Agents 16 (2000) 69–71.
26. P.S. Shirude, B. Paul, N. Roy Choudhury, C. Kedari, B. Bandodkar, B.G. Ugarkar, Quinolinyl pyrimidines: potent inhibitors of NDH-2 as a novel class of anti-TB agents, ACS Med Chem Lett 3 (2012) 736–740.
27. World Health Organization. ANTIBACTERIAL AGENTS IN CLINICAL DEVELOPMENT An analysis of the antibacterial clinical development pipeline, including tuberculosis. Geneva; 2017.
28. World Health Organization. Global tuberculosis report 2017. Geneva; 2017.
29. D. Murugesan, P.C. Ray, T. Bayliss, et al., 2-Mercapto-quinazolinones as inhibitors of type II NADH dehydrogenase and mycobacterium tuberculosis: structure-activity relationships, mechanism of action and absorption, distribution, metabolism, and excretion characterization, ACS Infect Dis (2018).
30. J.N. Blaza, H.R. Bridges, D. Aragão, et al., The mechanism of catalysis by type-II NADH:quinone oxidoreductases, Sci Rep 7 (2017) 40165.
31. J.M. Ostrem, U. Peters, M.L. Sos, J.A. Wells, K.M. Shokat, K-Ras(G12C) inhibitors allosterically control GTP affinity and effector interactions, Nature 503 (2013) 548.
32. J.H. Zhang, T.D. Chung, K.R. Oldenburg, A simple statistical parameter for use in evaluation and validation of high throughput screening assays, J Biomol Screen 4 (1999) 67–73.
33. J.B. Baell, G.A. Holloway, New substructure filters for removal of pan assay interference compounds (PAINS) from screening libraries and for their exclusion in bioassays, J Med Chem 53 (2010) 2719–2740.
34. D.A. Erlanson, S.W. Fesik, R.E. Hubbard, W. Jahnke, H. Jhoti, Twenty years on: the impact of fragments on drug discovery, Nat Rev Drug Discov 15 (2016) 605–619.
35. K. Zhu, K.W. Borrelli, J.R. Greenwood, et al., Docking covalent inhibitors: a parameter free approach to pose prediction and scoring, J Chem Inf Model 54 (2014) 1932–1940.