A Journal of the Gesellschaft Deutscher Chemiker

Angewandte International Edition Chemie

GDCh

www.angewandte.org

Accepted Article

Title: Small molecules targeting Mycobacterium tuberculosis type II NADH dehydrogenase with antimycobacterial activity

Authors: Michael B Harbut, Renhe Liu, Baiyuan Yang, Takahiro Yano, Catherine Vilcheze, Jonathan Lockner, Hui Guo, Scott G Franzblau, Mike Petrassi, William R Jacobs, Harvey Rubin, Arnab K Chatterjee, and Feng Wang

To be cited as: *Angew. Chem. Int. Ed.* 10.1002/anie.201800260
Angew. Chem. 10.1002/ange.201800260

Link to VoR: http://dx.doi.org/10.1002/anie.201800260
http://dx.doi.org/10.1002/ange.201800260

WILEY-VCH

10.1002/anie.201800260

WILEY-VCH

COMMUNICATION

Small molecules targeting Mycobacterium tuberculosis type II NADH dehydrogenase with antimycobacterial activity

Michael B. Harbut, Baiyuan Yang, Renhe Liu, Takahiro Yano, Catherine Vilchèze, Bo Cheng, Jonathan Lockner, Hui Guo, Chenguang Yu, Scott G Franzblau, H. Mike Petrassi, William R. Jacobs Jr., Harvey Rubin, Arnab K. Chatterjee, and Feng Wang*

Abstract: Generation of ATP via oxidative phosphorylation is an essential metabolic function for *Mycobaterium tuberculosis* (*Mtb*) regardless of the growth environment. The type II NADH dehydrogenase (Ndh-2) is the conduit for electrons into the pathway, and is absent in the mammalian genome, making it a potential drug target. Herein, we report the identification of two types of small molecules as selective inhibitors for Ndh-2 via a multi-component high-throughput screen. Both compounds block ATP synthesis, lead to effects consistent with loss of NADH turnover, and importantly, exert bactericidal activity against *Mtb*. Extensive medicinal chemistry optimization afforded the best analog with an MIC of 90 nM against *Mtb*. Moreover, the two scaffolds have differential inhibitory activities against the two homologous Ndh-2 enzymes in *Mtb*, which will allow precise control over Ndh-2 function in *Mtb* to facilitate assessment of this anti-TB drug target.

Mycobacterium tuberculosis resides in a variety of microenvironments, depending on the stage of infection, and to survive they are aided by a remarkable metabolic plasticity, which allows remodeling of energy utilization and biomass producing pathways, dependent on the environmental conditions and nutrient availability.[1] Recent work suggests that survival of *Mtb*, under a variety of conditions, is completely dependent on the existence of an energized plasma membrane, which couples nutrient oxidation to ATP synthesis via the production of an electrochemical gradient.[2] The discovery that TMC207 (bedaquiline) exerts its antimycobacterial activity via shutdown of ATP production through inhibition of the F-type ATP synthase validates oxidative phosphorylation as a pharmacologic target in *Mtb*.[3]

Electrons enter the respiratory chain from NADH oxidation, coupled to the reduction of menaquinone. The *Mtb* genome encodes both type I (*nuoA-N*) and two type II NADH:menaquinone oxidoreductases (Ndh-2: *ndh* and *ndhA*), though *nuo* appears dispensable for growth.[4] The role(s) for each individual copy of Ndh-2 are uncertain, but genetic knockout studies have suggested only *ndh* is essential for *Mtb* growth and persistence.[5] Importantly, while common among bacteria and protozoan parasites, these enzymes are absent from the mammalian genome, which further highlights their potential as drug targets.[6] A well-validated, specific Ndh-2 inhibitor would provide both a novel tool to study Ndh-2 function as well as enable preclinical pharmacological validation of Ndh-2 as a target for TB therapy.

We performed a high-throughput screen for compounds that reduced the production of ATP (via oxidative phosphorylation) through the inhibition of Ndh-2. We utilized membrane vesicles derived from mycobacteria and measured ATP production upon the addition of NADH. The NADH is then oxidized through a cascade of redox reactions, resulting in the formation of a proton gradient across the vesicle membrane. This proton gradient drives the synthesis of ATP from ADP and inorganic phosphate by the F_1F_0 ATP synthase. In this assay bedaquiline inhibits ATP synthesis with an IC_{50} of 6.9 nM (Figure S1).[7] In a miniaturized 1536-well plate format of this assay we screened over 800,000 compounds and identified over 7,000 primary hits (Figure S2). Hit compounds were progressed through a variety of triage assays to eliminate false positives, exclude cytotoxic compounds, and to determine the mode of inhibition in the pathway.

Compounds CBR-1825 and CBR-4032 each inhibited electron transport-dependent ATP production initiated by NADH, as well as NADH oxidation assayed in mycobacterium membranes with IC_{50} < 0.5 μM (Figure 1 and S3). Furthermore, they were inactive when assayed for succinate-initiated oxidative phosphorylation in membranes (Figure S4). This activity profile suggests that both small molecules block the electron transport chain at Ndh-2. Multiple active structural analogs were identified in the primary screen for both series. The major structural motif for CBR-1825 and the related compounds was the thioquinazoline (TQZ) core; CBR-4032 and like molecules possessed a tetrahydroindazole (THI) core. Importantly, both scaffolds showed growth inhibition of *Mtb* in liquid medium (MIC_{50} = 0.43 μM and 6.6 μM for CBR-1825 and CBR-4032, respectively) while displaying no gross toxicity towards mammalian cells (Figure 1 A & B).

[*] Dr. H. Guo, Prof. F. Wang
Institute of Biophysics, Chinese Academy of Sciences
Beijing 100101, China
E-mail: wangfeng@ibp.ac.cn
Drs. M. B. Harbut, B. Yang, R. Liu, B. Chen, J. Lockner, H. C. Yu, M. Petrassi, A.K. Chatterjee, F. Wang
California Institute for Biomedical Research
La Jolla, California 92037, USA
T. Yano, H. Rubin
Department of Medicine
University of Pennsylvania
Philadelphia, PA 19104, USA
C. Vilchèze, W. R. Jacobs Jr.
Howard Hughes Medical Institute
Department of Microbiology and Immunology
Albert Einstein College of Medicine
Bronx, NY 10461, USA
Scott G. Franzblau
Institute for Tuberculosis Research
University of Illinois at Chicag
Chicago, IL 60612, USA

Supporting information for this article is given via a link at the end of the document.

10.1002/anie.201800260
WILEY-VCH

COMMUNICATION

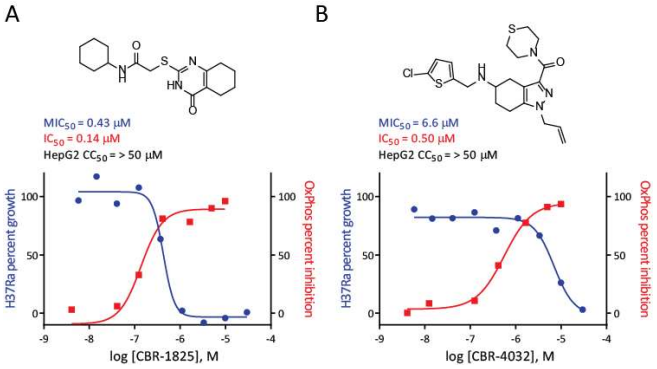


Figure 1. Representative hit compounds from the primary screen and their activities against *Mtb* H37Ra and oxidative phosphorylation assessed in mycobacterial membranes. A) CBR-1825, a representative of the thioquinazoline scaffold. B) CBR-4032, a representative of the tetrahydroindazole series.

We prioritized medicinal chemistry optimization for the TQZ series due to its high cellular potency. A few key structure-activity-relationships were observed during this process (Figure 2 and SI). Replacement of the thioether with oxygen, nitrogen, or methylene

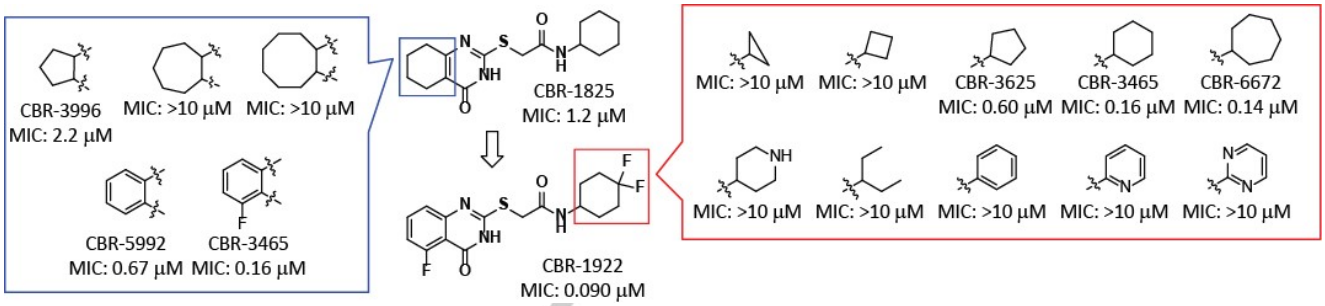


Figure 2. Selected SAR results of TQZ scaffold

significantly reduced activity, as did oxidation of the sulfide. Compared to saturated fused rings, a fused phenyl ring was preferred. CBR-5992, a quinazoline compound was identified with MIC of 0.67 μM, two-fold more active than the original hit CBR-1825. Further modifications of phenyl ring (various substitutions and heterocyclic rings) afford 5-fluoro substituted analog CBR-3465 with further improved activity (MIC = 0.16 μM). Next SAR study was conducted around cyclohexane amide side chain in the context of the optimized 5-fluoroquinazoline. While a lot of analogs are not active, fluoride, chloride, and methyl substituents are relatively tolerated. Among them, introduction of difluorines into to cyclohexane ring affords the most potent analog CBR-1922 against virulent *Mtb* with an MIC of 0.09 μM in growth inhibition assays. This compound compares favorably with the most advanced inhibitors from two recently described medicinal chemistry efforts targeting Ndh-2, the quinolinyl pyrimidines and a quinolone series.[8] The MIC_{50} of the most potent compounds from either of these two series ranged from 0.14-1.0 μM. Likewise, a suite of phenothiazine-derived compounds were unable to achieve an MIC_{50} below 10 μM against aerobic *Mtb*.[9]

Next, we assessed whether either Ndh-2 inhibitor series was bactericidal. Indeed, compounds from each scaffold produced over 1-log killing within the first three-day treatment, and greater than 4-log killing against cultures over a period of 32 days when tested at 10x the MIC (Figure S5). These data suggest that any assumed activity by the type I Ndh is unable to complement the loss of function of Ndh-2. Moreover, treatment of *Mtb* with complex I inhibitors, such as rotenone, has little effect on mycobacterial growth or NADH oxidation in purified membranes, suggesting that Ndh-2 is the dominant NADH dehydrogenase.[10]

Though our preliminary triage data suggested that both scaffolds block the electron transport chain (ETC) at Ndh-2, we initiated further target identification studies for confirmation. We attempted to identify the target(s) of the TQZ scaffold by isolating spontaneous arising mutants resistant to it. Single-step selection produced resistant mutants at a frequency of $3x10^{-8}$. Sequencing of 3 resistant mutants revealed a C-to-G mutation in the promoter region of *ndhA* (Rv0392c), 44 nucleotides upstream of the start codon. We analyzed gene expression levels of *ndh*, *ndhA*, and *nuoG*, in wild-type and one of the resistant strains and found that *ndhA* was expressed at a level approximately 50-fold greater than the wildtype strain (Figure 3A). Determination of MIC_{50} values of the resistant strains showed a greater than 10-fold decrease in the susceptibility to either scaffold suggesting resistance is conferred by compensatory upregulated expression of *ndhA*. (Figure 3B and S6).

The biochemical and genetic data suggested the activity of both scaffolds is mediated through one or both of the type II NADH:quinone oxidoreductases, yet those data do not specifically distinguish the protein target(s) of these inhibitors. The Ndh-2 enzymes Ndh and NdhA in *Mtb* share high homology (67% identity), are both expressed under aerobic culture conditions, and retain oxidoreductase activity.[11] Little is known though about their respective roles; in organisms with multiple copies of Ndh-2, their roles are often non-redundant.[12] To further examine target engagement, we utilized a temperature sensitive (TS) *Mycobacterium smegmatis* mutant to probe the activity of

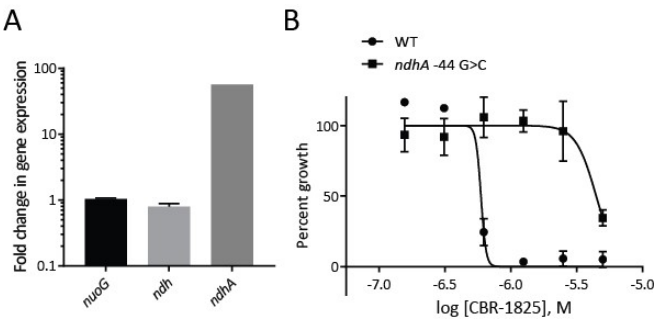


Figure 3. Upregulation of *ndhA* expression confers resistance to TQZ. A) Gene expression in the H37Ra *ndhA* -44 G>C background. B) Activity of TQZ against wild type and *ndhA* -44 G>C H37Ra strains.

each compound against Ndh and NdhA individually. When grown above the permissive temperature, this TS strain downregulates expression of *ndh* due to a base substitution of G250 to T of the *ndh* gene that alters amino acid Gly84 (GGC) to Cys (TGC) of Ndh (Yano and Rubin, unpublished data). When grown at 30°C this strain shows low susceptibility to either inhibitor series (similar to the WT *M.smegmatis*), but as the incubation temperature increases to 40°C, *ndh* expression correspondingly decreases, which results in an >8-fold shift in the MIC of the two compounds relative to their potency at the permissive temperature (Table S1 and Figure S7). In addition, the TS mutant can be complemented with *Mtb* malate dehydrogenase (*mdh*), which restores growth at 42°C by combining the *M. smegmatis* malate:menaquinone oxidoreductase (Mqo) activity with *Mtb* Mdh NADH recycling.[13] When grown at 42°C, a temperature where Ndh-2 activity is minimal and complemented by Mdh/Mqo activity, neither compound inhibited cell-growth at 100 μM, strongly suggesting their target is Ndh-2.

This strain also provides a biochemical means to assess inhibitor activity against either Ndh or NdhA utilizing isolated membrane vesicles. We used the mutant strain complimented with either *Mtb ndh* or *ndhA* and prepared membrane vesicles from cultures grown at the non-permissive temperature. In these membrane preparations, the complemented genes are the majority type II NADH dehydrogenases expressed. The TQZ analog CBR-1825 showed strong potency against both *Msmeg* and *Mtb* Ndh (IC_{50} = 10.7 nM and 64.7 nM, respectively) when expressed in temperature-sensitive background and grown under non-permissive conditions (Table 1). Its activity against *Mtb* NdhA was similarly potent. Unlike the TQZ scaffold, the THI inhibitor CBR-4032 appeared specific for Ndh, as its IC_{50} against NdhA was over 300-fold increased relative to Ndh.

Table 1: NADH oxidation in *M. smegmatis* membranes with complemented *Mtb ndh* or *ndhA* inhibited by TQZ and THI scaffolds.

Compound	*Mtb* Ndh IC_{50} (nM)	*Msmeg* Ndh IC_{50} (nM)	*Mtb* NdhA IC_{50} (nM)
TQZ	64.7	10.7	42.5
THI	25.0	44.3	15300

Taken these results together we felt confident that both scaffold series were potent and specific Ndh-2 inhibitors, and we next endeavored to characterize the phenotypic response to loss of function of Ndh-2 in mycobacteria.

The NADH:quinone oxidoreductases are the major enzymes that oxidize NADH to NAD^+, and we therefore hypothesized the immediate consequence of blocking Ndh-2 function would be the prevention of NADH oxidation, resulting in an increase in the ratio of NADH:NAD^+. It has previously been shown that increased levels of NADH cofactor can protect InhA from inhibition by isoniazid (INH), likely due to increased competition with the active form of INH, the INH-NAD adduct.[14] Indeed, CBR-1825 abrogated the anti-mycobacterial activity of INH when the two were tested in combination. Treating *Mtb* with a sub-lethal concentration of the Ndh-2 inhibitor completely reversed growth inhibition of isoniazid at concentration that normally prevents proliferation (Figure 4A). These results demonstrate that increase of NADH:NAD^+ ratio causes resistance to INH, consistent with the previous observations in type II NADH dehydrogenase mutants isolated during selection for INH resistance.[13b] Some strains with Ndh mutations are also auxotrophic for serine, possibly due to inhibition of the initial step in serine biosynthesis by elevated levels of NADH.[15] In corroboration of this, we found that addition of serine to Ndh-2 inhibitor-treated *Mtb* cultures could rescue bacterial growth (Figure 4B).

Shutdown of electron transport to the proton-pumping terminal electron acceptors will disrupt the proton motive force, which is utilized by the F_1F_0-ATP synthase to catalyze ATP synthesis.[16] Consistent with this notion, the inhibition of Ndh-2 also decreased intracellular ATP levels in a dose-dependent fashion (Figure 4C).

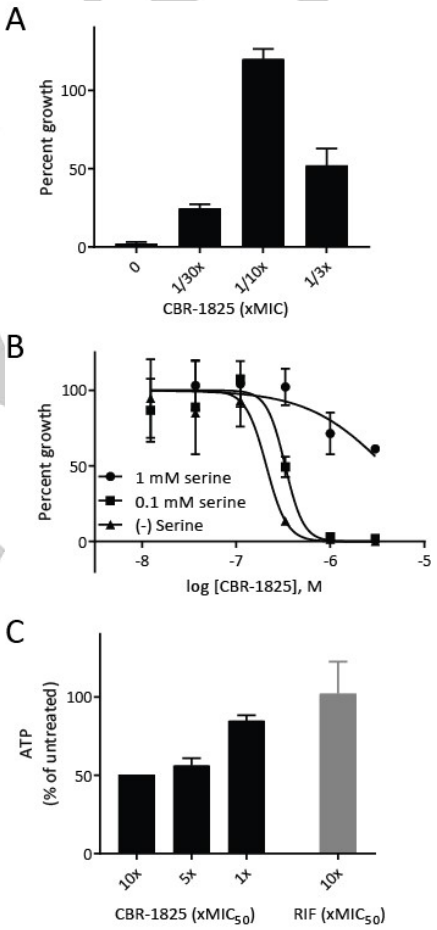


Figure 4. Metabolic and physiologic effects in *Mtb* produced by Ndh-2 inhibition. A) Treatment with sub-inhibitory concentrations of TQZ blocks growth inhibition by INH at a concentration (250 nM) that normally completely prevents replication in the absence of TQZ. Percent growth was normalized to untreated controls. B) Addition of exogenous serine rescues *Mtb* treated with an Ndh-2 inhibitor. C) Quantitation of ATP levels in *Mtb* treated with 10x, 5x, and 1x the MIC_{50} of the given inhibitor after 6 hr of treatment time.

The mycobacterium ETC and the ATP synthetic machinery-driven by it represent a hub of vulnerability for the pathogen.[17] Furthermore, due to the druggability of targets in this pathway, as evidenced here and elsewhere, it thus remains an opportunity for anti-TB drug discovery.[3, 18] Using a pathway-based screen in mycobacterial membranes we identified inhibitors that block oxidative phosphorylation and prevent *Mtb* growth via disruption

of Ndh-2 activity. Inhibition of Ndh-2 not only prevents ATP synthesis through blockade of the ETC, but also disturbs the intracellular redox balance which leads to an array of metabolic consequences. The identification of inhibitor from a biochemical screen that have cellular activity is especially encouraging, given past low success rates in these screens in the antimicrobial field.[19] The scaffolds identified here act on Ndh and NdhA differently even though their protein sequences are highly conserved. We believe it is reasonable to suggest that the TQZ and THI scaffolds, occupy the quinone and NADH binding sites, respectively, given their structural resemblance to the quinone and adenine molecules that normally reside in those positions.[20] Structural analysis coupled with kinetic enzymatic analysis of the inhibitors would be the best way to gain more insight into their inhibitory mechanism.[21] Future studies will also include utilizing our Ndh-2 inhibitors to explore the roles of Ndh-2 and Nuo *in vivo*, and will allow us to shutdown both or one copies of Ndh-2, respectively.

Acknowledgements

The authors thank Drs. Cliff Barry and Helena Boshoff for technical support or helpful discussion. This work was supported, in part, by National Key R&D Program of China 2017YFA0505400，Strategic Priority Research Program of CAS，XDPB0304, CAS Pioneer Hundred Talents Program, the Bill and Melinda Gates Foundation, the Global Alliance for TB Drug Development, and NIH Grants AI026170 (WRJ) and AI051519 (WRJ). We also ackowlwdge Ms. Jennifer Yano for her technical assistance.

Keywords: Ndh-2 • Oxidative phosphorylation • ATP production • Tuberculosis • Drug discovery

References

[1] V. Dartois, C. E. Barry, 3rd, *Bioorg Med Chem Lett* **2013**, *23*, 4741-4750.

[2] G. M. Cook, K. Hards, C. Vilcheze, T. Hartman, M. Berney, *Microbiol Spectr* **2014**, *2*.

[3] K. Andries, P. Verhasselt, J. Guillemont, H. W. Gohlmann, J. M. Neefs, H. Winkler, J. Van Gestel, P. Timmerman, M. Zhu, E. Lee, P. Williams, D. de Chaffoy, E. Huitric, S. Hoffner, E. Cambau, C. Truffot-Pernot, N. Lounis, V. Jarlier, *Science* **2005**, *307*, 223-227.

[4] C. M. Sassetti, D. H. Boyd, E. J. Rubin, *Mol Microbiol* **2003**, *48*, 77-84.

[5] D. Awasthy, A. Ambady, A. Narayana, S. Morayya, U. Sharma, *Gene* **2014**, *550*, 110-116.

[6] D. Bald, C. Villellas, P. Lu, A. Koul, *MBio* **2017**, *8*. [7] A. Koul, N. Dendouga, K. Vergauwen, B. Molenberghs, L. Vranckx, R. Willebrords, Z. Ristic, H. Lill, I. Dorange, J. Guillemont, D. Bald, K. Andries, *Nat Chem Biol* **2007**, *3*, 323-324.

[8] aP. S. Shirude, B. Paul, N. Roy Choudhury, C. Kedari, B. Bandodkar, B. G. Ugarkar, *ACS Med Chem Lett* **2012**, *3*, 736-740; bW. D. Hong, P. D. Gibbons, S. C. Leung, R. Amewu, P. A. Stocks, A. Stachulski, P. Horta, M. L. S. Cristiano, A. E. Shone, D. Moss, A. Ardrey, R. Sharma, A. J. Warman, P. T. P. Bedingfield, N. E. Fisher, G. Aljayyoussi, S. Mead, M. Caws, N. G. Berry, S. A. Ward, G. A. Biagini, P. M. O'Neill, G. L. Nixon, *J Med Chem* **2017**, *60*, 3703-3726.

[9] A. J. Warman, T. S. Rito, N. E. Fisher, D. M. Moss, N. G. Berry, P. M. O'Neill, S. A. Ward, G. A. Biagini, *J Antimicrob Chemother* **2013**, *68*, 869-880.

[10] S. P. Rao, S. Alonso, L. Rand, T. Dick, K. Pethe, *Proc Natl Acad Sci U S A* **2008**, *105*, 11945-11950.

[11] E. A. Weinstein, T. Yano, L. S. Li, D. Avarbock, A. Avarbock, D. Helm, A. A. McColm, K. Duncan, J. T. Lonsdale, H. Rubin, *Proc Natl Acad Sci U S A* **2005**, *102*, 4548-4553.

[12] S. S. Lin, U. Gross, W. Bohne, *Mol Microbiol* **2011**, *82*, 209-221.

[13] aD. Molenaar, M. E. van der Rest, A. Drysch, R. Yucel, *J Bacteriol* **2000**, *182*, 6884-6891; bC. Vilcheze, T. R. Weisbrod, B. Chen, L. Kremer, M. H. Hazbon, F. Wang, D. Alland, J. C. Sacchettini, W. R. Jacobs, Jr., *Antimicrob Agents Chemother* **2005**, *49*, 708-720.

[14] M. Nguyen, A. Quemard, S. Broussy, J. Bernadou, B. Meunier, *Antimicrob Agents Chemother* **2002**, *46*, 2137-2144.

[15] E. Sugimoto, L. I. Pizer, *J Biol Chem* **1968**, *243*, 2081-2089.

[16] K. Hards, J. R. Robson, M. Berney, L. Shaw, D. Bald, A. Koul, K. Andries, G. M. Cook, *J Antimicrob Chemother* **2015**, *70*, 2028-2037.

[17] G. M. Cook, K. Hards, E. Dunn, A. Heikal, Y. Nakatani, C. Greening, D. C. Crick, F. L. Fontes, K. Pethe, E. Hasenoehrl, M. Berney, *Microbiol Spectr* **2017**, *5*.

[18] K. Pethe, P. Bifani, J. Jang, S. Kang, S. Park, S. Ahn, J. Jiricek, J. Jung, H. K. Jeon, J. Cechetto, T. Christophe, H. Lee, M. Kempf, M. Jackson, A. J. Lenaerts, H. Pham, V. Jones, M. J. Seo, Y. M. Kim, M. Seo, J. J. Seo, D. Park, Y. Ko, I. Choi, R. Kim, S. Y. Kim, S. Lim, S. A. Yim, J. Nam, H. Kang, H. Kwon, C. T. Oh, Y. Cho, Y. Jang, J. Kim, A. Chua, B. H. Tan, M. B. Nanjundappa, S. P. Rao, W. S. Barnes, R. Wintjens, J. R. Walker, S. Alonso, S. Lee, J. Kim, S. Oh, T. Oh, U. Nehrbass, S. J. Han, Z. No, J. Lee, P. Brodin, S. N. Cho, K. Nam, J. Kim, *Nat Med* **2013**, *19*, 1157-1160.

[19] R. Tommasi, D. G. Brown, G. K. Walkup, J. I. Manchester, A. A. Miller, *Nat Rev Drug Discov* **2015**, *14*, 529-542.

[20] A. Heikal, Y. Nakatani, E. Dunn, M. R. Weimar, C. L. Day, E. N. Baker, J. S. Lott, L. A. Sazanov, G. M. Cook, *Mol Microbiol* **2014**, *91*, 950-964.

[21] T. Yano, M. Rahimian, K. K. Aneja, N. M. Schechter, H. Rubin, C. P. Scott, *Biochemistry* **2014**, *53*, 1179-1190.

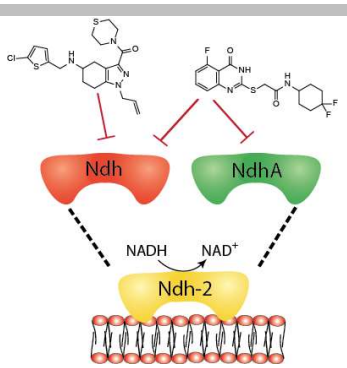
10.1002/anie.201800260

WILEY-VCH

Entry for the Table of Contents (Please choose one layout)

COMMUNICATION

A high-throughput screen against the *M. tuberculosis* oxidative phosphorylation pathway identified two inhibitors of the type II NADH dehydrogenase. The inhibitors are differentiated in their activity against one or both of the Ndh-2 isozymes. Treatment *Mtb* with the compounds leads to shutdown of ATP synthesis and bacterial killing.



Michael B Harbut, Feng Wang*

Page No. – Page No.

Small molecules targeting Mycobacterium tuberculosis type II NADH dehydrogenase with anti-mycobacterial activity